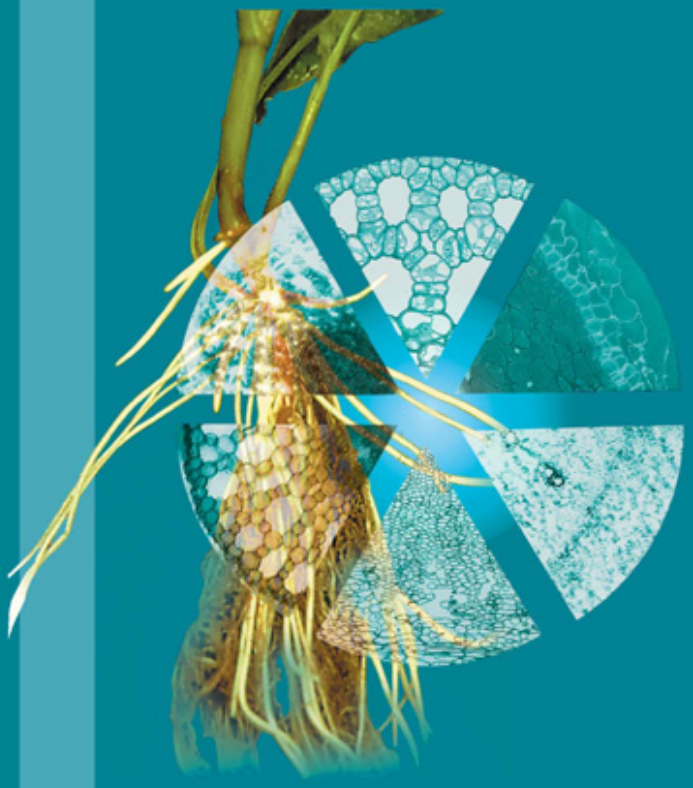
PLANT ECOPHYSIOLOGY

Root Physiology
From Gene to Function

Edited by
Hans Lambers
and
Timothy D. Colmer



Springer

ROOT PHYSIOLOGY: FROM GENE TO FUNCTION

Plant Ecophysiology

Volume 4

Series Editors:

Luit J. De Kok and Ineke Stulen

University of Groningen
The Netherlands

Aims & Scope:

The Springer Handbook Series of Plant Ecophysiology comprises a series of books that deals with the impact of biotic and abiotic factors on plant functioning and physiological adaptation to the environment. The aim of the Plant Ecophysiology series is to review and integrate the present knowledge on the impact of the environment on plant functioning and adaptation at various levels of integration: from the molecular, biochemical, physiological to a whole plant level. This Handbook series is of interest to scientists who like to be informed of new developments and insights in plant ecophysiology, and can be used as advanced textbooks for biology students.

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Reprinted from *Plant and Soil,* Volume 274 (2005).

Springer

DORDRECHT/BOSTON/LONDON

Library of Congress Cataloging-in-Publication Data

A C.I.P. Catalogue record for this book is available from the library of Congress.

ISBN-10 1-4020-4098-9 (HB)
ISBN-13 978-1-4020-4098-6 (HB)
ISBN-10 1-4020-4099-7 (e-book)
ISBN-13 978-1-4020-4099-3 (e-book)

Published by Springer,
P.O. Box 17, 3300 AA Dordrecht, The Netherlands

Sold and distributed in North, Central and South America
by Springer,
101 Philip Drive, Norwell, MA 02061, U.S.A.

In all other countries, sold and distributed
by Springer,
P.O. Box 322, 3300 AH Dordrecht, The Netherlands

Printed on acid-free paper

Printed in the Netherlands

Contents

ROOT PHYSIOLOGY: FROM GENE TO FUNCTION

Cover Photo: Images of root cross sections taken from the flooding tolerant *Rumex palustris* (whole root system in background). *R. palustris* has been used by groups at Nijmegen University and Utrecht University as a model species for studies of submergence- and flooding-tolerance, at the molecular-to-ecological levels (see Chapter 11). Images of root cross sections from Ankie Ammerlaan (Utrecht University) and whole root system from Eric Visser (Nijmegen University). Cover designed by Ankie Ammerlaan.

Plant and Soil (2005) 274:vii–xv
DOI 10.1007/s11104-005-0964-x

Root physiology – from gene to function

Preface

Introduction

In the last decade, enormous progress has been made on the physiology of plant roots, including on a wide range of molecular aspects. Much of that progress has been captured in the following papers, which highlight that the classical boundaries between physiology, biochemistry and molecular biology have vanished. Breakthroughs have been made possible through integration of molecular and whole-plant aspects. There has been a strong focus on a limited number of model species, including *Arabidopsis thaliana*. That focus has allowed greater insight into the significance of specific genes for plant development and functioning. However, many species are very different from *A. thaliana*, in that they are mycorrhizal, develop a symbiosis with N_2-fixing microsymbionts, or have other specialised root structures. Also, some have a much greater capacity to resist extreme environments due to specific adaptations, for example aerenchyma in the case of soil flooding, or are capable of tolerating a wide range of soil chemical constraints, such as acidity, salinity or heavy metal toxicities. Research on species other than *A. thaliana* is therefore pivotal, to develop new knowledge in plant sciences in a comprehensive manner. This fundamental new knowledge can be the basis for important applications in, e.g., agriculture and plant conservation. Although significant progress has been made, much remains to be learnt, especially for many aspects of root physiology. It is envisaged, however, that discoveries made in the recent past will likely lead to major breakthroughs in the next decade.

Resource acquisition and carbon metabolism

The discovery of the role of aquaporins in water transport in both animals and plants has been a major breakthrough for understanding plant water relations, in particular water uptake (Vandeleur et al., 2005). Aquaporins are water-channel proteins (Johansson et al., 2000; Maurel, 1997). Their name is somewhat unfortunate, since the term 'porin' is commonly used for proteins that allow the transport of large molecules in a fairly unspecific manner, whereas we now know that aquaporins, which belong to a class of proteins termed MIPs (membrane-intrinsic proteins), are rather specific. However, some can also transport other small molecules, e.g., glycerol (Zeuthen and Klaerke, 1999) or CO_2 (Uehlein et al., 2003). Knowledge on the regulation of aquaporins contributes to understanding plant responses to some abiotic stresses and might help in the design of new irrigation techniques to improve use of scarce water resources. Aquaporins are involved in the adjustment of the hydraulic conductivity of roots, and therefore in adjustments of the roots' physiology during both phases of wet/dry cycles in natural communities and in horticultural crops with irrigation management using partial root-zone drying (Vandeleur et al., 2005). Wider implications of the regulation of aquaporins for acclimation during abiotic stress include the recent finding that the well-known decrease in root hydraulic conductance during flooding involves gating of a root aquaporin, due to cytosolic acidosis (Tournaire-Roux et al., 2003). Moreover, it can be speculated that aquaporins are important in hydraulic redistribution of water in the root–soil system. Aquaporins would allow a plant control over the exit of water from its roots into a soil with a more negative water potential than that of the roots themselves. This phenomenon, which was first described by Caldwell and Richards (1989) and termed 'hydraulic lift', is quite common in desert species with access to deep water (Yoder and Novak, 1999) and re-hydrates the rhizosphere of surface roots during the night. In contrast to

'hydraulic lift', for trees growing in a soil with a low conductance between the top and deeper layers, water movement *down* the profile might rely on hydraulic redistribution via the taproot (Burgess et al., 1998). So far, there are no data to support any speculations on involvement of aquaporins in hydraulic re-distribution, but it is expected that such information will become available during the next decade.

Major progress has been made on the physiology of uptake and metabolism of nitrogen (Miller and Cramer, 2005) and phosphorus (Raghothama and Karthikeyan, 2005; Smith, 2003a). Genes involved in the transport of these nutrients have been identified, and we are beginning to learn when and where these are expressed. We have also gained a much better understanding of the functioning of specialised roots involved in nutrient acquisition, such as the "proteoid" or "cluster" roots of Proteaceae and *Lupinus albus* (Lambers et al., 2003; Shane and Lambers, 2005). These cluster roots produce and exude vast amounts of carboxylates. Carboxylate release is not restricted to species with cluster roots, but it is the combination of their capacity to release carboxylates in an exudative burst and their structure that allows build-up of high concentrations in the rhizosphere. This ability provides these species with an opportunity to substantially modify their rhizosphere and 'mine' the soil in their immediate vicinity; they are particularly effective in the acquisition of scarcely available phosphorus and micronutrients. Some species belonging to the Cyperaceae have 'dauciform' (=carrot-shaped) roots (Davies et a1., 1973; Lamont, 1974; Shane et al., 2005). In many ways, these dauciform roots function in much the same way as cluster roots; their development is suppressed at a high phosphorus supply and when formed they release citrate in an exudative burst (Shane et al., 2005; M.W. Shane and H. Lambers, unpubl.). The combination of biochemical/physiological and anatomical/morphological traits, which allows the build-up of high concentrations of exudates in the rhizosphere might be further exploited considering that the world's phosphorus reserves are dwindling (Vance, 2001).

Major phosphorus reserves are stored in agricultural soils, due to the application of phosphorus fertilisers ('phosphorus bank'). Depending on soil type and agronomic practices in the past, over 80% of applied phosphorus in fertilisers can be sorbed to soil particles, and hence, largely unavailable for most crop plants. We will need crop species with the root traits of Proteaceae and Cyperaceae to access that phosphorus bank. So far, only a limited number of species with cluster roots have been used in agriculture and horticulture, including *Macadamia integrifo1ia* (to produce nuts) (Firth et al., 2003), *Lupinus albus* (as a source of protein) (Gardner et al., 1981) and *Aspalanthus linearis* (to produce rooibos tea) (Ratshitaka et al., 2001). Most *Lupinus* species do not make cluster roots of the type produced by *L. albus*, but they do make structures of a similar function (Hocking and Jeffery, 2004). Other *Lupinus* species that are used as crop species, e.g., *L. angustifolius* make sand-binding roots (pers. observation), which may well serve a similar purpose. We are not aware of any species with dauciform roots being intentionally used in managed systems, although, given the relatively wide occurrence of dauciform roots in Cyperaceae (Shane et al., 2005) species with this root type might have been planted in some constructed wetlands. Making greater use of species with cluster roots or similar traits will be of economic benefit, since these plants will be able to access sparingly available phosphorus. In addition, there may be ecological benefits, because an improved capacity for phosphorus acquisition will decrease the need for high rates of phosphorus application, and hence potentially reduce run-off of phosphorus into streams and estuaries, thus limiting eutrophication.

Plants often acquire limiting resources in symbiosis with micro-organisms (Vessey et al., 2005). Our understanding of the legume–rhizobium symbiosis has increased dramatically, not in the least because of the powerful molecular techniques of transcriptomics and metabolomics. These approaches have made it possible to obtain a much improved overview of the metabolic differentiation during nodule development in *Lotus japonicus* (Colebatch et al., 2004). Approximately 860 genes are more highly expressed in nodules than in roots, including one third involved in metabolism and transport. More than 100 of the highly expressed genes encode proteins likely involved in signalling, or regulation of gene expression at the transcriptional or post-transcriptional level. The analysis showed clear signs

of hypoxia in root nodules, as expected; in addition, there were numerous indications that nodule cells also experience phosphorus limitation. Much less is known about other N_2-fixing symbiosis (Vessey et al., 2005).

Mycorrhizal associations can be found in most vascular plant species, and this field of research has developed enormously in the recent past (Graham and Miller, 2005). Molecular tools have revolutionised studies of mycorrhizal diversity and abundance, improving knowledge on host specificity of the symbionts (Graham and Miller, 2005) and highlighting relationships between mycorrhizal fungi diversity as influencing the structure of some communities of vascular plants (e.g., Reynolds et al., 2003). Like the recent discoveries of genes for transport of mineral nutrients in plants (Miller and Cramer, 2005), rapid advances to elucidate genes regulating exchanges of molecules between plant hosts and arbuscular mycorrhizal (AM) fungi are being made. Our views on 'mycorrhizal dependency' need to be revisited, with the discovery that the micro-symbionts can be responsible for most of the phosphorus uptake when there is no, or only a minor, growth response (Smith et al., 2003b).

Carnivory of small animals is a specialised strategy used by some plant species to acquire inorganic nutrients (Adlassnig et al., 2005). Carnivorous species inhabit a range of nutrient-impoverished environments, ranging from fire-prone sand plains to acid peat bogs and aquatic habitats, with, as one might expect, a wide range of root sizes and structures (Adlassnig et al., 2005). In contrast with information available on their traps formed by shoot organs (Juniper et al., 1989), comparatively little is known about the physiology of roots of carnivorous plants; several studies have demonstrated the importance of the roots in water and nutrient uptake for at least some carnivorous species, but not in several others.

Since two books (Day et al., 2004; Lambers and Ribas-Carbo, 2005) dealing with plant respiration have recently been published in Govindjee's series *Advances in Photosynthesis and Respiration*, no review dealing with this aspect of root physiology has been included. Over the last decade or so, we have gained a much better understanding of the respiratory costs of nutrient acquisition in fast- and slow-growing herbaceous species (Poorter et al., 1991; Scheurwater et al., 1998). When plants are grown at a high supply of nitrate, the costs associated with nitrate uptake are considerably greater for inherently slow-growing species, due to a major efflux component (Scheurwater et al., 1999). That passive efflux needs to be compensated by a greater active influx, and thus accounts for greater respiratory costs per unit N acquired. However, greater efflux is not the cause of slower growth; rather, slower growth leads to greater efflux (Ter Steege et al., 1999) and hence greater respiratory costs (Nagel and Lambers, 2002). Considerable information has also become available on the quantitative significance of cyanide-resistant root respiration (Millenaar et al., 2001). Major progress is to be expected on the physiological significance of the alternative path, which has puzzled plant scientists for quite some time. That progress can be expected, because a technique to assess the activity of this path in intact tissues has become more widely available (Ribas-Carbo et al., 2005).

Perception of the abiotic and biotic root environment

We have gained an appreciation of the fact that roots 'sense' their environment, and that the plant responds in a feed-forward and adaptive manner. The feed-forward response when roots sense adverse conditions in the soil allows acclimation before a major disturbance of the plant's metabolism (Davies et al., 1994). When roots perceive that the soil is flooded, they respond with an enhanced concentration of ethylene in both the roots and above-ground plant parts. Ethylene induces the formation of aerenchyma in roots (Visser and Voesenek, 2005), and also affects adaptive processes in above-ground organs (Voesenek and Blom, 1999). Similarly, roots can sense the availability of water (Davies et al., 1994) as well as nitrogen and phosphorus (De Groot et al., 2003), and signal this information to the shoot, which responds in a feed-forward manner. In the case of water stress, the signalling molecule is ABA (Davies et al., 1994; Schurr et al., 1992). Cytokinins are involved in signalling the plant's N and P status (De Groot et al., 2003; Kuiper et al., 1989; Lambers et a1., 1998). These are some of many examples showing

'signalling' between roots and shoots. More details about signals and signal-transduction pathways are included in the review by Dodd (2005), highlighting that the signals need not always be one of the classical phytohormones, but can, for example, be nitrate or sugars also. Internal signalling in plants, as dependent on plant development and environmental conditions is a rapidly developing field, where much progress can be expected. The challenge will be to integrate the new information into improved crop production systems.

Sensing does not only involve resources, but also other chemical factors in the environment. Aluminium-resistant genotypes must be able to sense the presence of aluminium to respond with the release of aluminium-complexing carboxylates (Kochian et al., 2005). When roots release either mainly citrate or mainly malate, depending on soil pH (Veneklaas et al., 2003), they must be sensing the soil pH or a factor closely associated with it. Sensing environmental conditions is obviously crucial to performance of plants, although we still lack a thorough understanding of the exact mechanisms involved.

Signalling in plants is not only important between different organs of a plant, but also between hosts and parasitic plants (Okubara and Paulitz, 2005). Holoparasitic species are entirely dependent on a host for the completion of their life cycle (Lambers et al., 1998). If their seeds were to germinate in the absence of a suitable host, that would be fatal, especially for those that have extremely small seeds. In fact, both germination (Siame et al., 1993) and the formation of haustoria (Estabrook and Yoder, 1998; Smith et al., 1990), which connect the parasite with its host, depend on chemical signalling between host and parasite. This is an exciting and rapidly developing field. Knowledge of these interactions may appear esoteric, but major applications can be expected, because some parasitic species (e.g., *Striga* and *Orobanche* species) belong to the world's worst weeds (Emechebe et al., 2004; Marley et al., 2004; Rodriguez-Conde et al., 2004). Others (*Cistanche* species) are grown to produce medicine in north-eastern China (Geng et al., 2004). Low-cost and safe signalling molecules that trigger the germination of the seeds of parasitic pest species before crops are sown might be of enormous benefit, especially to farmers in developing countries.

Signalling is also important between hosts and their symbiotic micro-organisms, e.g., rhizobia (Vessey et al., 2005) and mycorrhizal fungi (Graham and Miller, 2005). The intricate interactions that precede the establishment of a functional symbiosis are best understood for the rhizobium–legume symbiosis (Esseling and Emons, 2004). They must also play a role in other symbiotic systems that fix dinitrogen, but the progress in that area has been much slower (Rai et al., 2000; Vessey et al., 2005). Somewhat more is known on signalling between hosts and some mycorrhizal fungi (Graham and Miller, 2005), but much remains to be discovered.

Plants growing in soil with adverse abiotic or biotic conditions

Some species or ecotypes are capable of growing in soils that are naturally enriched with heavy metals, e.g., serpentine or ultramafic soils. The metals may be 'excluded' or absorbed, and stored in compartments where they do not harm the plant's metabolism (Meharg, 2005). Recently, progress has been made on the mechanisms accounting for internal transport and storage of heavy metals as well as metal 'exclusion' (Meharg, 2005). Species or ecotypes that accumulate heavy metals to very high levels are called metallophytes. These are not restricted to soils naturally enriched with heavy metals, but are also found on sites contaminated by heavy metals, e.g., due to mining. Such metallophytes are very important to stabilise contaminated soil, and stop it from spreading over a larger area. Metallophytes have been proposed as a method to clean contaminated soil (phytoremediation) (Meharg, 2005) or extract metals from soil with the intention to mine the metals (phytomining) (Li et al., 2003). To be economically viable options, the metallophytes have to accumulate metals to very high concentrations and produce a lot of biomass in a relatively short time. However, most metallophytes are inherently slow-growing, most likely because they were selected in low-nutrient environments, which are typically inhabited by slow-growing species (Lambers and Poorter, 1992). Genotypes that are both metal resistant and productive need to be selected. Major new discoveries are to be expected in the next

decade, but applications in the context of phytoremediation and phytomining would appear to be less promising than claimed when first proposed, unless combined with other profit-making operations, e.g., forestry (Robinson et al., 2003).

Mycorrhizas have been claimed to 'protect' higher plants from negative effects of heavy metals in soil (Leyval et al., 1997). It has also been shown that species that belong to a typically non-mycorrhizal family can be mycorrhizal if they are associated with soils with high levels of heavy metals. One example is for California serpentine grassland communities, where *Arenaria douglasii* (Caryophyllaceae) and *Streptanthus glandulosus* (Brassicaceae) were found to be mycorrhizal (Hopkins, 1987). Another example is for *Hakea verrucosa* (Proteaceae), occurring on nickel-containing ultramafic soils in Western Australia (Boulet and Lambers, 2005). These are exciting observations, from an evolutionary perspective as well as because of the possible implications for the rehabilitation of contaminated sites after mining.

Acid soils represent another stress to plant roots. It is not so much the low pH itself that causes the problems, but the fact that the solubility of specific metals strongly depends on pH (Kochian et al., 2005). In particular aluminium is considerably more soluble at low pH. Acid-resistant species typically are aluminium resistant. Resistance is at least partially based on 'exclusion', due to precipitation with citrate, malate or oxalate released from roots, depending on the species (Delhaize et al., 1993; Zheng et al., 1998). Some aluminium-resistant species also accumulate aluminium, and detoxify it internally as aluminium-carboxylate precipitates (Ma et al., 1997; Zheng et al., 1998). As for heavy metal resistance (Meharg, 2005), there is considerable genetic variation for aluminium exclusion (Delhaize et al., 1993; Kochian et al., 2005). This allows important application in agriculture, but care also has to be taken to minimise further soil acidification, which is a common trend in cropping systems (Lambers et al., 1998). Soil amelioration, e.g., using lime, remains important, but does not invariably address sub-soil acidity; therefore, aluminium-resistant genotypes would be important to develop.

Drought, salinity and flooding are abiotic stresses each of which influence plant species composition and productivity in natural and managed systems. Improved knowledge on physiological responses of roots to water stress, including root-to-shoot signalling (Dodd, 2005) should aid design of new irrigation techniques to improve use of scarce water resources (Vandeleur et al., 2005). Since various aspects of resistance to salinity have been reviewed recently (e.g., Munns, 2002; Tester and Davenport, 2003), and in a special issue dealing with dryland salinity in Australia (Lambers, 2003), salinity is not covered in this volume. Flooding regimes shape the composition of natural communities in some areas (Voesenek et al., 2004) and underpin rice production systems; soil waterlogging is also a problem in vast areas of irrigated and rainfed agriculture. Visser and Voesenek (2005) provide a comprehensive review on signals, and signal-transduction pathways, crucial to the perception and acclimation by plant roots to soil flooding. Hormones and signalling pathways that regulate traits for flooding resistance, such as adventitious rooting, aerenchyma formation for gas transport (Colmer and Greenway, 2005; Jackson and Armstrong, 1999) and root metabolism during O_2 deficiency (Gibbs and Greenway, 2003; Jackson and Ricard, 2003) are reviewed by Visser and Voesenek (2005). Substantial gains in knowledge in some areas are highlighted, as are emerging topics that are still poorly understood and will be priority areas for future research.

Roots frequently encounter adverse biotic conditions, due to the presence of microbial pathogens, nematodes, viruses and plant parasites (Okubara and Paulitz, 2005). Our understanding of these interactions has increased enormously, again, in part due to the development of new molecular tools. Developing resistance to root pests and diseases will continue to be important, as chemical protection is not always a desirable option.

Using new genotypes and combinations of crop species based on new ecophysiological information

Allelopathic interactions are very hard to demonstrate in nature, but they are very likely to occur, also in managed ecosystems (Lambers et al., 1998). The interactions may involve micro-organisms (Inderjit, 2005). Allelopathic interactions may account for the invasive nature of some

weeds (Ridenour and Callaway, 2001). There are major possibilities for applications in agriculture. Accessions of wheat (*Triticum aestivun*) differ widely in their potential to inhibit seed germination of ryegrass (*Lolium rigidumi*) (Wu et al., 2000a), a major weed in Australia (Powles and Shaner, 2001). That variation appears to be associated with the release of allelochemicals of a phenolic nature (Wu et al., 2000b), although so far the phenolics have only been assessed in root tissue, not in exudates. Making a crop more competitive, by enhancing its capacity for interference competition, would reduce the need for herbicides.

Facilitation refers to positive effects of one plant on another (Callaway, 1995). It is equally difficult to demonstrate in natural systems as allelopathic interactions are (Hauggaard-Nielsen and Jensen, 2005), but there are numerous examples of increased yields when combinations of crop species are used (Karpenstein-Machan and Stuelpnagel, 2000; Zuo et al., 2000). Such agronomic practices, usually called intercropping, are used in the low-input systems of the tropics, where crops are harvested manually (Willey, 1979), and are also common in China (Zhang et al., 2004) where new intercropping systems continue to be developed (Guixin et al., 2004). If reliable systems can be developed to mechanically harvest intercropped species at the same time, then this would stimulate the development of the practice for broad-area agriculture in other countries.

Pasture agronomy already uses combinations of species in broad-area agriculture, but further research might enhance productivity of pasture systems if the best combinations of species, and perhaps genotypes within species, can be further refined. For example, when seedlings of mycorrhizal and non-mycorrhizal species are grown together, they tend to have negative effects on each other which are not seen when either seedlings of mycorrhizal species or ones of non-mycorrhizal species grow together (Francis and Read, 1994). The chemical basis of this interference is not known, but the observation may have major implications for plant functioning in natural or managed systems. It may mean that combinations of mycorrhizal and non-mycorrhizal species are less desirable for intercropping, and this will need to be addressed to enhance productivity of intercropping and pasture systems.

Perspectives

Many new discoveries are to be expected in the ecophysiology of roots of native and crop species. One can envisage many applications of the new fundamental knowledge. One area that has not been reviewed in this volume, because it is too new to have generated many publications, is that of signalling in tritrophic below-ground interactions. Similar above-ground interactions are well documented for interactions between plants, their herbivores and 'bodyguards' (Alborn et al., 1997; Kessler and Baldwin, 2001; Sabelis et al., 2001). The first exciting information is now becoming available on interactions between roots of *Thuja occidentalis*, which release chemicals upon attack by weevil larvae (*Otiorhynchus sulcatus*), to attract parasitic nematodes (*Heterorhabditis megidis*), which then prey on the weevil larvae (Van Tol et al., 2001). Similar tritrophic interactions appear to occur in *Zea mays* (T.C.J. Turlings, pers. comm.). It is to be expected that improved knowledge in this area should provide opportunities for applications in plant management systems, similar to those existing for above-ground tritrophic interactions (Turlings and Wäckers, 2004).

Major progress on understanding numerous aspects of root physiology, and under several important environmental constraints, has been made possible by close interactions between ecophysiologists, biochemists and molecular geneticists. These close interactions will be important to achieve new breakthroughs in yield improvement. Such breakthroughs are vitally important, if we are to produce enough food and fibre for the worlds growing population in a sustainable manner.

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Plant and Soil (2004) 274: 1–36
DOI 10.1007/s11104-004-0965-1

Root nitrogen acquisition and assimilation

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Received 16 December 2003. Accepted in revised form 5 March 2004

Key words: ammonium, amino acid, fertiliser, metabolism, nitrate, reduction, soil, toxicity, transporter uptake

Abstract

Nitrogen (N) is the main mineral element in plant tissues and almost all of this nutrient is acquired from the soil by the roots. Nitrogen is available in many different forms in the soil, but the three most abundant forms are nitrate, ammonium and amino acids. The relative importance of these different soil N pools to a plant is difficult to measure and depends on many different environmental factors. Changes in the available amounts and imbalance in the supply of some N forms can even be toxic to plants and in extreme cases can lead to changes in the vegetation. However, the importance of this element for agriculture is reflected in the amounts of N-fertiliser applied to crops and this is a major cost (economic and environmental) for world agriculture. This review covers the molecular mechanisms that the plant uses for accessing these soil N pools and briefly includes consideration of the root N assimilatory pathways that exist in the plant. The soil forms of N that are used by plants depend on many factors, but a series of different transporter and assimilatory genes that can provide access to these pools have been identified. This information can now provide the molecular tools to identify the N sources accessed by a plant and the relative importance of these different pools.

Introduction

Plants require nitrogen (N) throughout their development. This N represents about 2% of total plant dry matter, and is a component of proteins, nucleic acids, coenzymes and numerous plant secondary products. Nitrogen is quantitatively the most abundant of the mineral elements in plant tissues, and enters the food chain mostly as NO_3^- or NH_4^+. The availability of N to plant roots is often an important limitation for plant growth, except where roots develop a symbiosis with N_2-fixing microorganisms (not reviewed). Only a tiny fraction (0.00024%) of planetary N is available to plants in the pedosphere (which includes plants, microbes, fauna, litter and soil). Plants cannot directly access either N_2, which comprises 2% of planetary N, or the 98% of planetary N that is immobilized in the geosphere (Rosswall, 1983). Atmospheric fixation of N_2 due to lightning is thought to account for between 0.5 and 30×10^{12} g N annum^{-1}, and biological N_2 fixation for 45 to 330×10^{12} g N annum^{-1}, 40% of which occurs in the oceans (Rosswall, 1983). The limited bio-availability of N and the dependence of crop growth on this mineral have spawned a massive N-based fertiliser industry worldwide, with annual N-fertiliser consumption currently close to 80×10^{12} g N (Figure 1). An increasingly large proportion of this N is currently applied in 'developing' countries, particularly in Asia, although, the extent of N application in the 'developed' world has declined over the last decade, resulting in a slowing in the rate of worldwide increase of N applications.

Nitrogenous fertilisers and associated contaminants accumulate in some situations to dangerous or even toxic levels, resulting in eutrophication of surface and ground water, and enriching the atmosphere with NH_3 and with N_2O. Considerable leaching of NO_3^- is caused, for example, by excessive application of nitrogenous fertilisers (inorganic and organic) to crops

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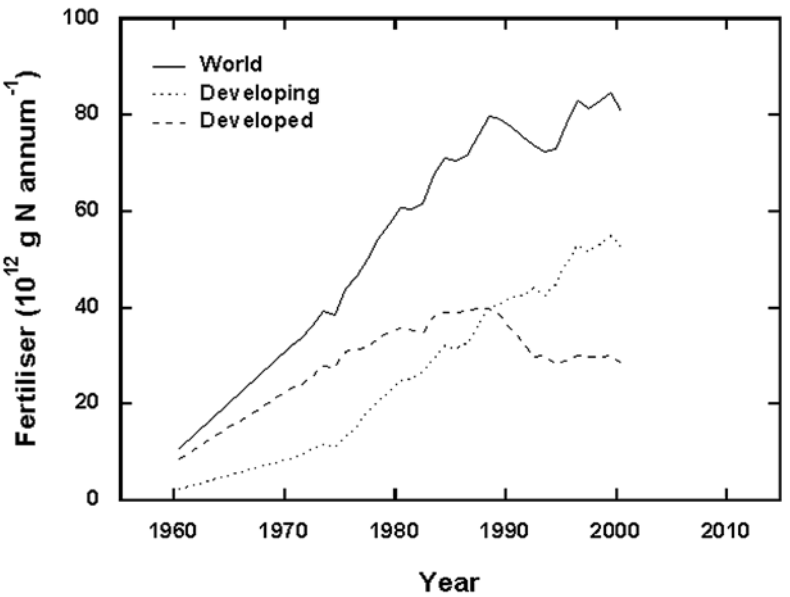

Figure 1. The consumption of N-containing fertilisers between 1961/62 and 2000/2001. The developing world was calculated from the data available for Central and South America, Africa (except South Africa), Near East (except Israel), South Asia, East Asia (except Japan), Socialist Asia and Oceania. Nitrogen-containing fertilisers include ammonium sulfate, urea, ammonium nitrate, calcium ammonium nitrate, ammonia direct application, calcium nitrate, sodium nitrate, ammonium chloride, calcium cyanamide, ammonium bicarbonate and combinations including ammonium phosphate, NP, NK and NPK (data plotted from that available from the International Fertiliser Industry Association, www.fertilizer.org/ifa/statistics.asp).

in an attempt to ensure maximum yields. Leaching also depends on soil characteristics, and the amount of water fluxing through the soil. Although less easily leached from soil, NH_4^+ is more toxic to plants than NO_3^- (Dejoux et al., 2000). However, conversion of NH_4^+ to NO_3^- (nitrification) can also contribute to the leaching of N from soils amended with NH_4^+ containing fertilisers. Estimates of total N loss by leaching from NH_4^+-based fertilisers range between 10 and 150 kg N ha^{-1} (International Fertilizer Industry Association, www.fertilizer.org/ifa/statistics.asp). Atmospheric pollution by NH_3 from organic manure, urea and ammonium sulphate might result from NH_3-volatilization. Although estimates of NH_3 volatilization are subject to a great deal of uncertainty, emissions estimates are between 15% and 25% of the applied amount of urea-N for Europe and for the tropics, respectively (Schjørring, 1998). Denitrification losses may be in the range of 5 to 10% of the applied N, of which about 10% is in the form N_2O (International Fertilizer Industry Association, www.fertilizer.org/ifa/statistics.asp), which is a greenhouse gas. Loss of NO_3^- through denitrification, both biological and chemical, occurs under reducing or anaerobic conditions (Haynes and Goh, 1978), and is especially important in fertilised fields where the loss of N may be enormous (Lewis, 1986). Thus excessive application of N fertilisers has enormous environmental costs, in addition to the economic and ecological costs of the production of the fertilisers. Most farmers are very aware that the excess use of fertiliser can cut their profit margins, but the yield penalties associated with application of too little N are potentially much larger, and it is therefore often economically not worth taking this risk. The balance between these two sides of the equation means that farmers cannot afford to skimp on their N-fertiliser applications, and the excess 'spare' N is deposited into the biosphere. This excess N and other man-made N pollution sources, such as factory and car exhaust, may have major environmental impacts as they supply additional growth potential to native plants. In some extreme cases this release of the growth limitation by N, whatever the source, can result in the invasion of new species, and a change in the landscape. This is the case, for example, with the N_2-fixing Australian *Acacia* spp. which have extensively invaded the 'Fynbos' biome in South Africa. Changes in forest species compositions and vegetation types as a result of agricultural pollution are now widely recognised (Nosengo, 2003), with reports of

changes in forests from the USA (e.g. Kochy and Wilson, 2001), Europe (e.g. Rennenberg et al., 1998) and changes in the UK flora (Pitcairn et al., 2003). This is not acceptable to most people who see this change in the environment as damaging the quality of life. Increasingly, farmers must be paid not just to produce food, but also to protect and maintain the environment.

The N accessed by plants exists in a variety of organic and inorganic forms within the soil. This influences the availability of the N and the uptake of the N by plants. A number of different transporters have been identified as being responsible for the uptake of inorganic (NO_3^- and NH_4^+) and organic N from the soil into roots. These multiple transport systems function under different circumstances, and are subject to complex regulation at the levels of transcription, translation and post-translation. Unlike many other mineral elements, N usually needs to be assimilated in order to participate in the biochemistry of the plant. This introduces a further level of complexity to the system with additional regulatory elements. Nitrate taken up by roots is either reduced *in situ* to NH_4^+ in the root, stored in vacuoles or transported to the shoot. The extent of shoot-based NO_3^- reduction varies between species and environmental circumstances (see below). Reduction of NO_3^- to NH_4^+ is achieved through participation of NO_3^- and NO_2^- reductases, with further assimilation of NH_4^+ into glutamate and glutamine by glutamine synthetase and glutamate synthase (see below).

Continued research and improved understanding of the chemistry of N in soils and the biochemistry of N uptake and assimilation may assist in development of improved management practices for natural and crop ecosystems, for example the reduction of N leaching (Spalding et al., 2001). The recognition that there is the possibility to breed improved genotypes capable of more efficient N uptake and utilization has become a new target for research. Most of the crop varieties grown in the developed world have been bred under conditions of high fertiliser input, approaching N saturation. There is an opportunity for the developed world to learn from the more sustainable agricultural systems in the developing world, and their cultivars will be a useful genetic resource in this effort. Improved efficiency of N recovery from soil and improved efficiency of utilization could allow crops to be grown with reduced N-fertiliser applications with contingent environmental and economic advantages. It is now timely and highly pertinent to review our current knowledge of the uptake and assimilation of N by plants. Much of the information on N metabolism is derived from studies on shoots which may or may not be pertinent for roots. This review attempts to provide an overview of N acquisition and assimilation in roots, while focusing on the latest findings relating to the molecular biology and the regulation of these processes.

Nitrogen acquisition

Nitrogen in the soil

Forms and origins of N

Nitrogen in the soil is present as a complex mixture of organic and inorganic forms, and, in addition to seasonal and diurnal changes, is also characterised by an extremely heterogeneous distribution. The transformation of one form into the other comprises what is known as the 'nitrogen cycle' involving the scavenging of organic N by microbial action and re-absorption by plants (Figure 2). Most of the N in soil is present in the form of complex organic molecules, which are converted to NH_4^+ by soil micro-organisms (bacteria and fungi) through mineralisation. Ammonium may then be oxidized via NO_2^- to NO_3^- through a process known as nitrification (*Nitrosomonas spp.*: $NH_3 + 1\ 1/2\ O_2 \rightarrow NO_2^- + H_2O + H^+$, *Nitrobacter spp.*: $NO_2^- + 1/2\ O_2 \rightarrow NO_3^-$). Nitrification is negatively influenced by low soil pH, anaerobic conditions, lack of soil water and temperatures below 5 °C and above 40 °C (Lewis, 1986). Nitrate can, in turn, be converted to nitrogen gases (N_2, N_2O, NO, NO_2) through use of NO_3^- as an electron acceptor in place of O_2 resulting in what is known as 'denitrification'. This occurs when the availability of O_2 is limited, the concentration of NO_3^- high, soil moisture is high, soil carbohydrates are available, and the temperatures are warm (Luo et al., 2000; Strong and Fillery, 2002).

Microbes also utilize inorganic N, and thus immobilize it, sometimes resulting in depletion of N available to plants if adequate carbon (C) is available to support the microbial biomass. The extent of competition between plants and microbes for soil N is complex, due to multiple pathways through which N cycles at variable rates and in varying amounts, and mycorrhizal symbiosis additionally complicates the picture (Hodge et al., 2000a). The availability of N to plants depends on the balance between the rates

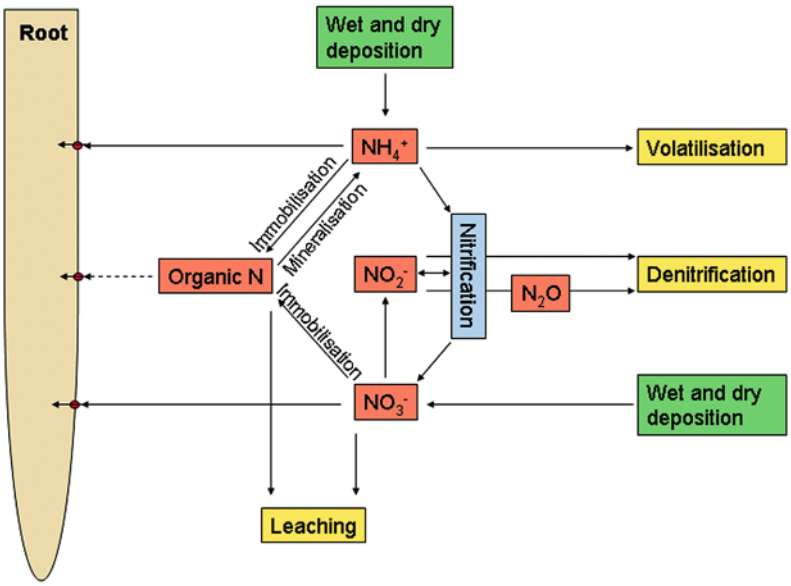

Figure 2. The main pools (boxes) and fluxes between pools (arrows) of N in terrestrial ecosystems, excluding both animals and inputs via N_2 fixation.

of mineralisation, nitrification and denitrification. The rate of mineralisation depends on factors influencing microbial activity such as water content of the soil, aeration of the soil and temperature (Lewis, 1986). If mineralisation is rapid, volatilisation of NH_4^+ to NH_3 can occur. This is favoured by alkaline soil pH and results in acidification (Dejoux et al., 2000). Primarily as a result of the biological component of N cycling, the availabilities of NO_3^- and NH_4^+ vary seasonally and the location and form of N within the soil profile varies with factors such as leaching, soil temperature and soil water status (Bloom, 1988).

The organic N fraction typically comprises 0.1 to 50% of total soil N (Barber, 1984). The current agricultural preference for urea-based fertilisers further contributes to the importance of organic N in the soil (see below). The organic N is in the form of peptides and proteins (ca. 99.5%, e.g., protein–humic complexes and peptides) and the remainder as free amino acids (Jones et al., 2002). Soil micro-organisms secrete proteases into the soil which facilitate the breakdown of proteins and peptides into their constituent amino acid units (Owen and Jones, 2001). The resultant amino acids do not bind strongly to the soil, and therefore do occur as free amino acids in the soil solution. The concentration of free amino acids in the bulk soil solution ranges from 0.1 to 50 mM, with the greatest concentrations in the surface horizons of soils rich in organic matter (Jones et al., 2002). Owen and Jones (2001) concluded that amino acid concentrations in agricultural soils generally range between ca. 1 and 100 μM. The largest source of amino N in the soil is vegetation, although, fauna, microbes and wet and dry deposition are also sources of varying importance. The concentration of amino acids in plant tissue is typically 1 to 10 mM making this an important source of organic N for the soil. Amino acids may be the dominant form of N in some high-latitude ecosystems. Since mineralisation is temperature dependent, cold anaerobic soils limit N mineralisation and aerobic nitrification, resulting in soils rich in amino compounds (Atkin, 1996). In contrast, many aerobic soils from warmer climes have little amino N since mineralisation proceeds rapidly. Jones et al. (2002) measured the free amino acid concentrations in soils from a range of ecosystem types in Southern Ireland (upland and lowland grasslands, forest, heathland and coastal saltmarsh) using centrifuge-drainage extracts combined with fluorometric assay of the amino acids. These authors found that free amino acids accounted for 24 $\pm$ 8 mM, NH_4^+ for 39 $\pm$ 14 mM and NO_3^- for 67 $\pm$ 42 mM N in the soil solution. Thus amino acids accounted for 10 to 40% of the total soil N in this survey. The possible roles of ecto- and endomycorrhizas in facilitating the uptake of organic N are briefly discussed below.

The inorganic N forms utilised by plants are NO_3^- and NH_4^+. Nitrite may arise in the soil from transfor-

mation of N compounds in the soil and rhizosphere, from organic wastes or from NO_3^--containing roots during low oxygen stress (Breteler and Luczak, 1982). However, NO_2^- uptake by plant roots is generally not considered to be of consequence as a result of the low levels of NO_2^- in the soil and the reported toxicity of this ion. Although in some soils NH_4^+ is more readily available than NO_3^-, in most agricultural soils the roots of plants take up N largely as NO_3^-. This is because NO_3^- generally occurs in higher concentrations than either NO_2^- or NH_4^+, and is free to move within the root solution due to the tendency for soils to possess an overall negative charge (Reisenauer, 1978). The high diffusion coefficient of NO_3^- in soil (Table 1) has the consequence that NO_3^- is not only readily available to plant roots, but that it is also easily lost from the root zone through leaching. Leaching may account for extremely high losses of up to 30% of soil inorganic N per growing season (De Willigen, 1986). The concentration of NO_3^- in many agricultural soils is in the millimolar range (1 to 5 mM, Owen and Jones, 2001). As a consequence of the ready use of NO_3^- by plants and micro-organisms and its leachability, concentrations of NO_3^- in the soil solution are usually very variable. In natural systems N is circulated relatively efficiently, with only small losses by denitrification and by leaching of NO_3^-, which is why water draining off natural ecosystems contains very low (e.g. ca. 5 μM) concentrations of NO_3^- (Hagedorn et al., 2001).

Ammonium concentrations in agricultural soils typically range between ca. 20 and 200 μM (Owen and Jones, 2001). However, low pH, low temperature, accumulation of phenolic-based allelopathic compounds in the soil, hydric and anaerobic soils inhibit nitrification and result in NH_4^+ accumulation (Britto and Kronzucker, 2002). Ammonium is relatively immobile in the soil, and less easily lost through leaching. Furthermore, human agricultural and industrial activities (pollution) have resulted in accumulation of NH_4^+ in many agricultural soils (see below). Thus, in some systems, NH_4^+ is the predominant form of N in the soil with concentrations averaging 2 mM in some forest soils up to 20 mM in some agricultural soils (Britto and Kronzucker, 2002). Such high concentrations of NH_4^+ are potentially toxic to some species, possibly due to problems with pH balance (Raven and Smith, 1976), anion/cation imbalance (Chaillou and Lamaze, 2001) and/or the energy drain resulting from the efflux of the ion (Britto and Kronzucker, 2002).

N Mobility in soil

Plants only rely extensively on 'root interception' for the uptake of sparingly soluble nutrients such as P; in contrast, N is mostly delivered to roots through a combination of mass flow and diffusion (De Willigen, 1986). Root interception, although a difficult concept to differentiate from interception combined with diffusion (Marschner, 1995), is thought to account for ca. 1% of N taken up (Barber, 1984). Mass flow relies on transpiration to draw water to the roots. If the rate of N delivery in the transpirational water stream is lower than the root demand for N, then diffusion also plays a role in uptake. Diffusion depends on the concentration gradient and the diffusion coefficient for the particular form of N. Although the diffusion coefficients for NO_3^- and NH_4^+ in water are similar (Table 1), the diffusion coefficients in soil are additionally determined by ion size and charge, viscosity of water, temperature, soil moisture, tortuosity and the soil buffer capacity. For NO_3^- the diffusion coefficient is ca. 1×10^{-10} m^2 s^{-1} (Barber, 1984), while that of NH_4^+ is ca. 10-fold to 100-fold less (Owen and Jones, 2001). This has the consequence that NH_4^+ is less readily leached from the soil than NO_3^-. The corollary of this is that NH_4^+ is also less available in the soil to roots for uptake, although when roots have access to NH_4^+ they take it up more readily than NO_3^- (Lee and Rudge 1986; Colmer and Bloom 1998). This preference for NH_4^+ is, however, modified by environmental factors such as temperature (Clarkson and Warner, 1979). For a maize (*Zea mays*) crop, N supplied by mass flow has been estimated to be ca. 4-fold greater than that supplied by diffusion (Barber, 1984), although this depends on many factors, including the activity of the roots.

Amino acids have strongly varying diffusion coefficients in water with lysine, glycine and glutamate having diffusion coefficients of ca. 1×10^{-12}, 1×10^{-11}, 1×10^{-11} m^2 s^{-1}, respectively (Owen and Jones, 2001). These low diffusion coefficients limit the rate of amino acid diffusion in the soil (less than 1 mm day^{-1}, Table 1) making it more likely that they will be consumed by microbes than taken up by roots, since the half-life of amino acids in soils is ca. 4 h. Thus, in practice, many plants may be unable to take up organic N compounds in competition with micro-organisms. This has been demonstrated by a lack of ^{13}C enrichments in the plant tissues supplied with ^{15}N–^{13}C-labelled organic substrates; however, ^{13}C taken up may also have been rapidly lost

Table 1. Calculation of the diffusion rates and sorption behaviour of inorganic N (NH_4^+, NO_3^-) and dissolved organic N (lysine, glycine, glutamate) in soil. The calculations are based upon the addition of 15.5 μM N-solute to the soil (modified from Owen and Jones, 2001)

	Unit	NO_3^-	NH_4^+	Lysine	Glycine	Glutamate
Diffusion coefficient in water	$m^2\ s^{-1}$	1.90×10^{-9}	1.96×10^{-9}	9.03×10^{-10}	1.05×10^{-9}	6.94×10^{-10}
Effective diffusion coefficient in soil	$m^2\ s^{-1}$	3.26×10^{-10}	2.70×10^{-12}	1.12×10^{-12}	9.03×10^{-12}	1.20×10^{-11}
Soil diffusion coefficient in soil relative to NO_3^-		1	8.23×10^{-3}	3.42×10^{-3}	2.76×10^{-2}	3.68×10^{-2}
Diffusion distance in 1 day	m	7.51×10^{-3}	6.80×10^{-4}	4.40×10^{-4}	1.25×10^{-3}	1.44×10^{-3}
Soil solution concentration	μM	77.3	0.62	0.55	3.87	7.73
Amount sorbed to soil	μmol L^{-1} soil	0.00	15.3	15.4	14.7	13.9
Total in soil	μmol L^{-1} soil	15.5	15.5	15.5	15.5	15.5
Percentage of N sorbed of total in soil	%	0	99.2	99.3	95.0	90.0

through respiration (Hodge et al., 2000b). The use of double-label isotopes seems to provide a reliable method for measurements of plant access to soil organic N sources. The low amino acid concentrations in agricultural soils, rapid microbial turnover of organic nitrogen, low diffusion coefficients and low uptake rates suggest that inorganic N will be the dominant N source available to crop plants (Owen and Jones, 2001). There is still some controversy as to the extent to which organic N is accessed by plants. Forest species *in situ* (*Deschampsia flexuosa, Picea abies, Vaccinium myrtillus*) in Sweden were found to take up intact ^{15}N-^{13}C labelled amino-compounds, which had been added to soils (Persson et al., 2003). In Arctic salt marshes, plant roots were found to take up between 5% and 11% of ^{15}N–^{13}C-labelled glycine supplied, and to contribute to the turnover of organic N in the soil (Hugh et al., 2003). To some extent the controversy in the literature over the degree to which organic N is accessed by plants may result from the use of different techniques and experimental conditions; however, soils also differ widely in their microbial flora. Variation in soil temperature and in microbial flora result in differences of the half-life of organic N in the soil, and thus the access that plants have to this organic N. The importance of factors like soil temperature for microbial activity may reduce the relevance of results obtained from pot experiments in the laboratory to the field situation. Furthermore, different plant species may also vary in their ability to intercept and to take up organic N.

Although there is some controversy as to whether plants do access organic N in soil, it is clear that plant roots do in general have the capacity to take up organic N. In a survey of 31 species from boreal communities using a GC-MS to measure ^{15}N-^{13}C-labelled amino acid uptake, it was found that all the plant species tested, representing a wide variety of plant types, had the ability to take up amino acids from a mixed solution containing 15 amino acids (Persson and Näsholm, 2001). In wheat (*Triticum aestivum*) roots exposed to amino acids at 100 μM, a concentration typical for agricultural soils, rates of net uptake of amino acids ranged between 3 and 33 pmol mm^{-1} root s^{-1}, depending on the amino acid in question (Owen and Jones, 2001). Following uptake, the amino acids enter the root pool of amino compounds, and may be directly incorporated into proteins, deaminated in the root or transported to the shoot.

Fertilisers

The fertilisers used currently include a diverse collection of compounds including organic sources of N, such as animal manures. The major synthetic fertilisers include: (1) ammonium fertilisers (ammonia, 80% N (w/w); ammonium sulphate, 21% N; ammonium

bicarbonate, 17% N); (2) NO_3^- fertilisers (calcium nitrate, 16% N; sodium nitrate, 16% N); (3) ammonium nitrate fertilisers (ammonium nitrate, 34% N; calcium ammonium nitrate, which is a combination of ammonium nitrate and calcium carbonate, 21 to 27% N; ammonium sulphate nitrate, 26 to 30% N); (4) amide fertilisers (urea, 46% N; calcium cyanamide, 20% N); (5) solutions containing more than one form of N (e.g., urea ammonium nitrate solution, 28 to 32% N); (6) slow-release fertilisers (which are either derivatives of urea, granular water-soluble N fertilisers encased in thin plastic film or other means of slow release such as sulphur-coated urea) and (7) multinutrient fertilisers containing N (NP, NK and NPK). There has been a dramatic increase in the utilisation of urea-based fertilisers over the last decades, so that urea is currently the predominant form of N fertiliser used (Figure 3).

In agriculture, application of urea may be used to enhance soil NH_4^+ contents because urea is readily hydrolysed to NH_4^+ in the soil (Harper, 1984), but it is not itself readily accessed by plants (Criddle et al., 1988). Urea is a popular form of N fertiliser due to its competitive price and high N concentration (46% of mass) reducing transport and distribution costs. However, N is lost from urea through conversion to NH_4^+ and then NH_3, although, this is less likely to occur from acidic soils with high cation exchange capacities. The enzyme urease converts urea to NH_4^+, and its activity is proportional to the microbial biomass, which in turn depends on the organic matter content of the soil, and on water present in the soil to solubilise the urea. Urease is a ubiquitous enzyme which is produced by micro-organisms in the soil and, because it is highly stable, persists in the soil after decay of the micro-organisms (Watson et al., 1994). Conversion of urea to NH_4^+ consumes H^+ and produces HCO_3^-, resulting in a net pH increase: $CO(NH_2)_2 + H^+ + 2H_2O \rightarrow 2NH_4^+ + HCO_3^-$. The fate of HCO_3^- is pH dependent. Due to the rapid equilibration of H_2CO_3 with CO_2 at acidic pHs it can be described as: $HCO_3^- + H^+ \leftrightarrow H_2CO_3 \leftrightarrow CO_2 + H_2O$ (pKa = 6.4). At more alkaline pHs: $HCO_3^- \leftrightarrow H^+ + CO_3^-$ (pKa = 10.3). Thus at acidic pH, two H^+ are consumed by formation of 2 NH_4^+ from urea, while at extremely alkaline pH there may be no pH implication of urea hydrolysis *per se*. However, at alkaline pH's volatilization of NH_4^+ can reduce soil pH: $NH_4^+ + OH^- \rightarrow NH_4OH \rightarrow NH_3 + H_2O$ (pKa = 9.3). If large amounts of urea are supplied to the soil, then the conversion of this to NH_4^+ can drive the pH up, with consequent promotion of volatilization; this has spurred the use of urease inhibitors to slow the breakdown of urea. However, nitrification of NH_4^+ derived from urea ($2\,NH_4^+ + 4\,O_2 \rightarrow 2\,NO_3^- + 4\,H^+ + H_2O$) can also cause severe pH decreases in some situations (Nohrstedt et al., 2000). Plant uptake of NH_4^+ derived from urea will further contribute to pH decreases. Thus the effect of urea on soil pH depends on several variables making the pH consequences uncertain.

The most common nitrogenous fertilisers used after urea are compounds containing NH_4^+. The application of NH_4^+-based fertilisers and those containing urea enhances soil NH_4^+ contents and the proportion of N available to the roots in this form. As a result of the high pKa (9.3) for conversion of NH_4^+ to NH_3, NH_4^+ is much more abundant in soil at acidic to neutral pH with only 0.5% of ammoniacal N in the form of NH_3 at pH 7. The utilisation of NH_4^+ has important implications for soil pH, since uptake of this cation results in a strong acidification of the soil. In contrast, uptake of NO_3^- results in net alkalinisation of the soil, albeit, at a much slower rate than that of acidification associated with NH_4^+ uptake. Furthermore, bacterial activity can rapidly convert NH_4^+ to NO_3^-. This nitrification also has an acidification effect, and consequently supply of NH_4^+-N can cause acidification regardless of whether the NH_4^+ is taken up by plant roots. The net acidification that occurs with NH_4^+ uptake and the net alkalinisation that occurs with NO_3^- uptake results in differences in solubility, concentration, ionic form, mobility and availability of N in the soil (Marschner, 1991). Since uptake of NH_4^+ by many crop plants is increased with increased pH, at high soil pH NH_4^+ toxicity may result, while at low soil pH, N starvation may occur (Findenegg, 1987).

Use of only one form of N fertiliser can drive soil pH away from the optimum. This can lead to deficiencies of elements such as K^+ (Findenegg, 1987) and P (Sentenac and Grignon, 1985) leading to interactions between N and the availability of other essential nutrients. Nitrogen-related changes in soil pH may also be responsible for the toxicity of certain elements. It may be argued that the extensive problems associated with Al toxicity may be related to the use of NH_4^+-containing fertilisers. On the other hand soil pH can be manipulated simply by modifying the form of N supplied, without the requirement for lime and without the risk associated with acids.

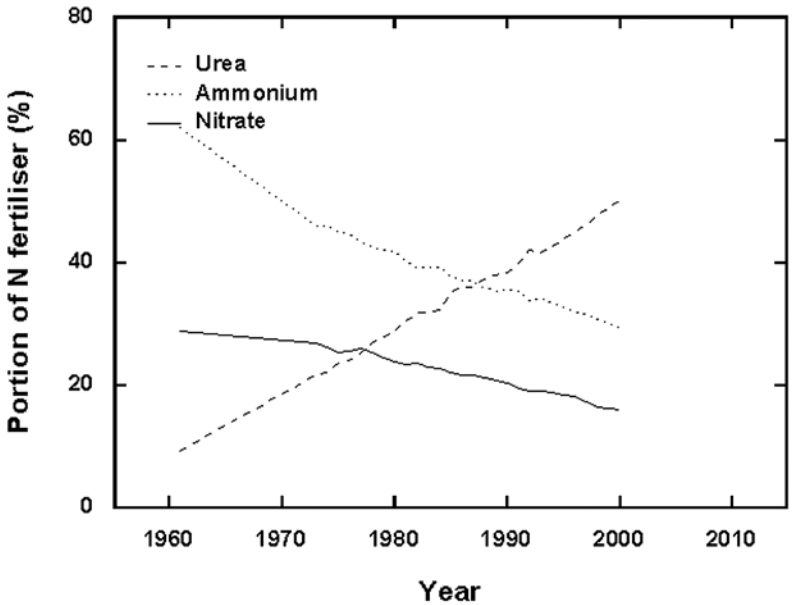

Figure 3. The proportion of total nitrogenous fertiliser applied containing urea, ammonium and nitrate or combinations of these (data plotted from that available from the International Fertiliser Industry Association, www.fertiliser.org/ifa/statistics.asp).

Although high concentrations of NH_4^+ can cause toxicity (see below), it has the benefits of (1) a smaller diffusion coefficient in the soil thus reducing loss of N through leaching, (2) higher specific N content, (3) lower costs, (4) plant incorporation of NH_4^+ avoids the carbon-intensive reduction of NO_3^- to NH_4^+. Thus NH_4^+ may be the N form of choice in some circumstances. However, conversion of NH_4^+ to NO_3^- by nitrification compromises some of these benefits. Nitrification inhibitors have been used in agriculture to enhance soil NH_4^+ contents (Adriaanse and Human, 1991; Bock, 1987). The availability of NH_4^+ within the soil may, however, also be severely limited, because it is tightly held by the micaceous clay minerals of the soil, and readily utilized by micro-organisms effectively removing it from the soil solution until mineralisation occurs (Lewis, 1986). The problem of limited availability of NH_4^+ may be partially overcome in agriculture through additional use of K^+ which increases the availability of NH_4^+ by occupying binding sites in the soil (Haynes and Goh, 1978), allowing more effective use of NH_4^+.

Root structure

The size and architecture of the root system is an important variable for ensuring adequate access to N. The architecture of the root is determined by the pattern of root branching. The species-specific size and architecture of root systems is also strongly determined by a wide range of physical, chemical and biological factors. In general, the size of the root (as measured by total mass, length or area) relative to the rest of the plant (e.g., as expressed by the shoot:root ratio or root mass ratio) increases when N is limiting. Nitrogen deprivation causes starch accumulation in leaves, and an increase in the proportion of photosynthate translocated to the root, resulting in a decline in the shoot:root ratios (Rufty et al., 1988). This enhanced allocation of C to the root was ascribed by these authors to a decreased utilization of sucrose in the shoot. Vessey and Layzell (1987) showed that only N in excess of the requirements of the root was exported to the shoot in *Glycine max*, suggesting that roots have the highest priority for N in times of N deficiency (Tolley-Henry and Raper, 1986), thus promoting root growth. However, there are now indications that root N availability controls developmental cues which in turn determine the demand for growth, thus controlling carbon allocation. Studies with tobacco (*Nicotiana plumbaginifolia*) deficient in NR (Scheible et al., 1997b) and in *Arabidopsis thaliana* (*Arabidopsis*) (Zhang et al., 1999) support the existence of a systemic signal elicited by NO_3^- accumulation that represses root growth. The notion that root growth is favoured by systemic signals under NO_3^- deficiency is also reinforced by the

observation that NO_3^-, but neither glutamine (Tranbarger et al., 2003) nor NH_4^+ (Zhang et al., 1999) supplied to the roots of *Arabidopsis* repressed root growth. Using macro-arrays, Tranbarger et al. (2003) identified transcription factors that were associated with the supply of NO_3^-, but not with glutamine supply. Furthermore, the studies conducted on the effect of N on root architecture suggest that the systemic signal regulating root growth in relation to N status is hormonal; auxin (Zhang and Forde, 2000) or abscisic acid (Signora et al., 2001). The function of decreased shoot:root ratios may be to compensate for N deficiency by increasing the N acquisition capacity of the plant (Brouwer, 1981; Khamis and Lamaze, 1990; Robinson, 1986; Rufty et al., 1990).

Apart from the total size of the root system, there are a large number of other attributes, which dictate its capacity and efficiency for N acquisition. Only a limited proportion of the root may actually be effective in the uptake of N (Robinson, 2001). The acquisition of N also depends on the distribution of the roots active in N uptake within the soil. Rooting depth, which varies greatly between species, determines the ability of a crop to intercept N, particularly NO_3^- during periods of leaching (Gastal and Lemaire, 2002). The construction costs of roots are also an important consideration; fine roots have a higher surface area to volume ratio than thick roots, and thus require less C for construction per unit root length, but may be more expensive for maintenance (per unit root weight). One of the most important attributes is the number, size and location of root hairs, which have an enormous impact on the absorptive surface area of the root.

Nitrogen in the soil is extremely heterogeneous on both a spatial and a temporal scale. Roots tend to proliferate in localized areas within the soil of high N content (Drew and Saker, 1975; Granato and Raper, 1989) and thus specific portions of the root may be exposed to high N concentrations while other parts of the root system are ineffective in N uptake. Plants may sense the soil N concentrations with specific sensors (see below), and also monitor and respond to their own internal N status (Malamy and Ryan, 2001). Many species respond to localised patches of NO_3^- by preferential lateral root proliferation within the nutrient-rich zones (Drew and Saker, 1975). In particular, the availability of NO_3^- affects both the number and location of lateral root initiation sites (Malamy and Ryan, 2001). The stimulatory effect of NO_3^- on root proliferation may seem contradictory to the inhibition of root development at high N concentrations. However, there seem to be two modes of action: inhibition of root development by a systemic inhibitory signal that results from the accumulation of NO_3^- in the shoot, and a localized stimulatory effect that depends on the local concentration of NO_3^- in the roots (Zhang and Forde, 2000). These authors provided evidence from NR deficient *Arabidopsis* that the localized stimulatory effect is a direct result of NO_3^- (i.e. not amino acids), probably in the leaf, acting on a NO_3^--inducible MADS-box gene (*ANR1*), which encodes a component of the signal transduction pathway linking the external NO_3^- supply to the increased rate of lateral root elongation. The systemic phloem-delivered signal, which is correlated with the N status of the plant, may depend on auxin or an auxin-related pathway for control of lateral root elongation, but not lateral root initiation (Zhang and Forde, 2000). Auxin localization appears to be a key factor in this nutrient-mediated repression of lateral root initiation (Malamy and Ryan, 2001). However, abscisic acid (ABA) applied exogenously inhibits *Arabadopsis* lateral root development through the operation of an auxin-independent pathway (De Smet et al., 2003). These authors showed that a mutation in the *ALF3* gene, which is part of the auxin-dependent regulatory pathway, did not alter the sensitivity of lateral root development to ABA, and that ABA suppresses auxin response in the lateral root primordia. De Smet et al. (2003) proposed a model in which different stages of lateral root initiation and development are regulated by both auxin and ABA.

The question has been posed as to why root proliferation in *Arabidopsis* occurs in localized patches of NO_3^-, which is a relatively mobile nutrient, whereas it does not respond to locally supplied NH_4^+ (Leyser and Fitter, 1998). Zhang and Forde (1998) have argued that this is because roots have evolved to use NO_3^- as a signal molecule, because it is relatively mobile in the soil. This may allow roots to proliferate towards areas where NO_3^-, other forms of N and P are localized within the soil. This ability to proliferate roots in areas with N may also be important in inter-specific competition for N or P (Hodge, 2002).

Plant-rhizosphere interactions

The availability of C in the rhizosphere is a major factor controlling the soil microflora and, consequently, N transformations in the soil (mineralisation, immobilisation, denitrification). A portion of the photosynthetic C is deposited in the soil in the form of root exudates (e.g., humic substances, sugars, organic acids,

amino acids), mucilage and sloughed cells and tissue (Marschner, 1995). Rhizodeposition is a major source of C and N for the soil and its inhabitants (Jensen, 1996). It is therefore of great importance for maintaining the level of microbial activity in the soil. Experiments with disturbed systems have indicated that total C input to agricultural soils can represent 15% to 33% of the C assimilated by plants (Qian et al., 1997). Using C_4 maize (which has a ^{13}C abundance which is distinct from that of C_3 plants), these authors were able to quantify the amount of C contributed to soils previously inhabited by C_3 plants by following changes in ^{13}C abundance. Between 5% (at maturity) and 12% (four-week old maize) of photosynthate was released to the soil as organic carbon. This release of organic C increased denitrification losses from soil by an average of 29% during the early growth stages.

Different portions of the root may exude different organic compounds. Bacterial biosensors were used to asses the exudation of tryptophan and sucrose from roots of *Avena barbata* (Jaeger et al., 1999). Tryptophan exuded from older portions of roots (0.12 to 0.16 m from the tip), while sucrose was most abundant in soil near the root tip. Nutritional circumstances have a significant impact on the type and concentration of exudation that occurs from roots. Al toxicity (Delhaize and Ryan, 1995) and P deficiencies (Shane and Lambers, 2004) strongly influence organic acid exudation. Exudation of carbohydrates and amino acids from roots of plants supplied with NH_4^+ is greater than that from roots supplied with NO_3^- (Cramer and Titus, 2001; Mahmood et al., 2002). This may partially be because plants supplied with NH_4^+ have higher root tissue concentration of amino acids (Cramer and Lewis, 1993), which may be exuded. The notion that carbohydrates simply 'leak' out of the roots has been challenged by work on kallar grass (*Leptochloa fusca*). Mahmood et al. (2002) found that 30-fold differences in sugar exudation between NO_3^-- and NH_4^+-supplied plants were not related to the internal root sugar concentration, or to the different root architecture, or to differential re-absorption of sugars. It was proposed that roots detected soil NH_4^+ concentrations as a signal for diazotrophic bacterial presence, and responded with enhanced sugar exudation. Thus soil exudation is not so much a passive event, but a means of manipulating the C content of the rhizosphere, and thus the soil microbial population.

While plants modify the rhizosphere and the environment for soil micro-organisms, these in turn modify plant physiology. Plant growth enhancement by plant growth-promoting bacteria involves diverse mechanisms including release of indoleacetic acid and cytokinin (Costacurta and Vanderleyden, 1995), reduction in ethylene levels (Wang et al., 2000), stimulation of the ion transport and enhancement of mineral availability (Bertrand et al., 2000). Several plant growth-promoting bacteria have been shown to stimulate root growth (Larcher et al., 2003), probably through hormone release. This modification of root growth has an important impact on N nutrition by increasing NO_3^- uptake capacity and possibly also by directly stimulating NO_3^- transport systems (reviewed by Mantelin and Touraine, 2004). The effects of plant growth-promoting bacteria on plant growth and the acquisition of N are usually greatest in low N fertility environments. Thus inoculation with plant growth-promoting bacteria could potentially have important consequences for enabling plant root growth for increased N acquisition under N deficiency.

Uptake and transport of N

Several recent reviews on the topic of NO_3^- and NH_4^+ transporters have been published (Forde, 2000; Forde and Clarkson, 1999; Touraine et al., 2001; Williams and Miller, 2001), and therefore only an overview of the main topics will be covered in this review. Less is known about uptake systems for other possible soil N sources, although genes encoding transporters for many types of N-containing organic molecules have been identified. The complete genome of *Arabidopsis* was the first to be published for a plant (Bevan et al., 2001), and so at present we have most molecular information for this species. *Arabidopsis* is a wild species and can grow and flower in low-N soils (Miller and Smith, unpublished results).

Nitrate transporters

Nitrate is actively transported across the plasma membranes of epidermal and cortical cells of roots, but net uptake is the balance between active influx and passive efflux. This transport requires energy input from the cell over almost the whole range of concentrations encountered in the soil (Glass et al., 1992; Miller and Smith, 1996; Zhen et al., 1991). It is generally accepted that the uptake of NO_3^- is coupled with the movement of two protons down an electrochemical potential gradient, and is therefore dependent on ATP supply to the H^+-ATPase that maintains the H^+ gradient across the plasma membrane (McClure et al., 1990; Meharg and Blatt, 1995; Miller and

Smith, 1996). Calculations of the energetic requirements for transport suggest that this co-transport is required for a wide range of extracellular NO_3^- concentrations (Miller and Smith, 1996; Siddiqi et al., 1990). For NO_3^- storage in the plant cell, transport at the tonoplast membrane requires a different mechanism and an antiport with H^+ has been suggested (Miller and Smith, 1992). Figure 4 is a schematic diagram that shows NO_3^- uptake and the associated proton-pumping ATPase (H^+-ATPase) that maintains the electrochemical potential gradient to drive the co-transport.

Physiological studies have shown the presence of both high- and low-affinity NO_3^--uptake systems operating at different external NO_3^- concentrations (Aslam et al., 1992; Glass and Siddiqi, 1995). There are believed to be two high-affinity transport systems (HATS) taking up NO_3^- at low concentration (generally below 0.5 mM with low transport capacity) and one low-affinity transport system (LATS) that transports NO_3^- at high concentrations (generally above 0.5 mM with high transport capacity) (Glass and Siddiqi, 1995). Numerous NO_3^- transporters have been cloned from a variety of species, and two distinct gene families, *NRT1* and *NRT2*, have been identified (Crawford and Glass, 1998; Daniel-Vedele et al., 1998; Forde, 2000; Forde and Clarkson, 1999; Williams and Miller, 2001). The Arabidopsis genome contains 52 *NRT1* and 7 *NRT2* family members; it was at first believed that NRT1 mediated the LATS and NRT2 the HATS (Forde and Clarkson, 1999; Zhuo et al., 1999). However, this tidy functional assignment in no longer valid, because in *Arabidopsis* the low-affinity NO_3^- transporter, *AtNRT1.1*, also functions in the high-affinity range (Liu et al., 1999), and these changes in the kinetics of transport are switched by phosphorylation of the protein (Liu and Tsay, 2003). A further complication for the *NRT1* family is that they belong to a much larger family of peptide transporters, the POT, or proton-dependent oligopeptide-transport family which is also known as the PTR or peptide-transport family (Paulsen and Skurray, 1994). Mammalian members of this family can transport peptides of varying sizes (Paulsen and Skurray, 1994). In *Arabidopsis* the pattern of tissue expression for much of the *NRT2* family has been mapped (Orsel et al., 2002; Okamoto et al., 2003). Some of the *NRT2* family require a second gene product for functional activity, but it is not known whether there is an interaction between the gene products (Galván et al., 1996; Zhou et al., 2000).

Some members of both *NRT1* and *NRT2* gene families are NO_3^- inducible and are expressed in the root epidermis, including root hairs, and in the root cortex. Members of both the *NRT1* and *NRT2* families are therefore good candidates for a role in the uptake of NO_3^- from the soil (e.g., Lauter et al., 1996; Lin et al., 2000; Ono et al., 2000; Nazoa et al., 2003). Some family members are constitutively expressed (see Okamoto et al., 2003 for details). For example, in *Arabidopsis AtNRT1.2* is constitutively expressed in the roots, particularly in root hairs and the epidermis (Huang et al., 1999). A detailed description of the tissue expression pattern of *AtNRT1.1* and *AtNRT2.1* has been provided by promoter-reporter gene fusions (Guo et al., 2001; Nazoa et al., 2003). These elegant papers show how much expression changes during development and reveal complicated tissue patterns. For example, *AtNRT1.1* was strongly expressed in the tips of primary and lateral roots but showed weak expression in the root cortex and epidermis (Guo et al., 2001). In contrast, the expression of *AtNRT2.1* was strong in the epidermis, cortex and endodermis of the mature parts of the root (Nazoa et al., 2003). The correlation between $^{13}NO_3^-$ influx and the expression of *AtNRT2.1* and *AtNRT1.1* has led to the suggestion that these two genes may be largely responsible for high and low affinity NO_3^- uptake (Okamoto et al., 2003). It has been suggested that the root cortex is the main site for the uptake of NO_3^- from the soil (Siddiqi et al., 1991), but it is difficult to reconcile this idea with the fact that some NO_3^- transporters are expressed in the epidermis. The expression of both families can be regulated by feedback from N metabolites in many plant species (Touraine et al., 2001). Various amino acids have been tested for their ability to alter the expression and activity of NO_3^- transporters through feedback regulation. Feeding amino acids to roots decreases the expression of NO_3^- transporters (Nazoa et al., 2003; Vidmar et al., 2000). However, identifying which amino acids are responsible for the feedback response is difficult, because they can be assimilated and converted into different amino acids. By using chemical inhibitors to block the conversion of amino acids into other forms, glutamine has been identified as an important regulator (Vidmar et al., 2000). Nitrate transporters are also diurnally regulated, undergoing marked changes in transcript levels and corresponding NO_3^- influx during day/night cycles, with high expression at the end of the light period (e.g., Ono et al., 2000). Sucrose supply in the dark rapidly increases the transcript levels (Lejay et al., 1999), and the diurnal increases in

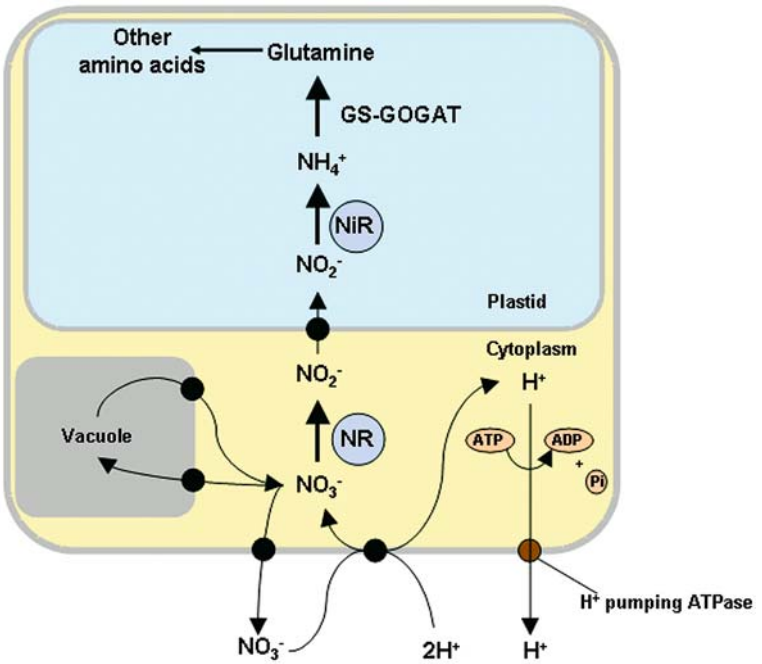

Figure 4. Schematic diagram of NO_3^- uptake and assimilation by plant cells. Key: nitrate reductase, NR; nitrite reductase, NiR; glutamine synthetase, GS; glutamate-2-oxoglutarate aminotransferase, GOGAT (redrawn from Crawford et al., 2000).

expression of root NO_3^-, NH_4^+ and SO_4^{2-} transporters seem to be linked to the changes in sucrose supply to the root which results from photosynthesis during the day (Lejay et al., 2003). These observations indicate the close co-ordination that exists between NO_3^- uptake and C metabolism.

The roles of both *NRT1* and *NRT2* genes in the uptake of NO_3^- from the soil have been demonstrated using mutant plants. A mutant *Arabidopsis* plant deficient in the expression of a *NRT1* gene led to the identification of the first member of this family, although, the original selection of the plant was made using chlorate which is a toxic analogue of NO_3^- (Tsay et al., 1993). Even stronger evidence is available for the *NRT2* family, where double mutant knock-outs of *NRT2* genes in *Arabidopsis* have demonstrated a clear role for these genes in the uptake of NO_3^- from the soil (Filleur et al., 2001). These mutants are deficient in both *AtNRT2.1* and *AtNRT2.2,* and they have lost almost all the NO_3^--inducible HATS, while LATS activity was not altered. Split-root experiments also showed that the double mutant has lost the ability to up-regulate uptake in one part of the root to compensate for N-starvation in another part of the root (Cerezo et al., 2001). In addition, the supply of NH_4^+ to the NO_3^--containing nutrient solution usually inhibits NO_3^- uptake in the wild-type, but this does not occur in the mutant (Cerezo et al., 2001). These elegant experiments illustrate the powerful use of gene 'knock-out' technology to identify the role of specific transporter genes in N uptake by roots. These results are also important for confirming the function of these genes as NO_3^- transporters, because almost all of the *in planta* expression studies have assumed function on the basis of sequence homology. Sequence similarities may be misleading, especially when a single protein can transport more than one type of ion or molecule, as is the case for both *NRT1* and *NRT2* transporter families. For example, some members of the *NRT1* family can transport amino acids and peptides, and both families can transport NO_2^- when the proteins have been expressed in foreign cells (Miller and Zhou, 2000; Zhou et al., 1998).

Efflux systems have been studied less than influx systems; however, it is known that efflux is protein-mediated, passive, saturable and selective for NO_3^- (Aslam et al., 1996; Grouzis et al., 1997). Anion channels seem the most obvious route for NO_3^- efflux, because the transport is thermodynamically downhill and genome analysis has identified several gene families that may fulfil this function. The NO_3^--efflux system is under a degree of regulation, induced by

NO_3^- (Aslam et al., 1996), and it is also proportional to whole-tissue NO_3^- concentrations (Teyker et al., 1988). We can therefore predict that the anion channel(s) responsible for NO_3^- efflux must be NO_3^- -inducible. Net NO_3^- uptake is regulated by whole-plant demand via shoot-derived signals transported in the phloem to the roots (Imsande and Touraine, 1994; Vidmar et al., 2000). The nature of these feedback signals seems to be amino acid concentrations in the phloem, specifically glutamine (Pal'ove-Balang and Mistrik, 2002; Tillard et al., 1998). Efflux of NO_3^- has been found to be associated with slow growth rates (Nagel and Lambers, 2002). This efflux is, however, a consequence rather than a cause of slow growth. Slow-growing plants from nutrient-poor habitats may simply not be able to exploit high concentrations of NO_3^-, which is then effluxed.

Ammonium transporters

Many plant NH_4^+-transporter (*AMT*) genes have been identified and their function has been confirmed by their ability to complement a yeast mutant deficient in normal NH_4^+ uptake (Ninnemann et al., 1994; von Wirén et al., 2000a). In *Arabidopsis* there are 6 *AMT* genes, while rice (*Oryza sativa*) has 10, and more detailed sequence comparisons have identified two distinct groups within the *AMT* family, denoted *AMT1* and *AMT2* (Shelden et al., 2001; Sohlenkamp et al., 2000). Like the NO_3^- transporters, some *AMT1*-type genes are expressed in root hairs, suggesting that they have a role in uptake of NH_4^+ from the soil (Lauter et al., 1996; Ludewig et al., 2002). Three *AMT1* genes show diurnal changes in expression in roots (Gazzarrini et al., 1999), and the changes in expression during the light period likely result from increases in sucrose availability from photosynthesis during the day (Lejay et al., 2003). More detailed information has been published about the *AMT1*- than about *AMT2*-type transporters, and a correlation between transcript (mRNA) level and NH_4^+ influx has been observed (Kumar et al., 2003), but the role of neither group in uptake from the soil has been clearly established. Although, *Arabidopsis* plants deficient in one of the root-expressed *AMT1* genes showed altered leaf morphology and a 30% decrease in NH_4^+ influx, there were no effects on growth when compared with wild-type plants in a range of conditions (Kaiser et al., 2002). Based on these observations it was suggested that redundancy within the *AMT*-family may compensate for the loss of this transporter. Similarly, inhibiting the mRNA transcript level of the single *AMT2* in *Arabidopsis* failed to significantly alter growth of the plant, although the actual uptake of NH_4^+ was not measured (Sohlenkamp et al., 2002). One of the *AMT2* transporters is constitutively expressed in the plasma membrane of most tissues including the nodules of a N_2-fixing species, suggesting that it may have a general role in the recovery of NH_4^+ effluxed from all tissues, not only the nodule (Simon-Rosin et al., 2003). Some *AMTs* are constitutively expressed (Suenaga et al., 2003), but for most the expression depends on the availability of NH_4^+ (von Wirén et al., 2000b). The expression of one tomato (*Lycopersicon esculentum*) *AMT1* gene was induced by the presence of N_2-fixing bacteria in the rhizosphere (Becker et al., 2002). In species like paddy rice that chiefly make use of NH_4^+ as a soil N source more of the *AMT1* genes show NH_4^+-induced expression when compared with *Arabidopsis* and tomato that chiefly use NO_3^- as an N source (Sonoda et al., 2003). However, in contrast to most situations for NO_3^-, the expression of some *AMTs* is repressed by the presence of NH_4^+, with the mRNA increasing when less NH_4^+ is available. As described for the NO_3^- transporters (Nazoa et al., 2003; Vidmar et al., 2000), the expression of an *AMT1* gene and NH_4^+ influx were suppressed when plants were supplied with glutamine, suggesting feedback regulation from downstream N metabolites (Rawat et al., 1999).

As for NO_3^-, NH_4^+ transport in plant cells can also be demonstrated by electrophysiology (Ayling, 1993; Wang et al., 1994). Electrophysiology can be used to determine the NH_4^+-transporter kinetics which suggested that NH_4^+ entry into cells may be mediated by cotransport with protons (Ayling, 1993; Wang et al., 1994). However, the energy requirements for uptake of a cation (e.g., NH_4^+) compared to an anion (e.g., NO_3^-) are different. The uptake of NH_4^+, like the uptake of K^+, could be through a channel, and chiefly driven by the negative membrane potential of the plant cell. Several examples of K^+ channels expressed in the root epidermis have been identified (e.g., Downey et al., 2000; Hartje et al., 2000) and gene knock-out studies could identify whether these have a role in NH_4^+ uptake. There is evidence from patch-clamp studies that NH_4^+ ions can enter cells through K^+ channels (White, 1996), and it may be that this is an important route for the entry of NH_4^+ into root cells. This topic is worth investigation using plants that have disrupted plasma-membrane K^+-channel activity, especially given the

lack of direct evidence for the role of *AMTs* in NH_4^+ uptake by root cells. More detailed functional analysis of the *AMT* genes, using heterologous expression, suggests that they may have a channel-type structure that can be composed of several different multiples of *AMT* protein units (Ludewig et al., 2003). The functional activity of the whole protein complex may be modified by altering the *AMT* component units. Electroneutral uptake of N as ammonia (NH_3) may occur by entry through membrane channels and aquaporins may provide a molecular route for this transport (Niemietz and Tyerman, 2000; Howitt and Udvardi, 2000). Aquaporins may also provide a route for efflux across the plasma membrane and for accumulation in the vacuole. The relatively alkaline pH of the cytosol will favour NH_3 flux both into the vacuole and into the apoplast.

The energetic requirements for pumping NH_4^+ out of cells has been identified as a possible cause for the toxic effect of the ion on some types of plants (Britto et al., 2001a, see below). The gene(s) responsible for this NH_4^+ efflux process have not yet been identified, but the thermodynamic mechanism for such a process requires an ATPase or an anti-port somehow exchanging H^+ and NH_4^+. It is not clear why K^+ entry and cytosolic concentration should be regulated while those of NH_4^+ are poorly regulated, but like Na^+ entry during salt stress, perhaps the plant cannot avoid this problem when exposed to high concentrations of these cations. Therefore accurate measurements of the soil concentrations of NH_4^+ may be important for answering these questions for plants growing in soil. The toxic effects of NH_4^+ depend on there being high external concentrations of the cation, perhaps greater than 20 mM (Britto and Kronzucker, 2002). As mentioned above, since the cytosolic pH is usually more alkaline than that of the vacuole and the apoplast, the chemical gradient for NH_3 favours passive exit of this molecule from the compartment. The plant *AMT* gene family function as high-affinity NH_4^+-uptake systems when they are expressed in yeast (von Wirén et al., 2000a). The requirement for an active efflux mechanism at high external NH_4^+ concentrations does not easily fit with the constitutive expression of some of these genes, so more expression analysis is needed to clarify this point.

N fluxes along the length of roots

Net uptake of NO_3^- and NH_4^+ along roots has been mapped using ^{15}N labelling of root segments (Lazof et al., 1992) and ion-selective microelectrode techniques (Henriksen et al., 1990; Taylor and Bloom, 1998). These measurements generally show that the site of most NO_3^- and NH_4^+ uptake is just behind the root meristem. In maize, NO_3^- elicited net H^+ uptake only at the root tip (0–1 mm), but H^+ extrusion in all regions (Taylor and Bloom, 1998). This correlates with symport of H^+:NO_3^- into the root tip. Rapid NO_3^- net uptake was found between 0 and 40 mm behind the root tip, decreasing between 40 and 60 mm. Ammonium-elicited H^+ extrusion was detected in all regions, except for the region 6 to 11 mm from the apex (Taylor and Bloom, 1998). In the region 11 mm from the apex there is hardly any elongation in maize primary roots (Sharp et al., 1988); it is possible that H^+ extrusion is already maximal, that NH_4^+ is stored rather than assimilated, or that NH_4^+ is translocated away from this region. Net uptake of NH_4^+ increased steadily with distance behind the root tip (measured up to 60 mm). When both NH_4^+ and NO_3^- were supplied, NO_3^- net uptake was suppressed at all locations along the root (Colmer and Bloom, 1998; Taylor and Bloom, 1998). Although there is a peak of N uptake just behind the root tip, it is sometimes overlooked that this represents only a 2- to 3-fold increase over that found in the older parts of the root further from the apex. Transporter gene expression studies suggest that mature parts of the root are also significant sites of uptake (Nazoa et al., 2003).

Organic N uptake

Gene families have been identified that are responsible for transporting amino acids (reviewed in Ortiz-Lopez et al., 2000), urea (Liu et al., 2003), oligopeptides (Koh et al., 2002; Steiner et al., 1994), purines (Gillissen et al., 2000), nucleosides (Li et al., 2003) and N-containing heterocyclic compounds (Desimone et al., 2002), but their role in uptake from the soil is still uncertain. This oligopeptide transporter (*OPT*) family is not related to the NTR1 (PTR) family described previously, but both that are able to transport peptides.

Arabidopsis has a large family of at least 46 putative amino acid/auxin transporters which can be sub-divided into some smaller groups based on sequence comparisons, but the functions of the family members are not well characterised. There is a smaller group of 9 related general amino-acid transporters, and some others specifically for auxin and amino acids such as lysine, histidine and proline. An amino-acid transporter, possibly for both histidine and proline, from *Mesembryanthemum crystallinum* is specifically

expressed in the roots, and most strongly expressed in the mature tissue (Popova et al., 2003). After salt treatment the expression pattern changed, with stronger expression in the root vascular system (Popova et al., 2003). Mycorrhizal associations with roots improve the N nutrition of many ericaceous plants, and this interaction has been shown to influence the function of amino-acid transporters in the plant (Sokolovski et al., 2002). The presence of a VA mycorrhizal fungus on the surface of barley (*Hordeum vulgare*) was also found to lead to changes in the expression of a H^+-ATPase in the plant root tissue (Murphy et al., 1997). Although this result was obtained for only a sub-unit of the H^+-ATPase, increased capacity of the pump might be needed to maintain the H^+ gradient for uptake of N through cotransport systems.

A family of 15 proton-cotransporters for purines and their derivatives has been identified (Gillissen et al., 2000), and these can have a high affinity (μM range) for these substrates, and are also able to transport cytokinins. The properties of this family would suggest that they could have a role in obtaining these substrates from the rhizosphere. In common with the auxin transporters described previously, they may be particularly important for root interactions with plant growth-promoting bacteria that can locally release these molecules and influence root development (Vessey, 2003). Similarly the family of high-affinity H^+-cotransporters for N-containing heterocyclic compounds (Desimone et al., 2002), such as uric acid, xanthine and allantoin may have a function in retrieving these molecules from the soil. In *Arabidopsis* there are 5 family members with at least one member having K_m values for these substrates in the μM range. A role for any of the 9 *Arabidopsis* oligo-peptide transporters in uptake from the soil has also not been demonstrated. Antisense to a peptide transporter that is usually expressed in the whole plant did not result in any changes in root transcript levels, and so the resulting significant phenotype was only explained by effects on peptide transport in the shoot (Song et al., 1997). There is clearly much scope for detailed studies using gene knock-out mutants to identify the role of these other forms of N in plant nutrition. Many of various different N transporters are likely to be involved in N transport within cells (plasma membrane and tonoplast) and inside the plant. As soil N is usually available as NO_3^-, and to a lesser extent as NH_4^+, some transporters are likely to only be important in environmentally extreme conditions or when N is in very short supply. One important exception might be the urea transporters, because this form of N is now a common form of fertiliser (Figure 3). As urea is rapidly broken down in the soil the direct uptake of this form of N from the soil is probably of minor importance, but for direct foliar applications of this fertiliser these transporters may be very important.

N sensors in the membrane

The induction or repression of transporter-gene expression requires that there is (are) some N-sensing system(s) within the cell, perhaps in the nucleus or at the cell surface. Membrane-associated proteins have been identified as possible sensors of soil N availability (Redinbaugh and Campbell, 1991; Forde and Clarkson, 1999). They provide a sensor at the root/soil interface that may be involved in sensing flux through the transporter protein and/or availability of particular forms of N at the cell surface. These sensors may have a role in regulating cellular N pools and/or detecting available pools of N both inside cells and in the soil around the root. Homeostasis of cytosolic NO_3^- pools requires some sensors to regulate these concentrations in this compartment (Miller and Smith, 1996; Van der Leij et al., 1998). There are fungal examples of 'transporter' proteins that seem to have this role for NH_4^+ and sugar sensing (Lorenz and Heitmann, 1998; Ozcan et al., 1998), but the situation in plants is less clear (Barth et al., 2003). The large numbers of particular types of some transporters (e.g., peptide transporters in the *PTR* family) may be ascribed to gene redundancy, but this may also be because some family members function as sensors. A family of membrane proteins that are related to known glutamate receptors have been identified in plants (Lam et al., 1998), and mutant plants with altered expression of the genes indicate they have a role in regulating C/N metabolism (Kang and Turano, 2003). A plant homologue of the bacterial N sensor PII has been identified, but this is not a membrane protein and it may actually be involved in sensing cytosolic energy and carbon status (Smith et al., 2003).

Xylem loading of N

The entry of NO_3^- into the xylem is likely mediated by anion channels (Kohler and Raschke, 2000) and these channels have been characterised for barley roots (Kohler et al., 2002), but their molecular identity has not yet been determined. Nitrate in the xylem exerts positive feedback on its loading into the xylem through a change in the voltage dependence of the channel.

Interestingly this effect was specific for NO_3^-, and was not found for Cl^-. By transport through this channel, NO_3^- efflux into the xylem can be maintained with high NO_3^- concentrations in the xylem sap; a situation that can occur during the night. There is a clear diurnal change in xylem sap concentrations (Siebrecht et al., 2003), related to changes in the transpiration rate. Concentrations of NO_3^- in the xylem sap can be quite high, especially in plants that transport most of the NO_3^- taken up to the shoot for reduction (e.g., maize 10.5 mM, Oaks, 1986; *Ricinus communis* 10 to 15 mM, Schobert and Komor, 1990; barley 27 to 34 mM NO_3^-, Lewis et al., 1982).

There has been some debate as to whether NH_4^+ is translocated in the xylem. Relatively low concentrations of NH_4^+ have been measured in the xylem (e.g., 0.4 and 2.6 mM in wheat and maize, respectively, supplied with 4 mM NH_4^+; Cramer and Lewis, 1993). Schjoerring et al. (2002) have re-evaluated the role of NH_4^+ transport in the xylem, and have shown that NH_4^+ translocation in the xylem does indeed occur. Using carefully checked methods, these authors found that NH_4^+ concentrations in the xylem sap of *Brassica napus* were < 1 mM when plants were supplied with 3 to 10 mM NO_3^-, and were 1 and 5 mM when plants were supplied with 3 and 10 mM NH_4^+, respectively. However, the latter represented less than 11% of the N in the xylem sap. The mechanism for loading of NH_4^+ into xylem has not been identified. This may, however, occur through transporters more usually used for K^+.

Amino acids are transported within the plant through both the xylem and phloem, and these two transport systems exchange contents to some extent *en route*. Promoter–GUS fusion analysis has revealed that the amino-acid transporter AtAAP2, which is a low-affinity transporter of neutral and acidic amino acids, is expressed in the vascular tissue, suggesting that this transporter may be responsible for xylem-to-phloem transfer (Fischer et al., 1998). The unloading/loading of organic N, results in extensive N cycling; in wheat the proportion of N cycling represented 18% of the total N in the plant (Simpson et al., 1982; Lambers et al., 1982). This has led to the hypothesis that there is only a single amino-N pool in both shoot and roots, and that it is this combined pool that regulates N uptake (Cooper and Clarkson, 1989). The entry of amino acids into the xylem could be mediated by a selective pore in the plasma membrane of xylem parenchyma cells, like that found in the chloroplast envelope (Pohlmeyer et al., 1997).

The major amino-acid components in the phloem and xylem include the amides, glutamine and asparagine, and the acidic amino acids, glutamate and aspartate (Hocking et al., 1984; Ta and Joy, 1984). The concentrations of total amino-N in the xylem are typically between 2 and 20 mM for wheat and maize (e.g., Cramer and Lewis, 1993). In plants that assimilate some NO_3^- in the root, or are supplied with NH_4^+ that is assimilated in the root, amino compounds are an important constituent of xylem sap. Ammonium nutrition compared with NO_3^- nutrition, enhanced xylem amino compound contents by 300% in maize (Murphy and Lewis, 1987) and 500% in barley (Lewis et al., 1982). The amides, with low C:N ratios, are the major xylem carriers of organic N. In barley fed NO_3^-, glutamine was the predominant amino compound, and its concentration was increased 3-fold by NH_4^+ nutrition (Lewis et al., 1982). In maize plants fed with NO_3^-, glutamine is the predominant amino compound in the xylem sap, whereas in NH_4^+-fed plants, asparagine levels exceed those of glutamine (Murphy and Lewis, 1987). This may reflect dependence on the phosphoenolpyruvate carboxylase (PEPc) reaction for C for assimilation of NH_4^+ into asparagine (Cramer et al., 1993), in which PEPc functions 'anaplerotically' to replace C drawn from the TCA cycle for amino acid synthesis (see below).

Future research for N transporters

Although the genes and their families have been identified the role of each in N nutrition has still to be determined. The transport function of relatively few proteins has been characterised in any detail, but where this has been done this was usually achieved by expressing the protein in yeast cells. This expression system has a disadvantage, which compromises its applicability to interpreting the likely functionality of the transporter in plant cells. The electrical energy across the yeast cell plasma membrane cannot easily be measured. However, expression of transporters in *Xenopus* oocytes allows the contribution of the membrane voltage to transport to be measured. These measurements have shown that both the affinity of a protein for the transported substrate and the electrical energy driving transport can be very sensitive to the membrane voltage (Miller and Zhou, 2000). These measurements suggest that accurate recordings of the resting membrane potential of roots cells in the soil and the factors that can change this cellular parameter are important for understanding nutrient uptake.

Using the relative numbers of each type of N transporter as an indicator of the relative importance of each type of soil N source may be misleading. More information is required to predict which of these transporters have roles in uptake of N from the soil. For a transporter to be assigned a role in nutrient uptake, it must be expressed in the plasma membrane of cells that are in contact with the soil solution. Part of the complexity, resulting in large numbers of N transporters being identified, is that some of the transporters are expressed at different stages of development, and that some of them are targeted to endomembranes, such as the tonoplast.

Biological N_2 fixation

Biological N_2 fixation is carried out by both free-living and endosymbiotic prokaryotes. This conversion of atmospheric N_2 gas to NH_4^+ is extremely important for both natural and crop systems (Vance, 2002; Vessey et al., 2004). The best characterised symbiosis is that between legumes and nitrogen-fixing endosymbiotic bacteria from the genera *Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium, Allorhizobium* and *Azorhizobium*, collectively termed rhizobia (Graham and Vance, 2000). Other organisms and symbioses which also contribute to N_2 fixation include actinorhizal symbioses (e.g., between *Casuarina* and *Frankia*), associative relationships (e.g., *Saccharum officinarum* with *Acetobacter*) (Graham and Vance, 2000) and cyanobionts in corraloid roots of cycads (Costa et al., 1999). The importance of symbiotic N_2 fixation for sustainable agricultural systems cannot be underestimated, and great potential exists for increasing the usefulness of leguminous crops through breeding for greater usefulness and palatability of legumes and through use of legumes as 'green' fertilisers. Furthermore, the association of free-living diazotrophic bacteria with plants could possibly also contribute to the N economy of a crop. This would be especially important in organic agriculture and for small-scale farmers who cannot afford the extensive application of fertilisers. There is a considerable amount of literature claiming significant contributions of associative endosymbionts to N budgets of various plants (e.g., Baldani et al., 2002; Döbereiner et al., 1972; James, 1999). While it is true that many heterotrophic bacteria in the soil are capable of fixing N_2, controversy exists about the significance of the contribution of endosymbionts to the N budgets of plants. It is attractive to explain the positive effect of plant growth-promoting bacteria on plant growth as being a consequence of N_2 fixation. However, there is little evidence for a significant contribution of plant growth-promoting bacteria to the N balances of plants (Mantelin and Touraine, 2004). In a review, Giller and Merckx (2003) assert that the contributions of associative endosymbionts to N budgets of grasslands and pastures are of only marginal significance.

Mycorrhizal N acquisition

Although the main benefit that plants derive from mycorrhizal associations is enhanced P interception, ectomycorrhizas and ericoid mycorrhizas also contribute to plant N nutrition (Chalot and Brun, 1998; Graham and Miller, 2005). This is through access to organic N that is not directly accessible to roots, due to slow diffusion and due to the requirement for degradation of polymeric forms of N. Ectomycorrhizas are capable of taking up organic N and of increasing the uptake of NH_4^+ via extensive growth of soil mycelia and circumvention of NH_4^+ depletion zones (Buscot et al., 2000). The ability of ectomycorrhizal fungi to take up NH_4^+ and transport N-containing solutes to their host plant is well established (Buscot et al., 2000). Evidence for direct, albeit inefficient, uptake of ^{15}N-^{13}C-labelled glycine by plants associated with ectomycorrhizal, ericoid or arbuscular mycorrhizal fungi in field-grown plants exists (Näsholm et al., 1998), although, the mycorrhizal partner may simply have facilitated greater mineralisation. The contribution of arbuscular mycorrhiza (AM) to plant N acquisition is indeed controversial (Hodge et al., 2000a). With complex organic substrates (labelled plant shoots) there was no direct uptake of intact organic N, although N uptake of mineralised organic material occurred (Hodge et al., 2000b). Thus AM may improve the competitive ability of roots with soil microorganisms for mineralised N and increase decomposition of organic N (Hodge, 2003). However, evidence for effective competitive ability of AM with soil microorganisms is also equivocal (Hodge, 2001). Thus the role of AM fungi in acquiring N is questionable, although they may facilitate mineralisation of organic N. The ability of ecto- or ericoid mycorrhizas to access organic N may arise from the evolution of these associations in N-poor environments.

Assimilation of N

Inorganic N

Nitrate is reduced and incorporated into cells by a series of assimilatory enzymes (illustrated in Figure 4; reviewed by Crawford et al., 2000). Nitrate ions are initially reduced to NO_2^- ions, via the enzyme **N**itrate **R**eductase (**NR**), further reduction to NH_4^+ occurs via the enzyme **N**itr**i**te **R**eductase (**NiR**). Ammonium is then added to C skeletons to produce a variety of amino acids via the GS/GOGAT cycle (the enzymes glutamine synthetase (GS) and glutamate synthase or glutamate-2-oxoglutarate amino-transferase (GOGAT). The activity of these N-assimilatory enzymes, like the transporters, can be regulated at a number of different levels; by the synthesis of both mRNA (transcription) and protein (translation) and activity of the enzyme (post-translation).

Nitrate reductase (NR)

NR is a complex, cytosolic enzyme made up of two identical subunits catalysing the transfer of two electrons (reduction) from NAD(P)H to a NO_3^- ion via several redox centres composed of three prosthetic groups: flavin adenine dinucleotide (FAD), heme (cytochrome 557) and a molybdenum-pterin cofactor (MoCo) (Solomonson and Barber, 1990). There are three main forms of NR in plants that are defined by their electron donor (reductant) source, either NADH, NADPH, or both; the most common form and that found in *Arabidopsis* is NADH-specific NR (Wilkinson and Crawford, 1993). Roots contain both the NADH and NADPH isoforms of NR. The activity of the NADPH isoform amounts to about 20% of that of the NADH-dependent NR in barley (Botrel and Kaiser, 1997). Stöhr and Ullrich (2002) have also described a form of plasma-membrane bound NR that can generate nitric oxide (NO), but the significance of this activity remains uncertain. Nitric oxide is a short-lived highly reactive molecule that is involved in responses to plant pathogens (Delledonne et al., 1998). NO can be detected in the soil (Gut et al., 1999) and may be part of a general signal response. There is also at least one other route for generating NO in plants. Guo et al. (2003) reported the existence of an arginine- and NADP-dependent nitric oxide synthase in *Arabidopsis* which is sensitive to Ca^{2+} modulation. The phenotype of *Arabidopsis Atnos1* mutants with impaired NO production could be restored by treatment with NO donors, and by expression of the *AtNOS1* gene, showing that this, rather than NR, is a crucial enzyme in NO synthesis for normal development.

Nitrite reductase (NiR)

NiR is a nuclear-encoded enzyme that is transported into the stroma of chloroplasts in green tissue, and into the plastids of roots, leaving behind a transit sequence of 30 amino acid (Wray, 1989). The enzyme has two redox centres, a siroheme and an iron-sulphur centre, and catalyses the transfer of 6 electrons from reduced ferredoxin (Fd) or a ferredoxin-like electron carrier to NO_2^-. In the chloroplast the reductant for NiR is Fd_{rd} derived from the light reaction, but in roots a non-haeme-iron-containing protein, similar to Fd, has been identified in maize which is thought to be the *in vivo* reductant for NO_2^- and functions with a pyridine nucleotide reductase, similar to Fd-NADP reductase from spinach (*Spinacea oleracea*) leaves (Suzuki et al., 1985). The NiR enzyme is inducible in the presence of both NO_3^- and NO_2^-, although the former is more effective (Barneix et al., 1984).

GS/GOGAT and GDH

Glutamine synthetase (GS) catalyses the ATP-dependent amination (adding -NH_2) of glutamate to produce glutamine, and glutamine:oxoglutarate aminotransferase (GOGAT) catalyses the transfer of an amide group from glutamine to 2-oxoglutarate to produce 2 molecules of glutamate. GS is one of several enzymes that can combine NH_4^+ with C-molecules; some other examples are asparagine synthase (AS) and glutamate dehydrogenase or GDH (reviewed by Brugière et al., 2001). Ammonium assimilation in higher plants was long thought to begin with the synthesis of glutamate by GDH, but the high K_m for NH_4^+ (ca. 5.8 mM) of GDH makes it unlikely that this enzyme could function *in vivo* for the assimilation of NH_4^+ (reviewed by Miflin and Habash, 2002). It is now believed that the major pathway for NH_4^+ assimilation is the GS-GOGAT pathway, and that GDH generally acts in a deaminating direction. However, a role in NH_4^+ detoxification would explain the increase in GDH expression under conditions that provoke high tissue NH_4^+ levels (Lancien et al., 2000).

In roots GS has two forms, one found in plastids (GS_2), the other in the cytosol (GS_1). In roots only the cytosolic forms are usually detected, but there are a few reports of the plastidic form (Brugière et al., 2001). In roots, GS_1 assimilates NH_4^+ derived directly from the soil or the reduction products of NO_3^- (Ireland and Lea, 1999). Within the GS1 gene family there

are several genes, some of which are specifically expressed within the root (Gebhardt et al., 1986). There are two types of GOGAT that can use either NADPH or reduced Fd as the electron donor; both are usually located in plastids. In roots, especially root tips, Fd-GOGAT is the major form present (Brugière et al., 2001). These two forms of the enzyme vary as tissues develop, but Fd-GOGAT is usually located in roots and is also located within plastids (Suzuki and Gadal, 1984). Two Fd-GOGAT genes, *GLU1* and *GLU2*, have been identified in *Arabidopsis* (Coschigano et al., 1998). *GLU1* expression is low in root tissues, and most abundant in leaves, while *GLU2* is constitutively expressed at low levels in leaves, and at higher levels in roots. NADH-GOGAT activity is 2- to 25-fold lower than that of Fd-GOGAT, being found mainly in non-photosynthetic tissues like nodules and roots (Ireland and Lea, 1999) where it is induced by NH_4^+ (Hirose et al., 1997) and by NO_3^- (Wang et al., 2000b). Thus a role for NADH-GOGAT has been suggested in primary N assimilation in roots and nodules.

Regulation of inorganic N assimilation

The regulation of N assimilation has been extensively studied, and reviewed; most of the work on this topic has focussed on green tissues (Comparot et al., 2003; Kaiser et al., 1999; Meyer and Stitt, 2001). Most information is available for NO_3^- assimilation and the regulation of the NR activity. NO_3^- is the primary signal, although other signals also influence the regulation of NO_3^- assimilation, e.g., light, sucrose, circadian rhythms, and the end-products of assimilation (Rothstein and Sivasankar, 1999). An outline of the regulation of the initial steps of N assimilation is given below, with special attention to these processes in the root.

Regulation of NR

Changes in root NR activity (NRA) may result from changes in gene expression or post-translational modifications to the protein. Like some of the N transporters, NR is induced by its own substrate, NO_3^-, and this induction is fast, occurring within minutes, and requires very low concentrations ($<10\ \mu$M) (Crawford, 1995; Sueyoshi et al., 1995). In leaves, light is required for optimal expression of NR and photosynthetic CO_2 fixation and sucrose synthesis (Cheng et al., 1992). NR transcript levels show diurnal variation, increasing during the night to a maximum in the early morning (Bowsher et al., 1991; Deng et al., 1991; Galangau et al., 1988). Again like the transporters, downstream N assimilation products such as amino acids (e.g., glutamine), together with C products from photosynthesis can feed back to regulate amounts of NR mRNA (Deng et al., 1991; Sivasanker et al., 1997; Vincentz et al., 1993). The diurnal changes are lost, and transcripts remain consistently high in mutants without functional NR (Cheng et al., 1989; Pouteau et al., 1989). The picture is further complicated by the differential expression of the two NR genes in *Arabidopsis* (Cheng et al., 1991; Yu et al., 1998), and the situation in roots has been studied much less.

The NR protein is relatively short-lived in cells (Li and Oaks, 1993), but tissue-extractable NRA does not match either amounts of NR protein or the rate of NO_3^- reduction *in vivo*, indicating that other regulatory mechanisms modify NRA (Lillo, 1994). NRA responds rapidly and reversibly to changing environmental conditions, such as light/dark transitions, CO_2 removal and anoxia (Glaab and Kaiser, 1993; Kaiser and Brendle-Behnisch, 1991; Kaiser and Förster, 1989). Rapid post-transcriptional modulation of NR was thought to prevent the accumulation of toxic NO_2^- (Kaiser and Huber, 1994), but it may also help regulate cellular pools of reductant (Kaiser et al., 2000). Activity of the NR protein is controlled by the reversible phosphorylation of the protein, and so the inactive phosphorylated state might be limited by ATP supply. Therefore post-translational activation of NR has been proposed as a beneficial effect for plants under anoxia; in anoxic roots NR activity may be important for recycling of reductant produced in glycolysis (Botrel and Kaiser, 1997). However, although the anoxic roots of NR-deficient tobacco mutants did show less cytosolic acidification when compared with wild-type plants, they showed no evidence of any limitation in recycling of reductant (Stoimenova et al., 2003). Phosphorylation also requires the presence of divalent cations (usually Mg^{2+}) and additionally a 'NR-inhibitor protein' (14-3-3 protein) is needed to inactivate the protein (reviewed by MacKintosh and Meek, 2001). The inhibitor protein was identified as a dimer of '14-3-3' proteins (Bachmann et al., 1996; Moorhead et al., 1996). 14-3-3 proteins were first known as abundant brain proteins, and have since been identified as a highly conserved protein family involved in many signalling systems in plant, fungal and mammalian cells (Sehnke et al., 2002).

Leaf extracts contain several protein kinases that can phosphorylate NR, and in spinach leaves there

are at least three kinases (PK_I, -II and -III) of differing dependence on Ca^{2+} (Douglas et al., 1997). There is evidence that PK_I and -III are also modified by phosphorylation (Douglas et al., 1997), indicating the considerable complexity of cascades mediating the regulation of NO_3^- assimilation in response to environmental stimuli. The extent to which these three kinases contribute to NR phosphorylation *in vivo* is unknown, and there is no information on the activity of these kinases in root tissues. NR is dephosphorylated by a type 2A protein phosphatase that is rapidly up-regulated on illumination of the leaf (MacKintosh, 1992). Type 2A protein phosphatases are involved in a range of signalling pathways in plants as well as animals (Smith and Walker, 1996). NR is also activated/dephosphorylated by a Mg^{2+}-dependent phosphatase *in vitro*, but the activity of this enzyme is much lower than that of the type 2A protein phosphatase (Kaiser et al., 1999). Once again these results were obtained using green tissues, and they have not been demonstrated in roots. Figure 5 shows a schematic summary of the activation/inactivation of NR by phosphorylation and 14-3-3 proteins.

Regulation of NiR

Overall, NiR and NR are similarly transcriptionally regulated. One *NiR* gene has been identified in *Arabidopsis* (Tanaka et al., 1994). The *Arabidopsis NiR* gene is strongly induced by NO_3^-, and is actually one of the most responsive genes to NO_3^-, probably to prevent the accumulation of toxic NO_2^- (Wang et al., 2000b). NiR induction in response to light is mediated by phytochrome, but the degree of dependence on NO_3^- and light for NiR induction varies considerably amongst species (Neininger et al., 1992; Seith et al., 1994). NiR mRNA levels are determined by different factors, solely by phytochrome in mustard (*Sinapis alba*) (Schuster and Mohr, 1990), by only NO_3^- in spinach (Seith et al., 1991) and by both in tobacco together with another specific plastidic factor (Neininger et al., 1992). In roots of the legume *Lotus japonicus* NiR mRNA levels were constant throughout the day and night (Orea et al., 2001). Soybean roots appear to have two types of NiR, one constitutively expressed, and the other induced by NO_3^- and light (Kim et al., 2001). NiR induction is also inhibited by the amino acids glutamine and asparagine (Sivasankar et al., 1997; Vincentz et al., 1993). The effect of carbohydrates on the induction of NiR differs between species; in maize sucrose enhances induction (Sivasankar et al., 1997), while in tobacco induction is unresponsive to glucose (Vincentz et al., 1993). These expression studies have mostly used leaf material.

NiR enzyme level and *NiR* gene expression correlate well in barley (Seith et al., 1994), whereas in spinach (Seith et al., 1991), mustard (Schuster and Mohr, 1990) and tobacco (Neininger et al., 1992) no quantitative relationship has been established. This suggests that NiR in spinach, mustard and tobacco is regulated at the protein level. NiR is also believed to be under post-transcriptional control. Plants grown on an NH_4^+-containing medium and constitutively expressing NiR show strongly reduced protein levels and activities compared with those grown on NO_3^- containing medium (Crété et al., 1997). The post-transcriptional control of NiR is different from that of NR. The reduction of NiR activity is due to a drop in the amount of NiR protein, and not protein inactivation of NiR. Post-transcriptional regulatory mechanism(s) remain(s) to be determined. It has been suggested that NiR translation or incorporation into the chloroplast could be the steps subject to post-transcriptional control (Crété et al., 1997).

Regulation of GS/GOGAT

GS is a multi-gene product under complex transcriptional control as a consequence of the many genes and promoters involved (Miflin and Habash, 2002). The cytosolic form of GS in the leaf is also post-translationally regulated by phosphorylation and interaction with a 14-3-3 protein (Finnemann and Schjoerring, 2000). The phosphorylation status of GS changed during light/dark transitions in senescing leaves. This mechanism of regulation is a common feature of key enzymes involved in N and C assimilation, and may be a factor in determining the lifetime of these proteins.

In rice roots NADH-GOGAT mRNA and protein accumulated within hours of supply of NH_4^+ (Hirose et al., 1997). Glutamine or its downstream metabolites, but not NH_4^+ itself, could be a signal substance for the accumulation of NADH-GOGAT mRNA in the roots (Hirose et al., 1997). Inhibition of protein phosphatases by okadaic acid results in accumulation of NADH-GOGAT mRNA, and indicates that phosphorylation is involved in the regulation of NADH-GOGAT gene expression; phosphorylation probably plays a role in the signal transduction pathway downstream from NH_4^+ (Hirose and Yamaya, 1999). In contrast, a protein kinase inhibitor inhibited the accumulation of NADH-GOGAT mRNA induced by both NH_4^+ and okadaic acid. Thus glutamine or its metabolites might stimulate the transcription of the NADH-

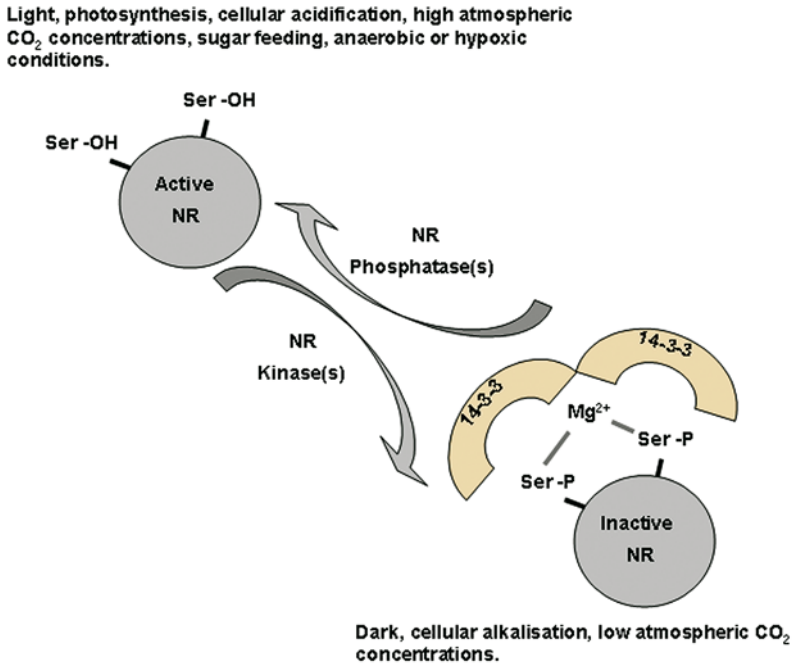

Figure 5. Schematic diagram of the environmental post-transcriptional regulation of nitrate reductase. For simplification the phosphorylation/dephosphorylation and the 14-3-3 binding steps have been shown together, but they actually occur as two separate steps. Key: nitrate reductase, NR; dephosphorylated serine residues, Ser-OH; phosphorylated serine residues, Ser-P (redrawn from Kaiser et al., 1999).

GOGAT gene by directly or indirectly inactivating protein phosphatases.

Diurnal changes in nitrogen assimilation

Tissue NR mRNA amounts decrease during the day and recover again during the night, whereas NR activity increases during the first part of the light period, and then decreases during the second part of the light period (Galangau et al., 1988; Geiger et al., 1998; Scheible et al., 1997a, 2000). Whole leaf tissue NO_3^- concentration, presumably in the vacuole, decreases during the light, and recovers at night (e.g., Steingröver et al., 1986). By using mutant plants with altered expression of specific assimilatory enzymes under controlled environmental conditions the regulation of the diurnal changes in N assimilation can be teased apart. In this way changes in the expression of NO_3^- transporters and assimilatory enzymes can be used to explain the distinct pattern of diurnal changes in cellular N pools (Geiger et al., 1998; Scheible et al., 1997b, 2000) and when a mixture of both NO_3^- and NH_4^+ is supplied (Matt et al., 2001). During the first part of the light period NO_3^- reduction is twice as fast as NO_3^- uptake, and exceeds the rate at which reduced N is assimilated. Later in the diurnal cycle, NR expression and activity declines, transporter expression and NO_3^- uptake remain high, and NO_3^- is accumulated in the leaf again. The regulatory network that underlies these changes is still not well understood, but NO_3^- (Scheible et al., 1997a,c) and possibly cellular pools of certain amino acids (Scheible et al., 2000) may be the feedback signal for regulation. In tobacco roots NO_3^- assimilation is differentially regulated from that in leaves with a different pattern of diurnal changes in activities of the NR, NiR and GS (Stöhr and Mäck, 2001). Two distinct peaks of NRA were detected in the roots; one during the light period was the soluble NR of the cytosol while a second peak occurred in the dark period for the plasma-membrane bound NR. The NH_4^+ generated during the diurnal cycle could be assimilated by the cytosolic GS (Stöhr and Mäck, 2001).

Variability in the site of NO_3^- reduction and assimilation in the plant

The site of NO_3^- reduction and subsequent assimilation of NH_4^+ in the plant may vary between the root and the shoot tissue depending on the species, the developmental stage and the environment. There are also developmental gradients of NRA along the length of

the root, with peak reduction and assimilatory activity often occurring just behind the root tip (Fedorova et al., 1994; Tischner et al., 1993). The largest effect on NRA is the local availability of NO_3^-. The advantages of foliar- as opposed to root-based NO_3^- reduction may be due to the benefits of NR having access to photosynthetic reductant in the shoot, rather than reductant derived from glycolysis and the oxidative pentose phosphate pathway (Andrews, 1986a). If NO_3^- is retained in the root tissue to some extent, then the ability to reduce the NO_3^- might prevent losses due to efflux, and thus contribute to the energy efficiency of the uptake system (Mata et al., 2000, Scheurwater et al., 2002).

Whether NO_3^- is reduced in the shoot or the root varies between species. For example, in rice (Yoneyama and Kumazawa, 1975) and *Quercus suber* (Mata et al., 2000), NO_3^- was found to be reduced in the root. In contrast, in barley (Lewis et al.. 1982, Ashley et al., 1975), maize (Murphy and Lewis, 1987), *Lupinus albus* (Cen et al., 2001) and *Glycine max* (Cen and Layzell, 2003), and eight naturally occurring monocotyledonous species examined by Scheurwater et al. (2002), NO_3^- is predominantly reduced and assimilated in the shoot. As a consequence of shoot reduction, considerable quantities of NO_3^- are accumulated and transported in the xylem sap. The extent of translocation of NO_3^- via the xylem from root to shoot also depends on the external NO_3^- concentrations (Oaks, 1986).

It appears that once the NO_3^- reduction capacity of the shoots is exceeded, NO_3^- accumulates in the shoot (Oaks, 1986) or root (Schobert and Komor, 1990). Thus the variable proportion of NO_3^- reduced within the root may be a function of the NO_3^- concentration supplied and the limited reduction capacity of the root (Andrews, 1986b). The location of NR in the root tissue, and possibly the extent of apoplastic transport of NO_3^- through root tissue to the stele effectively partitioning the NO_3^- away from the NR enzyme, also determine the extent of root-based NO_3^- reduction (Lewis et al., 1982). Root tissue NRA shows both diurnal and seasonal changes, with activity matching the times of maximal growth and NO_3^- supply (Lillo, 1983). The diurnal changes in NRA may be partially due to changes in transpiration rates, which coordinate the delivery of NO_3^- to the shoot (Cen and Layzell, 2003; Rufty et al., 1987). The reduction of NO_3^- within the root may be further dependent on the availability of carbohydrate in the root (Pate, 1980). Salinity also results in relatively more NO_3^- reduction in the root as compared with non-treated plants (Cramer et al., 1995; Peuke et al., 1996), presumably due to restriction of NO_3^- uptake.

The relative amounts of NRA in roots and shoots are variable, but in general most herbaceous plants have much more activity in the shoots while woody species have most activity in the roots (Pearson et al., 2001). However, there are exceptions, and, for example, poplar (*Populus tremula* × *P. alba*) trees show more NRA in leaves than in the roots (Black et al., 2002). The question has been raised as to whether there are systematic differences between the sites of NO_3^- reduction in plants differing in growth rate. Slow-growing plants could reduce a greater proportion of their NO_3^- in the roots than in the shoots. This proposal was tested by comparing a range of grass species differing in growth rates (Scheurwater et al., 2002) and also by comparing slow- and fast-growing tomato plants (Cramer et al., 1995). In both cases the reduction of NO_3^- was predominantly shoot based, and a similar proportion of NO_3^- was reduced in the roots. However, the NR activity was correlated with the growth rate, and thus slower growth could be associated with a greater efflux of NO_3^- when NO_3^- is freely available.

Urea assimilation

Urea is quantitatively the most important N fertiliser (Figure 3), and is also an important source of animal-derived N. It has generally been assumed that the bulk of the urea is converted to NH_4^+ by urease in the soil, and that the NH_4^+ is taken up by plants. However, plants are able to utilise urea applied to foliage (e.g., Leacox and Syvertsen, 1995), and it has been known for some time that plants grown in sterile culture are able to utilize urea as their sole N source (Harper, 1984). Tomato plants grown in hydroponics with urea in the nutrient solution as the sole N source were able to take up significant quantities of ^{15}N-urea (Tan et al., 2000). However, between 84% and 94% of the ^{15}N taken up was recovered in the form of urea, indicating that urea metabolism in tomatoes is slow relative to root uptake. Urease is a ubiquitous enzyme in plants responsible for the recovery of urea from arginine catabolism. Urease has been claimed to be induced by urea, although this may also be attributed to increased bacterial urease activity (Witte et al., 2002). Urease was not induced by the application of foliar urea to potato (Witte et al., 2002) or to *Brassica napus*

(Gerendás and Sattelmacher, 1999). However, Witte et al. (2002) found a correlation between metabolism of applied urea and the activity of urease in plants with antisensed urease. Similarly, Gerendás and Sattelmacher (1997) found that Ni deficiency inhibited both urease activity, which requires Ni for activation, and urea metabolism in zucchini (*Cucurbita pepo*). The activity of urease is, however, increased in older tissue which might indicate that it normally plays a role in recovering N from urea in senescing leaves (Witte et al., 2002). Since urea is rapidly hydrolysed in the soil and is therefore generally inaccessible to roots, it is unlikely that plant urea assimilation is important in primary N acquisition from the soil.

Interaction between nitrogen and carbon metabolism

C and N metabolism are linked by shared intermediates and products (Figure 6), and also by a complex network of cross-talking signal pathways. This regulation has been better documented for shoots than for roots (e.g., review by Coruzzi and Bush, 2001). It is known that information on the C and N status of the plant is used to regulate gene expression and enzyme activity, but the nature of the signals communicating N status to various component metabolic systems are still unclear. Gene expression is altered by NO_3^- supply in tobacco with very low levels of NRA, implicating NO_3^- as a signal molecule (Coruzzi and Bush, 2001). However, the presence of NR activity does modify gene expression further, also implicating the involvement of downstream products of NO_3^- assimilation. A role for glutamine has, for example, been indicated by repression of NH_4^+-transporter genes in *Arabidopsis* (Rawat et al., 1999). A further layer of complexity is introduced by the fact that carbohydrate metabolism is also implicated in the control of N metabolism. Two of the main points of regulation in inorganic N reduction and assimilation are NR and PEPc; the control of these two enzymes contributes greatly to the integration of C and N metabolism.

Two recent studies demonstrate the complexity of N metabolism. Microarray analysis identified 1,280 genes in tomato roots which responded within 1 to 96 h to resupply of NO_3^- after N deprivation for 48 h (Wang et al., 2001). In *Arabidopsis* roots, microarray analysis detected 1,176 genes that rapidly (20 minutes) responded to the switch from NH_4^+ to NO_3^- nutrition, whereas only 183 genes responded in the shoot (Wang et al., 2003). Amongst the genes that responded (up or down) in these studies were genes associated with N/P/K transporters, NO_3^- and NO_2^- reductases, amino-acid synthesis, oxidative pentose phosphate pathway, glycolysis, trehalose synthesis/catabolism, and water channels. A large number of regulatory genes were also identified (e.g., protein kinases/phosphatases and transcription factors) as well as stress response proteins and ribosomal proteins. These results indicate the complexity of N metabolism and implicate many divergent processes.

Roots depend on the organic compounds delivered via the phloem for most of their C requirements. The phloem sap contains carbohydrates (mostly sucrose in most species), organic acids and amino acids. Some additional C may be taken up by the roots in the form of organic N or from other sources, and some C is assimilated through the activity of PEPc and other carboxylating enzymes. The C available in the root is utilised for the provision of reductant and for C skeletons for amino-acid synthesis. Shoot-derived sucrose is metabolised in glycolysis to yield reductant. The C products of glycolysis (malate and pyruvate) are then available to the mitochondria. Amino acid synthesis via GS/GOGAT requires 2-oxoglutarate derived from the TCA cycle as a C source (Figure 6). Since the TCA cycle requires stoichiometric parity between acetyl Co-A and oxaloacetate, removal of oxaloacetate or any of its precursors disrupts the cycle (Hill, 1997). 'Anaplerotic' synthesis of malate redresses imbalances that may occur as a result of consumption of TCA-cycle intermediates for amino-acid synthesis or other processes. Malate is derived from carboxylation of glycolytic PEP by PEPc to yield oxaloacetate; subsequent reduction of the oxaloacetate yields malate. This results in PEPc having a key role in N metabolism, which consumes organic acids for amino acid synthesis (Cramer et al., 1993). PEPc activity in leaves is regulated by reversible protein phosphorylation catalysed by a kinase and a phosphatase, rendering the enzyme more sensitive to activation by phosphorylated allosteric effectors (e.g., Glc 6-P), and less sensitive to allosteric inhibition by certain organic acids (e.g., malate). Furthermore, the kinase is induced by NO_3^- and its reduction products, with associated reduction in C flux to sucrose (reviewed by Foyer et al., 2003). PEPc also plays a critical role in symbiotic N_2 fixation in nodules (Vance et al., 1994), where it supplies anaplerotic C (malate or succinate) for the assimilation of NH_4^+ into amino compounds. The nodular PEPc is also subject to post-translational regulation by

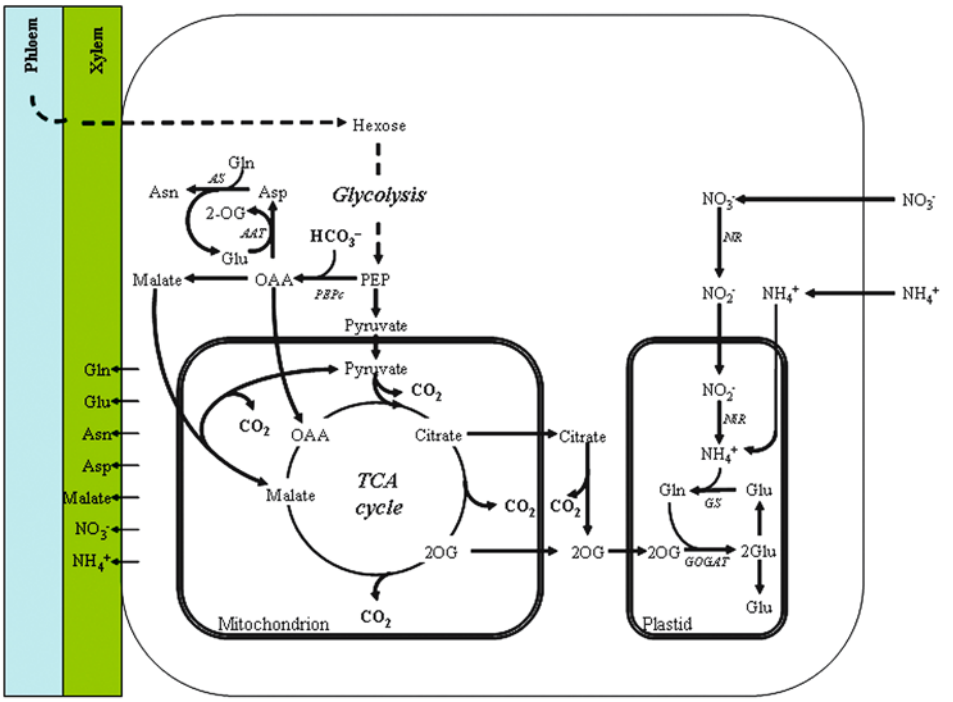

Figure 6. Simplified scheme of some C and N interactions in plant roots highlighting the role of anaplerotic C provision to the TCA cycle in plant roots. C enters the TCA cycle through pyruvate and through oxaloacetate (OAA) or malate, which may be derived from carboxylation of PEP. OAA may also be transaminated to yield aspartate and asparagine. The TCA cycle provides citrate for synthesis of glutamate and glutamine. Key enzymes associated with N metabolism are identified as glutamine synthetase, GS; glutamine:oxoglutarate aminotransferase, GOGAT; aspartate aminotransferase, AAT; asparagine synthase, AS; nitrate reductase, NR; nitrite reductase, NiR; phospho*enol*pyruvate carboxylase, PEPc. Note that although GS is indicated in the plastid it may also occur in the cytosol (Tobin and Yamaya, 2001).

kinases (Vance et al., 1994), which are in turn regulated by carbohydrate status in the root nodule (Xu et al., 2003). Similar regulatory mechanisms apparently operate on PEPc in white lupin (*Lupinus albus*) cluster roots (Uhde-Stone et al., 2003), cucumber (*Cucumis sativus*) roots (De Nisi and Zocchi, 2000) and sugar beet (*Beta vulgaris*) roots (Andaluz et al., 2002).

Comparison of plant responses to NO_3^- and NH_4^+ nutrition

Although NH_4^+ is a common form of N accessed by plants, and in many cases the preferred source of N, in many natural and agricultural circumstances it may also be toxic. A continuum of plant types exist ranging from those that prefer exclusively NO_3^- to those that prefer exclusively NH_4^+. Martins-Loução and Cruz (1999) surveyed reports for a wide range of species, and found that NH_4^+ inhibited the growth of 55% of species surveyed, relative to equimolar concentrations of NO_3^-. Many studies have shown that plants benefit from a mixture of both NO_3^- and NH_4^+, although the optimal ratios of NO_3^- to NH_4^+ and N concentration vary. The optimum mixture of NO_3^- and NH_4^+ depends on the species of plant, plant age and the pH of the growth medium (Haynes and Goh, 1978).

Even closely related species vary greatly in their sensitivity to NH_4^+; this sensitivity depends on the edaphic environment to which the plants are adapted. Many crop plants are sensitive to NH_4^+ toxicity and the concentration that is toxic varies greatly depending on the species (Chaillou and Lamaze, 2001; Britto and Kronzucker, 2002). However, it is not so much the concentration of NH_4^+ that is crucial, as the relative amounts of NO_3^- and NH_4^+ (Chaillou and Lamaze, 2001). Sensitivity to NH_4^+ has been used as an explanation for successional changes in forests from Douglas fir (*Pseudotsuga menziesii*) and aspen (*Populus tremuloides*) to spruce (*Picea glauca*) (Kronzucker et al., 2003). Since urea and NH_4^+-based N fertilisers are common, this toxicity of NH_4^+ also has major implications for agriculture.

Symptoms of NH_4^+ toxicity are variable but include visual symptoms such as chlorosis, growth inhibition, increased shoot:root ratios and wilting (water stress) (Cramer and Lewis, 1993). These changes are associated with decreased concentrations of inorganic cations (apart from NH_4^+) and increased concentrations of inorganic (Cl^-, SO_4^{2-} and PO_4^{2-}) and organic (carboxylic acid) anions in the tissue. The cation/anion imbalance that results from switching N sources from NO_3^- to NH_4^+ is thought to be a major factor in generating toxicity and is known as 'ammoniacal syndrome' (Chaillou and Lamaze, 2001). Another of the characteristics of NH_4^+ toxicity is the accumulation of amino acids in the tissue. When supplied with NH_4^+, many plants take up large quantities. Bloom (1988) quaintly suggested that the interaction between plants and NH_4^+ is like that between children and candy: when offered large quantities, they eat more and become sick. This was once thought to be because of the equilibrium of NH_4^+ with NH_3 allowing free diffusion of neutral NH_3 into the root tissue. However, since the pKa of NH_4^+ is ca. 9.3, it is unlikely that NH_3 would exist at concentrations high enough to play a role in uptake (Britto and Kronzucker, 2002). Indeed, extensive evidence is available that NH_4^+ and not NH_3 is the major form of ammoniacal N taken up, although, passive efflux of NH_3 could occur. The uptake of NH_4^+ from high concentrations in the medium is mediated by a LATS system that is apparently not down-regulated by high NH_4^+ concentrations (Britto and Kronzucker, 2002). These authors speculated that competitive exclusion of K^+ by NH_4^+ could result in over-expression of K^+ channels, which also transport NH_4^+, leading to runaway NH_4^+ accumulation. This might explain extensive accumulation of NH_4^+ in tissue, and the requirement for secondarily ATP-dependent NH_4^+ efflux systems, which, since operating to eject NH_4^+ against the membrane potential, would be relatively inefficient. Kronzucker et al. (2001) have speculated that high costs associated with regulation of internal NH_4^+ concentrations through NH_4^+ efflux against the unfavourable membrane potential for cation efflux may explain toxicity of NH_4^+ to some species (e.g., barley). In other species such as rice, depolarisation of the membrane potential upon exposure to NH_4^+ may reduce the energetic impact of NH_4^+ efflux on the root system. The toxicity of NH_4^+ may thus arise from the likelihood that most plants evolved in an environment in which NH_4^+ concentrations were rarely high enough to be toxic. Thus mechanisms for the exclusion of NH_4^+ may not have developed in many plant species, since toxic concentrations of NH_4^+ may be a man-made phenomenon in most situations.

Uptake of NH_4^+ results in rhizosphere acidification, possibly as a means of maintaining charge balance within the plant to compensate for NH_4^+ uptake. Although many authors have claimed that acidification of the rhizosphere is a primary cause of NH_4^+ toxicity, toxicity has also been observed in situations where the pH has been controlled. Another candidate possibly causing toxicity associated with NH_4^+ is the supposed change in cytosolic pH induced by the release of H+ from NH_4^+ during assimilation into amino acids (reviewed by Raven and Smith, 1976). While H^+ production undoubtedly accompanies this process, there are many reactions associated with the uptake and assimilation of NH_4^+ which also have pH implications, including the provision of C skeletons through 'anaplerotic' PEPc activity, amino-acid synthesis and NH_4^+ uptake itself (Britto and Kronzucker, 2002). The activity of PEPc, which responds positively to provision of NH_4^+ nutrition (Arnozis et al., 1988), has in the past also been assigned the role of a 'pH-stat' due to the consumption of HCO_3^- derived from hydration of CO_2. Although the observed organic acid synthesis, which accompanies NO_3^- nutrition could counteract the production of excess OH^-, this does not make much sense in the context of NH_4^+ metabolism, since it would exacerbate the acidification effect. With NH_4^+ nutrition, the organic acids produced by PEPc activity are depleted by amino acid synthesis, indicating that PEPc activity is an important source of organic acids, rather than a pH-stat.

The dogma that, despite symptoms of NH_4^+ toxicity, free NH_4^+ does not accumulate in plant tissue has been challenged by findings of millimolar concentrations of NH_4^+ in the cytosol *Chara corallina* (Wells and Miller, 2000) and in a range of other plants (reviewed by Miller et al., 2001) and in the xylem sap of *Brassica napus* (Husted and Schjoerring, 1995; Schjoerring et al., 2002). However, there is no evidence for the much-touted electron-transport uncoupling explanation for NH_4^+ toxicity in intact or suitably isolated (e.g., chloroplast) systems (reviewed by Britto and Kronzucker, 2002). Thus it seems that NH_4^+ is not toxic *per se*, but rather its consequences for metabolism result in its toxicity. This is likely to arise from the energetic costs of NH_4^+ efflux (Britto et al., 2001b) and of NH_4^+ assimilation. The high shoot:root ratios and accumulation of NH_4^+ observed

with NH_4^+ nutrition in C_3 plants, but not in C_4 plants, has led to the explanation that the higher capacity of the C_4 photosynthetic system allows the plant to meet the challenge of NH_4^+ assimilation better than that of the C_3 system (Cramer and Lewis, 1993). This was associated with greater partitioning of shoot-derived C to amino acids in the roots of plants supplied with NH_4^+ nutrition than in those supplied with NO_3^- nutrition (Cramer and Lewis, 1993). The notion that NH_4^+ toxicity is associated with competition between NH_4^+ efflux/assimilation and other C-requiring processes may be criticised on the basis that NH_4^+ toxicity is often more pronounced at high light intensities where photosynthesis rates are likely to be high, and thus C more readily available. Furthermore, one may expect photosynthetic activity to be higher with greater demand for C for assimilation of NH_4^+ in the roots, whereas NH_4^+ has often been shown to suppress photosynthetic CO_2 acquisition (reviewed by Britto and Kronzucker, 2002). However, NH_4^+ toxicity is associated with reduced leaf moisture contents and water potentials, and suppression of photosynthesis by NH_4^+ under these circumstances is possibly the consequence of reduced stomatal conductance (Cramer and Lewis, 1993). Thus NH_4^+ toxicity is probably the product of several processes, including the requirements for NH_4^+ efflux, assimilation and interactions with photosynthesis.

Concluding remarks

Our understanding of N acquisition and assimilation has changed radically over the past decade, as a consequence of molecular techniques and the increasing number of known gene sequences. This has led to the identification of genes encoding most of the steps involved in N acquisition and metabolism. This sequence information allows the design of PCR primers that make the identification and isolation of closely related genes in other species fairly easy. Increasingly, it is becoming evident that there are families of different genes, and that individual members may be expressed in diverse tissues and at different stages of development. The use of microarrays now allows expression changes in the whole genome to be measured and the key genes at each developmental stage and in different parts of the plant to be identified, although, their actual functions may still be unknown. This technology is likely to be used with increasingly smaller quantities of tissue, and even at the level of single cells. Changes in gene expression may be a useful tool to identify soil N sources, for example, the increased expression of an NH_4^+ transporter was used to identify the presence of N_2-fixing bacteria on the root surface (Becker et al., 2002). The molecular tools are available to identify, using the changes in expression of N transporters, which soil N sources a root is accessing, thus accounting for the complexities of inter-conversions between N-forms in the soil. However, this does require the application of plant molecular techniques to soil-grown root material, and this is not a simple matter. Other valuable tools include *in situ* mRNA hybridisation (e.g., Lin et al., 2000; Vuylsteker et al., 1998), immuno-localisation and promoter-Gus/GFP fusion (Guo et al., 2001, 2002; Nazoa et al., 2003), allowing the identification of the expression patterns and localisation of gene products within tissue types as well as within cells. For instance, it is possible to identify the sites of expression of enzymes/transporters associated with N acquisition and metabolism along the length of the root, allowing better interpretation of localised changes, without these being swamped by either converse or no changes in neighbouring tissues. Similarly, micro-scale electrophysiology allows the monitoring of localised changes in N pools close to or within root cells. The combined application of these techniques provides an opportunity for detailed tissue mapping of cellular heterogeneity, but the challenge is to apply these techniques to plants growing in soil.

The identification of many members of some gene families (e.g., 52 peptide and NO_3^- transporters) is described as 'redundancy' but this can be misleading as the function may only become apparent when the plant is subject to specific environmental stresses. The plasticity of plants, their ability to adjust and reproduce in the location where a seed lands, requires a reserve of genes whose expression may only be needed under very specific conditions. This gene 'redundancy' is a design feature that enables the complex system to function in an environment of multiple requirements. However, in the headlong rush to identify sequences associated with various systems, it must be remembered that rigorous identification of function is required. Thus, for example, the multiple genes associated with NO_3^- transporters may reflect the requirements for a diversity of functions, including NO_3^- transport but also NO_3^- sensing in the soil, NO_3^- transport on many endo-membranes and the transport of other substances.

Association of a particular activity with a gene product does not necessarily imply that this was its

selected function in an evolutionary context. Thus efflux of NO_3^- and NH_4^+ could be the consequence of accumulation of these substances to levels in cells which are artificially high for the plant system and they reflect the cellular response to maintain cytosolic homeostasis (Miller and Smith, 1996). Wild plants grow in N-limited environments that must have selected for optimisation of N interception and acquisition, but when these plants are placed in high N environments an imbalance between influx, growth and storage capacity occurs that results in efflux. These energetically wasteful leakage processes may occur through non-specific mechanisms that cannot be bypassed, such as leakage of NH_3 through aquaporins or NO_3^- through anion channels. One of the lessons learnt over the past decades is that (plant) metabolism is under strict control at a variety of levels, both transcriptional and post-transcriptional. For example, experiments to manipulate the gene expression of both N- and C-assimilatory enzymes have failed to give major changes in the phenotype until some environmental or nutritional stress is applied to the plants. Efflux may thus be a consequence of high rates of N fertilisation to which the plants are not adapted.

The prime importance of N for plant growth has led to the suggestion that N, or specific forms such as NO_3^-, may function as plant growth regulators (Trewavas, 1983) and/or part of signalling systems (Scheible et al., 1997a, c). Increasingly, it has been recognised that NO_3^- and its assimilation products do play a role as signalling molecules. This is, for instance, the case in the induction of lateral root formation by localised concentrations of NO_3^- (Drew and Saker, 1975) and the classic induction of NR. Although NO_3^- and other N forms may play a role as environmental signals, and even in inducing aspects of N metabolism, there is little evidence for a role as a 'phytohormone'. There is increasing realisation of the complexity of control through mechanisms such as multi-gene enzymes (e.g., GS) subject to transcriptional control through multiple promoters combined with post-translational control through mechanisms such as enzyme phosphorylation. Much more attention needs to be devoted in the future to understanding and interpreting these controls, as well as the enzymes themselves.

The below-ground portion of plants is not the favoured research material of plant biologists. Not only are one's finger nails at risk, but the root is difficult to free of contamination from the soil. This has resulted in the use of hydroponically grown plant material, and a preference for the use of shoots in metabolic work. Although hydroponics has many advantages, there are some important differences from a soil environment including: (1) higher water content than that in most soils; (2) nutrients are uniformly available and not in patches; (3) the gas environment (e.g., O_2, CO_2, NO) is very different; (4) root exudates are readily lost from the rhizosphere; (5) soil micro-flora/-fauna are absent; (6) mycorrhizal infection is compromised; (7) nodules are often absent from legumes. For these reasons, hydroponically grown plants are likely to be a better model for intensive agriculture than for ecology, because roots are growing in a saturated system that can leach nutrients and where the rhizosphere is much less diverse. There is far more information available on shoot N metabolism than on root metabolism. In some cases the assumption is made that the root metabolism is similar to that of the shoot. While this is partially true (e.g., NR regulation in leaves and roots shares common elements), the specifics may vary greatly with different enzyme isoforms and regulatory networks.

High-input agriculture has presented crop plants with a novel set of challenges, which genetic engineering may be able to help the plant to meet. Many possibilities exist for the manipulation of N acquisition and metabolism ranging from management-based to biotechnological manipulations. Crop management can be used to control soil N concentrations, N forms, soil pH, rates of N supply, timing of N supply, foliar applications, plant demand (for instance, by manipulating sink strength), to list but a few. Biotechnological or breeding manipulation of root density, volume of soil exploited, affinity of transporters for N, reduced efflux and greater efficiency of N utilisation (e.g., through increased specificity of Rubisco for CO_2 over O_2) are just some of the possible approaches to improving crop use of soil N. However, the complex regulation and strong interactions with other components of plant metabolism make the potential for transgenic modification of plants for greater N-use efficiency or capacity somewhat daunting. Over-expression of homologous genes seldom benefits plants, since key points in metabolism are under strict feed-back control. Expression of heterologous genes may bypass the normal regulatory systems, but there needs to be careful consideration of the target tissues, the membranes to which transporter gene products are targeted, and the timing of expression (i.e., promoters). Manipulating any of the physiological attributes of N acquisition

and assimilation is likely to be complex, with many implications for the physiology of the whole plant.

Acknowledgements

The authors are grateful for useful comments from the anonymous reviewers. AJM is supported by grants from DEFRA (AR0910) and the EU (HPRN-CT-2002-00247) and Rothamsted Research is grant-aided by the Biotechnology and Biological Sciences Research Council of the UK. Funding for collaborative research from the British Council is gratefully acknowledged.

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Plant and Soil (2005) 274:37–49
DOI 10.1007/s11104-004-2005-6

Phosphate acquisition

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Received 3 May 2004. Accepted in revised form 9 August 2004

Key words: genes, homeostasis, mineral nutrition, phosphate, phosphate transporters, rhizosphere, roots, tissue-specific expression

Abstract

Phosphate (Pi) is considered to be one of the least available plant nutrients in the soil. High-affinity Pi transporters are generally accepted as entry points for Pi in the roots. The physiological, genetic, molecular and biochemical analysis of phosphate starvation response mechanisms highlight the ability of plants to adapt and thrive under phosphate limiting conditions. These responses help them enhance the availability of Pi, increase its uptake and improve the use-efficiency of Pi within a plant. Enhanced ability to acquire Pi appears to be regulated at the level of transcription of high-affinity phosphate transporters. These transporters are encoded by a family of small number of genes having characteristic tissue and organ associated expression patterns. Many of them are strongly induced during phosphate deficiency thus providing plants with enhanced ability to acquire and transfer phosphate. In addition, plants also activate biochemical mechanisms that could lead to increased acquisition of phosphate from both inorganic and organic phosphorus sources in the soil. Furthermore, altered root morphology and mycorrhizal symbiosis further enhance the ability of plants to acquire Pi. Interestingly most of these responses appear to be coordinated by changes in cellular phosphate levels. It is becoming apparent that phosphate acquisition and utilization are associated with activation or inactivation of a host of genes in plants. In this article we describe molecular, biochemical and physiological factors associated with phosphate acquisition by plants.

Introduction

Phosphorus is an integral part of energy metabolism, a constituent of nucleic acids and membranes. Major biochemical processes such as photosynthesis and respiration are activated by inorganic phosphate (Pi) or its organic derivatives. Phosphate esters in general act as energy carriers in various metabolic pathways. Phospholipids play an important role in membrane integrity and function. In addition, phosphorylation and dephosphorylation of proteins are crucial for signal-transduction pathways in plants. Furthermore phosphate homeostasis in the chloroplast regulates the transport of phosphorylated sugars across the membrane and synthesis of starch.

Plants acquire phosphorus as phosphate anions from the soil solution. It is probably one of the least available plant nutrients found in the rhizosphere. Inorganic fixation and formation of organic complexes of available phosphate in the soil are the primary reasons for its low availability. In this context, phosphorus deficiency is considered to be one of the major limitations for crop production, particularly in the tropics. Interestingly the ability of plants to acquire Pi increases significantly under Pi deficiency. This

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increase appears to be regulated, at least in part, at the transcriptional level. This review will provide an overview of Pi acquisition by plants. Readers are referred to many other informative reviews on phosphorus nutrition and physiology for obtaining comprehensive information on this subject (Abel et al., 2002; Franco-Zorrilla et al., 2004; Harrison, 1999; Lynch, 1995; Marschner, 1995; Plaxton, 1996; Plaxton and Carswell, 1999; Poirier and Bucher, 2002; Raghothama, 1999; Raghothama, 2000a,b; Raghothama, 2002; Rausch and Bucher, 2002; Ryan et al., 2001; Schachtman et al., 1998; Smith et al., 2000, 2003; Vance et al., 2003).

Phosphorus availability; a major factor determining crop productivity

There is a great disparity in distribution of Pi between plant cells (mM) and soil solution (μM). Extremely low levels of available phosphorus in the rhizosphere, makes it one of the major growth-limiting factors in many ecosystems (Barber et al., 1963). The concentration of available phosphate (Pi) in soil seldom exceeds 10 μM (Bieleski, 1973). This problem is further heightened in highly weathered and volcanic soils of the humid tropics and subtropics, and sandy soils of the semi-arid tropics (Sanchez et al., 1997). It is estimated that 5.7 billion hectares of land worldwide is deficient in Pi for achieving optimal crop production (Batjes, 1997). Phosphate fixation increases significantly in acid soils, which accounts for nearly 26% of the world's soils (Eswaran et al., 1997). As a consequence of organic and inorganic fixation, nearly 80% of applied Pi may be unavailable to plants (Holford, 1997). This problem is especially acute in tropical regions, particularly Africa, where production of crops without fertilizer application is resulting in continuous mining of essential nutrients by plants. Furthermore, at the current world-wide rate of application of fertilizers, the readily available sources of high-grade phosphate rocks may be depleted within the next 60 to 90 years (Runge-Metzger, 1995). Increasing population and extension of agriculture to low- and marginal-fertility lands will further increase the demand for the precious supply of phosphate fertilizers.

Phosphate uptake is a highly regulated process in plants

A combination of efficient uptake and translocation of Pi is essential for plants to maintain adequate levels of cellular Pi necessary for normal functioning. Plants have to acquire Pi against a steep concentration gradient (three orders of magnitude or greater) across the plasma membrane. In addition electrical gradients also play a major role in nutrient uptake. Many uptake models have been proposed to explain the plant's ability to acquire Pi both under deficiency and sufficiency conditions (Borstlap, 1983; Epstein et al., 1963; Kochian and Lucas, 1982; Nissen, 1971). A dual uptake model for ions involving both the high- and low-affinity uptake mechanisms is widely used to explain the concentration-dependent acquisition of Pi (Amijee et al., 1991; Epstein et al., 1963; Kochian and Lucas, 1982; Sentenac and Grignon, 1985; Ullrich-Eberius et al., 1984). Both soil and plant factors such as Pi supply to roots by mass flow and diffusion, changes in root geometry and size, Pi uptake in relation to Pi concentration at the root surface, and root competition influence Pi uptake by plants (Barber and Cushman, 1981; Barber and Silberbush, 1984; Silberbush and Barber, 1983). Now there is genetic and molecular evidence for the presence of transporters operating efficiently at both high and low concentration of Pi in the medium. Interestingly, the low-affinity transport system appears to be expressed constitutively in plants; in contrast, the high-affinity uptake system is strongly enhanced during Pi deficiency (Furihata et al., 1992).

In general the orthophosphate ion ($H_2PO_4^-$) is the preferred form of phosphate for translocation by transporters. The low levels of available Pi in the soil solution (around 5 μM) dictate the uptake by low-K_m, high-affinity Pi transporters (Dunlop et al., 1997; Sentenac and Grignon, 1985; Shimogawara and Usuda, 1995; Ullrich-Eberius et al., 1984). Phosphate is acquired by an energy-mediated co-transport process driven by protons generated by plasma-membrane associated H^+ATPases (Sakano et al., 1992; Ullrich-Eberius et al., 1984). Depending on Pi availability and tissue Pi concentration the transport process may be energized by the symport of 2 to 4 $H^+/H_2PO_4^-$J transported (Sakano, 1990).

Active uptake of Pi results in a transient decrease in the cytosolic pH and membrane depolarization (Bowling and Dunlop; 1978; Ullrich-Eberius et al., 1984). Dissipation of proton gradients across the membranes by metabolic inhibitors can suppress Pi uptake. In addition to Pi/H^+ symporters, there is also evidence for the presence of a Na^+/Pi-mediated transport system in algae. In the interaodal cells of giant alga *Chara corallina*, a Na^+-coupled Pi uptake system induced by Pi deficiency has been reported (Mimura et al., 2002; Reid et al., 2000). Interestingly, presence of Na^+ in the external medium was found to be essential for the induction of the transporter during Pi deficiency. Although several genes sharing sequence homology with the mammalian Na^+/Pi transporters have been identified in the *Arabidopsis* and other plant genomes, at present there is no experimental evidence for their involvement in Pi uptake.

Phosphorus homeostasis

Phosphorus is an essential element for all energy-mediated processes in plants. Maintaining the cellular Pi homeostasis is essential for normal functioning of the organism (Plaxton and Carswell, 1999). Phosphate acquisition, homeostasis and metabolism are highly regulated and well coordinated to maximize the use of this often-limiting nutrient. Cells have mechanisms to maintain cytosolic levels of Pi, in spite of large fluctuations in the external concentrations (Glass and Siddiqi, 1984). Higher plants acquire and store excess Pi in the vacuole, which is crucial for cytosolic Pi homeostasis. Under Pi sufficiency conditions, most of the cellular Pi (85 to 95%) can be found in the vacuoles (Anghinoni and Barber, 1980; Natr, 1992). ^{31}P-NMR studies showed an 80% increase in the vacuolar Pi under Pi sufficiency, whereas the level in the cytosol was relatively constant (Tu et al., 1990). The cytosolic Pi levels are maintained at the expense of vacuolar Pi during short-term deficiency (Bieleski, 1973; Lee and Ratcliffe; 1993, Mimura et al., 1996; Sakano et al., 1992; Tu et al., 1990).

Multiple transporters may be involved in maintaining both cellular and whole-plant Pi homeostasis. Maintenance of cytosolic Pi homeostasis is bound to involve bi-directional movement of the nutrient across the tonoplast. There is evidence for ATP-dependent Pi transport across the tonoplast (Mimura et al., 1990; Sakano et al., 1995). A decrease in the ATP levels in the cytosol results in suppression of Pi transport across the tonoplast membranes (Sakano et al., 1992). It is likely that the tonoplast-associated H^+-translocating pyrophosphatase or H^+-ATPase may provide required energy for maintaining an electrochemical potential gradient of H^+ across the tonoplast to facilitate Pi transport. Kinetic analysis of Pi uptake in intact vacuoles further confirmed the stimulation of transport process by both ATP and pyrophosphate (Massonnearu et al., 2001). The apparent high *Km* (5 mM) of the vacuolar Pi-uptake system indicates that low-affinity, high-flux transport mechanisms are operating in the tonoplast. At present details about this transport process are lacking as none of the tonoplast Pi transporters are characterized at the molecular or biochemical level.

In addition to storage of Pi in the vacuole, Pi efflux from roots may also help plants to maintain ion homeostasis (Cogliatti and Santa Maria, 1990; Elli-ott et al., 1984). Under Pi excess, increased Pi efflux by roots nearly compensates for the higher Pi influx (Cogliatti and Santa Maria, 1990).

Plant phosphate transporters

Phosphate transporters are integral membrane proteins responsible for acquiring Pi from the rhizosphere or transferring it to different tissues and organs. They may exhibit different uptake kinetics and tissue and organellar localization. Molecular studies provide evidence for the presence of both high- and low-affinity Pi transporters, thus supporting the data obtained in many physiological experiments (Daram et al., 1998; Muchhal et al., 1996). Genes representing several high-affinity Pi transporters have been isolated from several species (Raghothama, 1999). The function of many of these genes has been confirmed by functional complementation of yeast mutants lacking high-affinity Pi uptake (Daram et al., 1998; Leggewie et al., 1997; Muchhal et al., 1996). The first *in planta* evidence for the role of high-affinity Pi transporters came from ectopic-expression of an *Arabidopsis thaliana* transporter

in tobacco (*Nicotiana tabacum* cells (Mit-sukawa et al., 1997). In *Arabidopsis thaliana* nine high-affinity Pi transporter genes have been identified and characterized (Karthikeyan et al., 2002; Muchhal et al., 1996; Mudge et al., 2002; Okumura et al., 1998; Smith et al., 1997). There is growing evidence suggesting that plants in general have a small family of high-affinity Pi transporters (Muchhal et al., 1996; Mudge et al., 2002; Mukatira et al., 2001; Uta et al., 2002).

Plant Pi transporters are structurally similar

Phosphate transporters are proteins consisting of 12 membrane spanning regions, separated into two groups of six by a large hydrophilic charged region. They belong to the Major Facilitator Super family (MFS) of transporters, comprising sugar, antibiotic, ion and amino acid transporters (Pao et al., 1998). This is one of the largest families of single-polypeptide facilitated carriers capable of transporting various solutes utilizing the chemiosmotic ion gradients. The similarity between the first and second half of deduced Pi transporters suggests that they may have evolved by a tandem intragenic duplication of the original structural unit of a six membrane-spanning protein (Pao et al., 1998). Furthermore, several conserved phosphorylation and N-glycosylation sites are found in the Pi transporters isolated from different plant species. Plant Pi transporters are of similar size (approximately 518–587 amino acids), and all utilize the H^+ gradient to drive the transport process.

Phosphate transporters are involved in nutrient acquisition and translocation in plants

Phosphorus is considered as one of the mobile nutrients in plants. The acquisition and mobility of Pi in plants is quite complex indicating the involvement of multiple transporters (Raghothama, 1999). Most of the Pi acquired by roots is rapidly loaded into the xylem and it moves upward in shoots where it is unloaded into growing sinks. Since Pi moves readily in both xylem and phloem, a constant loading and unloading of the nutrient into different organs may occur. Furthermore, it is recycled from old and senescing tissues, a process that is enhanced during Pi deficiency. Not much is known about transporters involved in xylem and phloem loading, although two mutants of *Arabidopsis, pho1* and *pho2*, provide evidence for specific transport events occurring at xylem and phloem interfaces. *The pho1* mutant *of Arabidopsis* lacks the ability to load Pi into the xylem (Poirier et al., 1991). This appears to be due to a mutation in a gene coding for a membrane-associated protein similar to the mammalian receptor for murine leukemia retrovirus (Rcml) (Hamburger et al., 2002). Since the *Pho1* mutation did not map to any of the known Pi transporters, the deduced protein may have a regulatory role in Pi transport. The *pho2* mutant has higher levels of Pi in leaves than wild type due to defective regulation of Pi loading into shoots (Delhaize and Randall, 1995). There is also growing evidence supporting the involvement of high-affinity Pi transporters in internal mobilization of Pi (Karthikeyan et al., 2002; Mudge et al., 2002). The *in planta* evidence for the involvement of high affinity Pi transporters in Pi acquisition under both low and high concentrations of the nutrient came from analysis of *Arabidopsis* mutants (Shin et al., 2004). The double mutant of two highly expressed *Arabidopsis* Pi transporters (*pht1;1* and *pht1;4*) showed severe reduction in phosphate uptake rate and symptoms of Pi deficiency. This study provided genetic and functional evidence for the involvement of high-affinity phosphate transporters in phosphate acquisition.

Transcriptional regulation of Pi transporters

Survival of plants in a naturally Pi-deficient condition may require coordinated expression of Pi transporters. In order to maintain Pi homeostasis, the expression and function of Pi transporters need to be coordinated with changes in the availability of Pi. The high-affinity Pi transporters are preferentially expressed in the roots, and many of them are induced under Pi starvation (Daram et al., 1998; Liu et al., 1998; Muchhal et al., 1996; Raghothama, 1999; Raghothama, 2000a). In addition to inducibility under Pi deficiency both temporal and spatial patterns of regulation have been reported for Pi transporters (Karthikeyan et al., 2002; Mudge

et al., 2002). Two of the tomato (*Ly copersicon esculentum*) Pi transporters (*LePT1* and *LePT2*) are induced in a temporal and concentration-mediated manner, both in roots and cell cultures. A rapid induction of transcripts was observed within 3 to 6 hours of exposing the cell cultures to Pi deficiency. The Pi-transporter protein accumulation was observed within 12 to 24 hours of Pi starvation in plants (Liu et al., 1998). The LePT1 transporter is localized in plasma membranes and enriched in epidermal layers of Pi-deficient roots. These studies provided evidence that increased transcription of genes during Pi deficiency is at least in part, responsible for increasing the capacity for Pi uptake (Muchhal and Raghothama, 1999). This also could explain the observed increases in V_{max} in many physiological experiments with P-deprived plants (Anghinoni and Barber, 1980; Drew and Saker 1984; Shimogawara and Usuda, 1995). Both transcript and protein analysis confirmed that Pi transporters are induced and rapidly turned over in roots in response to changes in Pi supply (Liu et al., 1998; Shimogawara and Usuda, 1995). This rapid turnover may enable plants to modulate nutrient uptake without leading to toxic levels of Pi. Furthermore, presence of conserved phosphorylation and glycosylation sites in the high-affinity Pi transporters also points to potential post-translational modifications that could affect uptake.

Split-root studies have shown that even if Pi is supplied to a portion of the root system, expression of Pi transporters in other portions of roots exposed to Pi deficiency does not increase as long as the internal requirement of Pi is satisfied (Baldwin et al., 2001; Liu et al., 1998). The divided-root-system studies suggest that shoot Pi levels play a role in the regulation of P-deficiency-induced responses, including its uptake by roots (Anghinoni and Barber, 1980; Baldwin et al., 2001; Drew and Saker, 1984; Liu et al., 1998). In addition, foliar sprays of Pi suppressed the production of proteoid roots of white lupin (*Lupinus albus*) under Pi deficiency (Gilbert et al., 1997). Analysis of the *Arabidopsis* mutant *Pho2*, impaired in regulating shoot Pi levels, also lends support to the hypothesis that signals originating in shoots control Pi uptake by roots (Dong et al., 1998,1999; Smith et al., 1997).

Tissue- and organ-specific expression of Pi transporters

The physiological uptake studies point to the role of root-associated high-affinity Pi transporters in Pi acquisition from the rhizosphere. The high-affinity Pi transporters in general are expressed in roots under Pi deficiency (Leggewie et al., 1997; Liu et al., 1998; Muchhal et al., 1996). Furthermore they are enriched in the epidermis and root hairs to enhance Pi uptake (Daram et al., 1998; Liu et al., 1998). In addition, transporters such as *LePT1* are expressed both in the epidermis and in cortical cells. Recent studies using transgenic *Arabidopsis* plants expressing reporter genes driven by promoters of the members of the *Pht1* family revealed strong expression of four members of the family (*Pht1.1, Pht1.2, Pht1.3* and *Pht1.4*) in the root-hair producing trichoblast cells of the epidermis (Karthikeyan et al., 2002; Mudge et al., 2002). There is also evidence for expression of high-affinity transporters in other plant parts, including flowers, leaves, cotyledons and tubers (Karthikeyan et al., 2002; Leggewie et al., 1997; Liu et al., 1998; Mudge et al., 2002). Promoter/reporter-gene fusion analysis in *Arabidopsis* revealed the expression *of Pht1.5* in the cotyledons of young seedlings as well as in senescing mature leaves. The reporter activity was strongest in the vascular bundle, especially in the phloem. Weak expression of some of the members of the family was also observed in hydathodes (*Pht1.3* and *Pht1.4*) and in axiliary buds, senescing anther filaments and silique bases (*Pht1.4*) (Karthikeyan et al., 2002; Mudge et al., 2002). Interestingly, expression of all these genes was strongly induced under Pi deficiency. Expression of high-affinity Pi transporters in tissues other than roots point to their role in mobilization of Pi within the plants. One of the Pi transporters (*LaPT1*) is expressed at a higher level in Pi-deficient proteoid roots of white lupin (*Lupinus albus*) than in non-cluster roots of Pi-deficient plants (Liu et al., 2001). This pattern of expression points to spatial targeting of Pi transporters to specialized proteoid roots involved in soil Pi solubilization and acquisition.

The transcriptional regulation of Pi transporters is likely mediated by interaction of proteins (*trans* factors) with promoter elements of the

genes. Genes coding for MYB transcription factors in both algae (*PSR1*) and higher plants (*PHR1*) appear to regulate the expression of many Pi-starvation-induced genes, including some Pi transporters (Rubio et al., 2001; Wykoff et al., 1999). Conserved MYB-factor-binding sequences have been identified in promoter regions of several P-starvation-inducible genes. In addition DNA-protein interaction studies revealed the existence of specific protein-binding domains (*cis* elements) in P-deficiency-induced genes including a Pi transporter (Mukatira et al., 2001). The DNA protein interaction was observed only under Pi sufficiency conditions, suggesting that some of the Pi-starvation-induced genes may be under negative regulation. There is also evidence for activation of a *bZIP*-like transcription factor and a MAP kinase when the Pi content in a tobacco cell culture is altered (Sano and Nagata, 2002; Wilson et al., 1998).

Low-affinity Pi transporters are involved in internal mobilization of Pi

The uptake-kinetics data provided physiological evidence for the presence of both high- and low-affinity Pi transporters in plants (Marschner, 1995). These studies also pointed to the constitutive nature of low-affinity Pi-transporter expression under varying concentrations of Pi. The intracellular/organellar movement of Pi in vacuoles, chloroplasts and mitochondria very likely involves the low-affinity transporters. The first gene coding for a low-affinity Pi transporter (*Pht2.1*) was cloned from *Arabidopsis*. *Pht*2;1 encodes a 64 kD membrane-associated protein structurally similar to that of high-affinity transporter family members. This is predominantly expressed in the green tissues (Daram et al., 1999). Functional analysis indicated that Pht2;l protein is a H^+:Pi symporter with an apparent *Km* of 0.4 mM. Interestingly, Pi deficiency does not seem to increase the transcript levels as observed in with high-affinity Pi transporters. Sequences similar to a chloroplast transit peptide are present in Pht2;l, indicating that this transporter might be associated with the chloroplast membranes (Versaw and Harrison, 2002). The chloroplast envelop targeting of the protein was demonstrated using GFP (green fluorescent protein) tagged Pht2;l protein. In this context, Pht2;l like proteins may be working in concert with the triose:phosphate shuttle in mobilizing Pi across the chloroplast membranes.

Phosphate acquisition is associated with modified root system

The low Pi in many soils forced plants to develop mechanisms to scavenge for the nutrient. This is evidenced by altered root morphology and preferential proliferation of roots over shoots under Pi deficiency conditions. Root-associated factors such as root morphology, architecture, root-hair density and length, nutrient-absorption rate, ability to modify the rhizo-sphere and mycorrhizal symbiosis may strongly influence Pi acquisition (Raghothama, 1999). Distinct changes in both root growth and architecture are observed under Pi deficiency (Lynch, 1995). Increase in the root mass ratio, results in enhanced root surface area available for soil exploration and acquisition of Pi, relative to that of leaf surface, where most of the Pi is utilized. Interestingly Pi-acquisition efficient genotypes of beans possess highly branched and relatively fast growing root systems (Lynch and Beebe, 1995).

In *Arabidopsis* elongation of the primary root is suppressed, whereas the secondary root branching, and root-hair formation and elongation are enhanced, under Pi deficiency. Phosphate uptake is strongly influenced by increased root hair density in Pi-acquisition efficient plants (Föhse et al., 1991; Ga-hoonia and Nielsen, 1998). Root-system modeling studies have shown that the combined, synergistic effects of coordinated root-hair response on Pi acquisition were 371% greater than their additive effects (Ma et al., 2001). In many cases root hairs may contribute up to 70% of the total surface area of roots, thus increasing the surface area of the root cylinder nearly 27-fold (Jungk, 2001). In *Arabidopsis*, extra root hairs produced under Pi deficiency are often located in places where non-root hair cells are found under Pi-sufficient conditions. The genetic evidence for the role of root hairs in Pi acquisition was obtained with a hair-less mutant of *Arabidopsis* (Bates and Lynch, 2001). Although the mutant (rhd2) had no difference in Pi uptake or growth compared to wild-type plants under Pi sufficiency, both Pi acquisition and growth were significantly

lower under Pi deficiency. There is growing evidence suggesting that newly formed root hairs become targets for expression of Pi transporters induced under Pi deficiency (Daram et al., 1998; Karthikeyan et al., 2002; Mudge et al., 2002).

Role of hormones in P-starvation induced changes in roots

Many of the P-starvation responses observed in root morphology are similar to those caused by auxin and ethylene, thus pointing to a potential involvement of hormones in acclimation to P-deficiency. Studies on hormone mutants in *Arabidopsis* have provided a genetic link between altered root morphology and hormone action or transport (Vance et al., 2003). Exogenous application of auxin to P-sufficient white lupin resulted in cluster-root formation similar to that under Pi deficiency (Gilbert et al., 2000), whereas auxin-transport inhibitors had the opposite effect, suggesting a role for auxin in nutrient deficiency response. Interestingly 10- to 100-fold higher concentrations of auxin were required to inhibit primary root growth, induce lateral-root development and increase lateral-root density under high Pi (1 mM) condition than in low Pi (1 μM) conditions. Based on these results with white lupin, and those obtained with auxin-transport mutants of *Arabidopsis*, it has been suggested that increased sensitivity to auxin may play an important role in the modification of root architecture during Pi starvation (Lopez-Bucio et al., 2002). There are also reports suggesting the involvement of an auxin-independent pathway in low-P mediated root morphological changes (Schmidt, 2001; Williamson et al., 2001).

In addition to auxin the phytohormone ethylene also influences Pi-deficiency mediated root morphological changes (Borch et al., 1999; Schmidt, 2001). However, studies with ethylene-signaling mutants of *Arabidopsis* and the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) showed that ethylene might not be directly involved in lateral-root formation during Pi starvation (Lopez-Bucio et al., 2002). Application of hormones such as cytokinin and auxin suppressed the expression of some of the Pi-deficiency-induced genes including a Pi transporter in *Arabidopsis* (Karthikeyan et al., 2002; Martin et al., 2000). In addition several genes associated with hormone metabolism or actions are induced in proteoid roots formed under Pi deficiency (Uhde-Stone et al., 2003). The available data suggest that both hormone-dependent and independent regulatory pathways may be operating under Pi deficiency, leading to increased Pi uptake and acclimation to Pi deficiency.

Mycorrhizae: an integral part of Pi uptake by plants

In response to persistent deficiency of Pi in the soil many plants have developed a symbiotic relationship with mycorrhizal fungi. Readers are advised to refer to the article by Graham and Miller in this issue for detailed information on role of mycorrhizae in Pi nutrition of plants. Recent molecular analyses of Pi transporters in both monocotyledonous and dicotyledonous species have revealed the activation of specific Pi transporters during mycorrhizal symbiosis. The rice (*Oryza sativa*) Pi transporter, OsPT11, is specifically induced in response to mycorrhizal symbiosis (Uta et al., 2002). Similarly, expression of high-affinity Pi transporters of potato (*Solanum tuberosum*) (*StPT3*) and *Medicago truncatula* (*MtPT4*) is distinctly associated with arbuscule-forming root cells (Harrison et al., 2002; Rausch et al., 2001). The level of induction is correlated with the increasing degree of colonization of roots by mycorrhizal fungi. The spatial and temporal expression of these genes is consistent with their presumed role in acquiring Pi released by the fungus.

Biochemical modification of the rhizosphere to enhance Pi availability

It is obvious that in many ecosystems the low availability of Pi is a major factor determining plant productivity. Plants growing in these environments have to obtain Pi from adsorbed Pi, sparingly soluble Pi, and organic P complexes. Many plants, especially those adapted to low-phosphate conditions, have developed elegant biochemical mechanisms to solubilize inorganic

Pi complexes. They produce and secrete organic acids into the rhizosphere (Shane and Lambers, 2005, in this volume). Organic acids, such as malate, citrate and oxalate release Pi from inorganic complexes in soil by the ligand exchange mechanism. Species such as buckwheat (*Fagopyrum esculentum*), oilseed rape (*Brassica napus*) and legumes are quite efficient in utilizing phosphate rock as a source of Pi by releasing organic acids (Hinsinger, 2001; Holland et al., 1989).

This phenomenon has been thoroughly studied in members of Proteaceae and white lupin that are capable of forming cluster roots or proteoid roots (Dinkelaker et al., 1995; Johnson et al., 1996; Shane and Lambers, 2005, in this volume; Vance et al., 2003). These specialized roots produce and exude large quantities of organic acids into the rhizosphere. Production and secretion of organic acids by proteoid roots concur with the production of phosphatases, extrusion of protons, and increased uptake of Pi by expressing high-affinity Pi transporters (Gilbert et al., 1999; Miller et al., 2001; Sas et al., 2001; Tadano and Sakai, 1991; Vance et al., 2003; Yan et al., 2002). Although proteoid roots excrete large quantities of organic acids, not much is known about the proteins involved in the secretary pathway. Anion channels such as chloride channels and MATE (Multidrug And Toxin Extrusion) proteins have been suggested as potential candidates for organic acid excretion (Vance et al., 2003). Analysis of ESTs from Pi-starved proteoid roots of white lupin identified a putative protein exhibiting homology to a transmem-brane protein belonging to the MATE protein family. This Pi-starvation induced MATE gene has been suggested as potential candidate for organic acid secretion during Pi starvation (Vance et al., 2003). Involvement of anion channels in organic acid excretion was further confirmed by the action of anion channel blockers that decreased carboxylate exudation by nearly 50% (Neumann et al., 1999). Recently, an aluminum activated malate transporter has been described in wheat (Sasaki et al., 2004). In addition to organic acids, phenolic compounds such as piscidic acid and alfafuran are secreted by pigeon pea (*Cajanus cajan*) and alfalfa (*Medicago sativa*), respectively (Ae et al., 1990; Masaoka et al., 1993).

Acquisition of phosphate from organic sources

A major portion of Pi in soil may be present in organic forms. Organic P complexes such as phytic acid may contribute to significant portions (20–80%) of P in soil (Jungk et al., 1993; Richardson, 1994). The organic P complexes need to be broken down by enzymatic activity before the inorganic Pi is released into the rhizosphere. Phosphatases are a group of enzymes produced in higher amounts in Pi-starved plants. Phosphatases produced by plants and microbes may be involved in degradation of organic-P complexes. Induction of phosphatases during Pi deficiency is a universal response in higher plants (Duff et al., 1994). It is presumed that phosphatases secreted into the rhizosphere along with those targeted to the apo-plastic region are involved in releasing Pi from organic sources.

Different types of phosphatases produced by plants are presumed to liberate Pi from organic materials present in intracellular compartments or extracellular spaces (Duff et al., 1994; Goldstein, 1992; Lefebvre et al., 1990; Ueki, 1978). Acid phosphatases (APs) are involved in pH-dependent hydrolysis of monoester soil organic P in the rhizosphere. Extracellular acid phosphatases generally exhibit broad substrate specificity, whereas intracellular APs are much more specific in their: function (Duff et al., 1991). Increased phosphatase activity under Pi deficiency appears to be correlated with enhanced expression of genes coding for phosphatases (Baldwin et al., 2001; Miller et al., 2001; del Pozo et al., 1999; Wasaki et al., 1999). In proteoid roots of white lupin Pi deficiency increased the expression and secretion of phosphatases (Miller et al., 2001; Wasaki et al., 1999). At present not much is known about the quantitative contribution of plant phosphatases to Pi nutrition. However the molecular and biochemical studies suggest that phosphatases are an integral part of Pi starvation response mechanism.

Purple acid phosphatases (PAPs) are among the commonly observed phosphatases secreted into the rhizosphere during Pi deficiency. They represent a distinct class of nonspecific acid phosphatases consisting of binuclear transition metal (Fe(III)-Fe(II), Fe(III)-Mn(II) or Fe(III)-Zn(II)) centers. A family of 29 genes sharing

conserved domains of purple acid phosphatases (PAP) has been identified in the *Arabidopsis* genome. Two members of this family (*AtPAP11* and *12*) were induced under Pi-deficiency conditions (Li et al., 2002). The purple acid phosphatase (*AtACP5*) of *Arabidopsis* is strongly induced under Pi deficiency (del Pozo et al., 1999). Two secreted PAPs have been isolated and characterized from tomato cell suspension cultures (Bozzo et al., 2002). These monomeric proteins exhibit phosphatase activity against a host of phosphate-esters. Increased secretion of these proteins during Pi deficiency suggests that they may be a component of a coordinated Pi-starvation rescue mechanism. In some instances there is no direct correlation between increased phosphatase activity and adaptation of the plants to Pi deficiency (Yan et al., 2001). This highlights the complexity of plant responses to nutrient stress. Some of the phosphatases induced under Pi deficiency may also be involved in protein dephosphorylation, an important component of signal transduction. In this context one of the tomato genes *LePS2*, induced under Pi deficiency may represent this category of phosphatases (Baldwin et al., 2001). Induction of the *LePS2* gene is specific to Pi deficiency and reversible upon replenishment of Pi. A search of genome databases indicates that homologs of *LePS2* are found in many plant species. Three closely related members can be identified in *the Arabidopsis* genome. The deduced peptide contains motifs similar to those found in the members of the HAD and DDDD superfamilies of phosphohydrolases. Even though the substrates for this phosphatase are not yet identified, recent data suggest that LePS2 is likely to act as a phosphoprotein phosphatase.

RNases are another group of enzymes that are presumed to be involved in mobilization of Pi from organic sources during Pi deficiency (Bariola et al., 1994; Green, 1994; Kock et al., 1995). Suppression of *RNS1* and *2* of *Arabidopsis* resulted in higher levels of anthocyanin accumulation in transgenic plants, a common feature observed under Pi deficiency (Bariola et al., 1999). In addition, tomato cells also produce extracellular cyclic nucleotide phosphodiesterases that are thought to function in concert with RNases in releasing Pi from nucleotides (Abel et al., 2000). Activity of several phosphohydrolases increases during Pi deficiency (Bosse and Keck, 1998). Apyrases capable of releasing Pi from extracellular ATP are also induced during Pi deficiency (Thomas et al., 1999).

Concluding remarks

Phosphate deficiency is one of the major factors limiting plant growth and productivity in many ecosystems. Most of the adaptations and acclimations are targeted towards increasing availability, uptake and utilization efficiency of Pi. Marked changes in root morphology and architecture under Pi deficiency help plants to explore soil for the nutrient. Enhanced capacity for uptake of the nutrient is directly correlated, at least in part, to transcriptional activation of high-affinity Pi transporters localized in the plasma membranes of roots and root hairs. The high-affinity Pi transporters belong to a small family of genes showing interesting tissue- and organ-specific expression patterns in plants. In addition to nutrient acquisition these transporters also appear to be involved in mobilization of internal Pi in both root and shoot. Isolation and characterization of low-affinity Pi transporters point to their potential role in transport of the nutrient between organs and organelles.

The enhanced capacity of plants to obtain Pi is coordinated with changes in expression of genes, and activity of proteins involved in Pi mobilization. These include, but are not limited to phosphatases, RNases, nucleases, apyrases and organic acid exudates. Interestingly all these responses are triggered by Pi deficiency. This type of highly coordinated response points to an efficient signal-transduction pathway operating in plants. The search for the components of the signaling pathway is in progress in several laboratories. This process is aided by the availability of a large number of Arabidopsis mutants and microarray analysis of genes expressed under Pi deficiency (Hammond et al., 2003, Uhde-Stone et al., 2003, Wang et al., 2002, Wasaki et al., 2003, Wu et al., 2003). Analysis of Pi-deficiency associated mutants and dissection of the Pi-signaling pathway could help identify key regulators of Pi acquisition and utilization in plants.

Acknowledgments

Research in the first author's laboratory is supported from grants from United States Department of Agriculture (# 2003-35100-13402) and US-Israel Binational Agricultural Research and Development (US-3231-01R). We sincerely appreciate the support of the members of the first author's laboratory.

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Plant and Soil (2005) 274:51–78
DOI 10.1007/s11104-005-5881-5

Root-based N_2-fixing symbioses: Legumes, actinorhizal plants, *Parasponia* sp. and cycads

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Received 28 November 2003. Accepted in revised form 9 March 2004

Key words: actinorhizal plants, coralloid roots, cyanobacteria, cycads, *Frankia*, legume, mutualism, N_2 fixation, *nod* genes, *nif* genes, nodulation, *Nostoc*, *Parasponia*, *Rhizobium*, symbioses

Abstract

In the mutualistic symbioses between legumes and rhizobia, actinorhizal plants and *Frankia*, *Parasponia* sp. and rhizobia, and cycads and cyanobacteria, the N_2-fixing microsymbionts exist in specialized structures (nodules or cyanobacterial zones) within the roots of their host plants. Despite the phylogenetic diversity among both the hosts and the microsymbionts of these symbioses, certain developmental and physiological imperatives must be met for successful mutualisms. In this review, phylogenetic and ecological aspects of the four symbioses are first addressed, and then the symbioses are contrasted and compared in regard to infection and symbio-organ development, supply of carbon to the microsymbionts, regulation of O_2 flux to the microsymbionts, and transfer of fixed-N to the hosts. Although similarities exist in the genetics, development, and functioning of the symbioses, it is evident that there is great diversity in many aspects of these root-based N_2-fixing symbioses. Each symbiosis can be admired for the elegant means by which the host plant and microsymbiont integrate to form the mutualistic relationships so important to the functioning of the biosphere.

Introduction

The ability of a plant to supply all or part of its N requirements from biological nitrogen fixation (BNF) in its roots can be a great competitive advantage over non-N_2-fixing neighbours. BNF is the conversion of atmospheric N_2 to ammonium, a form of N that can be utilized by plants. However, BNF is in the sole domain of certain bacteria (diazotrophs), which contain nitrogenase, the enzyme complex that catalyzes the conversion of N from the gaseous to the combined form. Occurrence of N_2-fixing bacteria with higher plants is not uncommon, but in most case these are only 'associations', in which relatively free-living bacteria grow in the rhizosphere, on the rhizoplane, or more rarely, in non-specialized intercellular spaces in plants (Vessey, 2003). The transfer of fixed N from the bacterium to the plant in these associations is relatively low, and the relationship between the two organisms could be viewed as opportunistic rather than mutualistic. However, in a much smaller proportion of cases across the plant world, the association between plant and bacterium is much more intimate, with the N_2-fixing bacterium being housed within specialized plant organs. In these truly mutualistic symbioses, the genetics and physiology of the plant and bacteria are integrated to the extent that the two organisms can appear to function nearly as one.

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On several occasions, symbioses have evolved between terrestrial plants and N_2-fixing bacteria, leading to the existence of specialized organs on the host plants that provide excellent environments for the prokaryotes to infect, live, and fix N_2. Although the genetic backgrounds and physiological functioning of these symbioses can be seen as very diverse, there are several developmental and physiological 'imperatives' that must be met for successful symbioses between host plants and their N_2-fixing microsymbionts. These imperatives include:

- the ability of the microsymbiont to infect and colonize host plant organs;
- the ability of the host plant to supply energy and nutrients to the microsymbiont;
- the ability of the host plant and microsymbiont to regulate O_2 flux;
- the ability to transfer the fixed N from the microsymbiont to the host.

Four such extant plant root symbioses are the partnerships between legumes and rhizobial bacteria, actinorhizal plants and *Frankia* bacteria, *Parasponia* and rhizobial bacteria, and cycads and cyanobacteria. In this chapter we will compare and contrast these four symbioses using these criteria. However, we will first address evolutionary and ecological aspects of the symbioses. A challenge in comparing these symbioses is the unbalanced level of knowledge that exists for the four symbioses; i.e. the legume–rhizobium symbiosis is very well studied, there is a relatively good level of knowledge on the actinorhizal–*Frankia*, information on the *Parasponia*–rhizobia symbiosis is limited except on some topics (e.g. hemoglobin), and knowledge about the cycad–cyanobacterial symbiosis is scant. Nonetheless, we will see that despite quite varied genetic makeup and evolutionary backgrounds, each of the four symbioses has developed equally elegant means to meet the physiological and developmental imperatives for a successful symbiosis.

Evolutionary and ecological considerations

The four symbioses addressed here represent wide ranges in both evolutionary/phylogenetic and ecological contexts. However, the legume, *Parasponia*, and actinorhizal plant symbioses can be seen as more closely related to each other compared to the cycad symbiosis. This is quite obvious given that the legumes, *Parasponia* and actinorhizal plants are angiosperms (and may even have had a common ancestor, see below), whereas cycads are gymnosperms. From the microsymbiont perspective, there is an extreme diversity with rhizobia (Gram negative) being members of the α-subgroup of the phylum Proteobacteria, *Frankia* (Gram positive) from the high-GC subgroup of the phylum Actinobacteria, and the Cyanobacteria (Gram negative) representing their own phylum of photoautotrophic non-proteobacteria.

Cycads represent an ancient life form, have a unique placement in terrestrial plant evolution, and are among the most primitive extant seed-plants (Brenner et al., 2003; Schneider et al., 2002). They are evergreen and have a palm-like appearance with a thick, often columnar stem and rosettes of long pinnately compound leaves (see Costa and Lindblad, 2002; Lindblad and Bergman, 1990). They may range in height from about 0.2 to 20 m. The presence of prominent reproductive cones (male and female) reveals their true gymnosperm nature. Extant cycads may be divided into three families: Cycadaceae with the genus, *Cycas*; Stangeriaceae with the genera, *Stangeria* and *Bowenia*; and Zamiaceae comprising eight genera, *Zamia*, *Chigua*, *Ceratozamia*, *Dioon*, *Encephalartos*, *Lepidozamia*, *Microcycas* and *Macrozamia*. Together, about 240 extant species have been identified within the order Cycadales. Nucleotide sequencing of the *rbcL* gene suggests that the cycad genera cluster monophyletically, and that the genus *Cycas* forms a basal group (Treutlein and Wink, 2002). Molecular data also imply that extant cycad genera and species may not have evolved until within the last 50 million years. Cycads dominated the Earth's forests from Greenland and Alaska to Antarctica about 250–65 Myr ago, long before the advent of angiosperms (Brenner et al., 2003; Schneider et al., 2002). During this period the climate was warmer, wetter and low in seasonality. As CO_2 levels were also higher, this may have enhanced plant growth and the N demand, which in turn may have stimulated the development and maintenance of the cycad–cyanobacterial symbiosis. Hence, the well-developed symbiotic relationship between cycads and cyanobacteria may be due to a long-lasting co-evolution between the organisms. As the

cyanobacteria are ancient organisms that arose some 3 Byr ago (Schopf et al., 2002), symbiotically competent cyanobacteria may have been widespread long before the cycad started to dominate the global terrestrial vegetation.

Today, the ecological distribution of cycads is considerably more limited. Many cycads are endangered due to the drastic climate change, commercial exploitation, and anthropogenic activities. Species within the *Cycas*, *Encephalartos* and *Macrozamia* genera are still well represented, predominantly in warmer and more humid tropical and subtropical regions. Species within the genera *Macrozamia* and *Zamia* are also common in dryer soils of low fertility in Australia, and often comprise an understorey vegetation of *Eucalyptus* forests (Grove et al., 1980). More than 35 species of the genus *Encephalartos* (endemic to Africa) constitute the large cycad flora in South Africa, while *Stangeria* is endemic to its coastal regions (Grobbelaar et al., 1986). Due to their highly decorative appearance, cycads are widely cultivated and used as ornamental plants in greenhouses and in private and public botanical gardens world-wide.

The capacity of cycad–cyanobacteria symbioses to fix N_2 has been confirmed in a limited number of field and laboratory studies (see Rai et al., 2000, 2002). Grobbelaar et al. (1986) also demonstrated that all 33 species of *Encephalartos* and one *Stangeria* tested, using the acetylene reduction technique as well as ^{15}N-enrichment, were capable of fixing N_2. In the field, two *Macrozamia riedlei* stands growing naturally in Western Australia in *Eucalyptus* forests fix 19 kg N ha^{-1} yr^{-1} (Halliday and Pate, 1976) and about 8 kg N ha^{-1} yr^{-1} (Grove et al., 1980), respectively. These rates are essentially similar to those of free-living cyanobacteria, while much lower than those recorded for legume–rhizobia symbioses (see below). However, the much lower growth rate of cycad tree species compared to annual legume crop species should be taken into consideration when comparing N_2 fixation rates between these two symbioses. The proportion of cycad N is derived from fixation of atmospheric N (%NDFA) is not known. Additional field studies are needed to fully estimate the N_2-fixing capacities and ecological significance of cycad–cyanobacterial symbioses.

In contrast to the cycads, legumes, *Parasponia* and actinorhizal plants are flowering plants encompassing families all found within the Eurosid I clade of the Eudicots (Soltis et al., 2000). Legumes are within the order Fabales and represented by a single family, the Fabaceae (formerly the Leguminosae); however, most of the more than 650 genera in the family contain species that can form rhizobial root nodules. *Parasponia* is one of the 18 genera of the Ulmaceae (order Rosales), and is the only genus outside the legumes known to enter an N_2-fixing symbiosis with rhizobia. There are only five identified species of *Parasponia* (Becking, 1992), with *P. andersonii* appearing to be the most commonly studied. In contrast, actinorhizal plants (which are simply defined as a group by their ability to be nodulated by *Frankia* bacteria) are more taxonomically diverse. They encompass 25 genera, in eight different families, in three different orders: the Betulaceae, Casuarinaceae and Myricaceae of the order Fagales; the Rosaceae, Rhamnaceae and Elaeagnaceae of the order Rosales; and the Coriariaceae and Datiscaceae of the order Cucurbitales (Gualtieri and Bisseling, 2000).

It was once thought that apparent taxonomic diversity among legumes, *Parasponia* and actinorhizal plants indicated that N_2-fixing symbioses in flowering plants evolved independently several or possibly many times (Baker and Mullin, 1992). However, nucleotide sequencing of the *rbcL* gene of 99 taxa in the Eurosid I lineage infers that all eight families capable of N_2-fixing symbiosis exist within a 'nitrogen-fixing clade' (Soltis et al., 1995). These data suggest a single origin of the predisposition for nodulating, N_2-fixing symbioses in flowering plants. If there was only one origin, then the fact that not all extant taxa in the N_2-fixing clade are capable of N_2-fixing symbiosis implies a loss of the capacity in those taxa. This loss might have been due to high metabolic cost to the plant for N_2 fixation compared to assimilation of combined N from the soil (Layzell, 1990). However, although the potential for nodulation may have a common ancestry, analysis of symbioses-related genes appears to indicate that actual symbioses have arisen multiple times within the clade, at least among both the legumes and actinorhizal plants (Doyle, 1998; Swensen, 1996).

Origins of the legume, *Parasponia* and actinorhizal plant symbioses are highly likely to be much more recent than the cycad–cyanobacteria symbiosis given that angiosperms did not evolve until

250–150 Myr ago (Sprent and Raven, 1992), a period when cycads were already the dominant flora on the planet. Today, legumes are the third-largest family of flowering plants with over 18,000 species; however, not all species are capable of forming N_2-fixing symbiosis. Legumes are an incredibly morphologically and ecologically diverse group of plants, ranging from small forbs to large trees, and occur from the Arctic tundra, to tropical rainforests, to arid deserts (Allen and Allen, 1981). The legumes represent many of our most important grain and forage crops including soybean (*Glycine max* L. Merr.), common bean (*Phaseolus vulgaris* L.) and alfalfa (*Medicago sativa* L.). Peoples et al. (2002) have shown that across a wide range of environments and species, legumes commonly fix approximately 25 kg N per tonne of aboveground dry matter. With root biomass also taken into account, the amount of N_2 fixed by legumes can easily be in the range of 300–400 kg N ha^{-1} yr^{-1} (Kelner et al., 1997; Peoples et al., 2002).

Actinorhizal plants represent approximately 200 species of woody shrubs and trees predominantly in temperate climes but also extending into the tropics, especially the Casuarinaceae. Like the wild (non-crop) legumes, actinorhizal plants have a propensity to grow in marginally fertile soils, and many as early-successional plants (Benson and Silvester, 1993). Hence actinorhizal plants play extremely important roles in the N cycle of forests and in the re-vegetation of various landscapes (Benoit and Berry, 1990; Schwencke and Caru, 2001). Rates of N_2 fixation in the range of 30–50 g N $tree^{-1}$ $season^{-1}$ are possible, but actual rates in the field are often lower due to environmental stresses such as drought, nutrient limitation, or nematode predation (Dommergues, 1995). For example, Mariotti et al. (1992) found N_2 fixation rates of only 15 kg N ha^{-1} yr^{-1} for a 3-year old *Casuarina equisetifolia* stand in Senegal. Actinorhizal plants have been used in erosion control, soil reclamation, agroforestry and dune stabilization, as well as in fuel wood, pulp and timber production. For instance, Casuarinaceae are utilized in stabilizing desert and coastal dunes (i.e. in shelter belts), and in the reclamation of salt-affected soil as well as in intercropping systems (Diem and Dommergues, 1990).

It was only 30 years ago that Trinick (1973) first reported the N_2-fixing, root-nodule-forming symbiosis between *Parasponia* (initially classified as *Trema* sp.) and rhizobia. The *Parasponia*–rhizobia symbiosis is represented by only five species of tropical tree which can grow up to 15 m and are native to the Indo-Malaysian Archipelago and the Pacific Islands, from Sumatra in the east to as far west as Polynesia (Becking, 1992; Webster et al., 1995a). As with the other symbiosis these trees are often pioneering species in very nutrient-poor soils (e.g., volcanic ash). Despite its microsymbionts being rhizobia, *Parasponia* is taxonomically and phylogenetically closer to members of the actinorhizal members of the Rosaceae, Rhamnaceae and Elaeagnaceae than to the legumes (Soltis et al., 1995). Quantification of N_2 fixation in *Parasponia* are rare and no doubt reflect the challenges in making such measurements in trees species, as is the case in many actinorhizal plants. However, acetylene reduction assays of intact and detached *Parasponia* nodules generally indicate that nitrogenase activity per unit mass of nodule is lowers than that seen in legumes by 0.5–1.0 orders of magnitude (see Becking, 1992). It is particularly interesting that when rhizobial isolates from *Parasponia* were used to infect the legumes *Vigna* sp. (Becking 1983a) and *Macroptilium atropurpureum* (Price et al., 1984), the nitrogenase activity per unit mass fresh weight of the legume nodules was approximately three times that of the *Parasponia* nodules. These results suggest that *Parasponia* as a host is more responsible for the lower levels of nitrogenase activity (compared to the legumes) than its microsymbiont. Nonetheless, from observations of its growth rate under optimal conditions in a glasshouse, Trinick (1981) estimated that a plantation of 6-month-old *Parasponia* might fix as much as 850 kg N ha^{-1} yr^{-1}.

On the microsymbiont side, the phylogenetic relationships between rhizobia, *Frankia*, and cyanobacteria can be considered even more distant than those of their plant hosts, with each group coming from separate eubacterial phyla. Rhizobia is a generalized term referring to bacteria from the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*. Individual legume species are often infected by a single species of rhizobia, but in some cases multiple species, even in some

cases from multiple genera, infect a single host species (e.g., soybean [*Glycine max*] is infected by *Brayrhizobium japonicum*, *B. elkanii*, and *Sinorhizobium fredii*). *Parasponia* is infected by rhizobial strains belonging to the *Bradyrhizobium* and *Rhizobium* genera. Although there is cross-inoculation between rhizobial isolates from *Parasponia* and some legumes (e.g., *Macroptilium* sp. and *Vigna* sp.) (Becking, 1992), *Parasponia* strains tend to display specificity for *Parasponia* as a host. For example, although *Bradyrhizobium* strains isolated from a wide range of legume hosts could induce nodules on *Parasponia*, the nodule morphology was commonly abnormal, and there was little or no nitrogenase activity (Becking, 1983a; Trinick and Galbraith, 1980).

Actinorhizal plants are only nodulated by strains of actinomycetes from the genus *Frankia*. Whereas 'host-specificity' (the selectivity of the plant host for specific species, or even subspecies, of microsymbiont) is useful in species designation in the rhizobia, this phenomenon is not nearly as effective in *Frankia*, and even with the abundance of molecular tools at our disposal, identification of speciation in the genus is still a difficult task (Benson and Silvester, 1993; Clawson and Benson, 1999).

All cycad genera are capable of forming well-developed root-symbioses with cyanobacteria, and filamentous heterocystous species within the genus *Nostoc* are the dominant microsymbionts (Rasmussen and Nilsson, 2002), although others have been identified (e.g. *Calothrix* sp.; Grobbelaar et al. 1987). Considering the large variety of terrestrial cyanobacterial genera, the dominance of the genus *Nostoc* in cycad and other plant symbioses implies that specific symbiotic characters are held by this genus. In spite of the relatively narrow diversity among the cyanobacterial cycad colonizers, a recent study using PCR fingerprinting with primers derived from short tandemly repeated repetitive sequences demonstrated that a single root cluster can be colonized by several strains of the cyanobiont (Zheng et al., 2002).

The phylogenetic diversity among the microsymbiont groups in the four symbioses under discussion is not surprising. Although the structure, function, and amino acid sequence of nitrogenase is highly conserved throughout the domain, the ability to fix N_2 is 'sprinkled' throughout many taxa of the Eubacteria (Reinhold-Hurek and Hurek, 1997). This situation of a highly conservative genetic trait being widely and diversely spread among taxa is more comprehensible if horizontal (lateral) transfer of aspects of this trait were at play (Zehr et al., 2003). Another possibility is that widely spread symbiosis genes have been lost in some organisms through evolution (e.g., due to increases in N contents of soils and water), and that only those in which N_2 fixation was a competitive advantage for survival retained them. Analysis of partial *nifH* gene products (a common marker for the enzyme nitrogenase reductase) demonstrates a very broad phylogenetic range among N_2-fixing prokaryotes (Figure 1). Interestingly enough, rhizobia, *Frankia*, and cyanobacteria cluster together more closely than many other diazotrophs (Reinhold-Hurek and Hurek, 1997; Zehr et al., 2003). Many nitrogenase-encoding genes (*nif* genes) as well as genes related to enabling symbiosis with host plants (e.g., *nod* genes) are clustered in 'symbiotic islands' on the chromosome or on plasmids as in rhizobia and *Frankia* (Lavire and Cournoyer, 2003). There is strong evidence for lateral transfer of some symbiosis genes within rhizobia (Parker et al., 2002; Qian et al., 2003; Suominen et al., 2001) and some evidence for such transfer in *Frankia* (Lavire et al., 2001; Lee et al., 2001).

An interesting evolutionary convergence among rhizobia, *Frankia* and cyanobacteria is the occurrence of NiFe hydrogenase-uptake systems in all four microsymbionts. The reduction of protons to H_2 gas by nitrogenase is an obligatory process in N_2 fixation in all four symbioses. For example, in legume-rhizobia symbioses, commonly 25–60% of electron flow through nitrogenase may be used to produce H_2 (Layzell, 1990). The ATP and reductant utilized to form this H_2 by-product can be seen as lost energy, as there is no apparent useful function for H_2 in the microsymbiont or the host. In some diazotrophs scavenging systems have evolved, so-called uptake hydrogenases (Hup+), which oxidize the H_2 produced by nitrogenase, recovering the ATP and reducing power. Uptake hydrogenase is widespread in rhizobia (Baginsky et al., 2002), and the small and large subunits of this heterodimeric enzyme are coded for by the *hupS* and *hupL* genes, respectively. Specifics on the uptake hydrogenase of the *Parasponia*–rhizobia symbiosis are not known, but the system is known to be active due to the negligible levels of H_2 evolution from nodules as a proportion of total nitrogenase activity

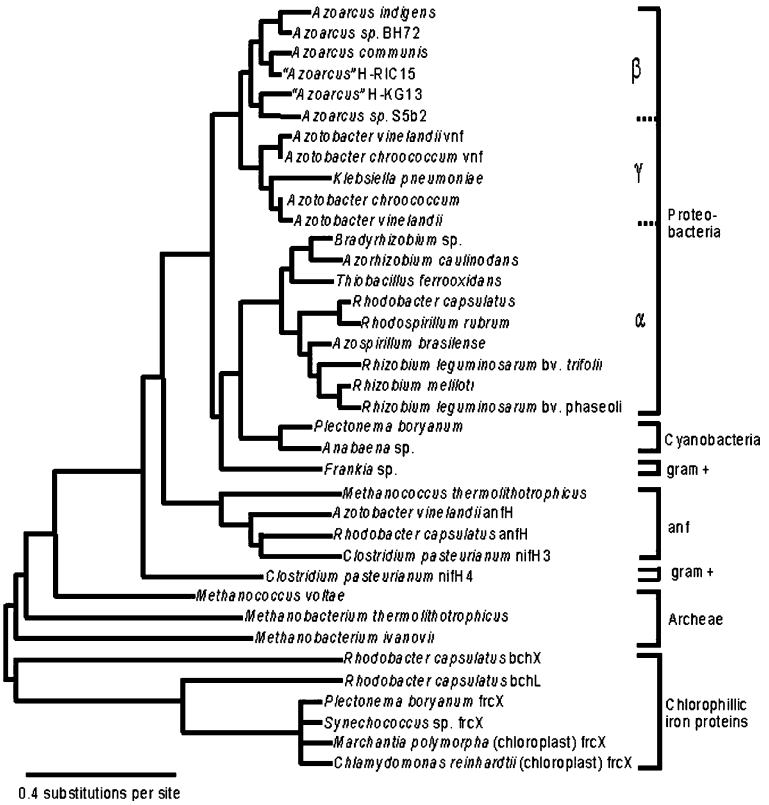

Figure 1. Phylogeny of partial NifH protein sequences from a wide range of diazotrophic bacteria and Archea. Also included are partial sequences of NifH-like iron–sulfur proteins associated with chlorophyll (bchXL and frxC). Designations 'vnf' and 'anf' refer to sequences associated with the vanadium and the iron-only (non-Mo) forms of nitrogenase, respectively. Adapted from Heinhold-Hurek and Hurek (1997).

(Becking, 1983b). Likewise, uptake hydrogenase activity is widespread in *Frankia* (Benson et al., 1980; Mattsson and Sellstedt, 2002; Roelofsen and Akkermann, 1979). Antisera raised against the large subunit of *Bradyrhizobium japonicum* hydrogenase recognized this protein in *Frankia* KB5 (Mattsson et al., 2001). Recently the two filamentous heterocystous cyanobacteria *Anabaena* strain PCC 7120 and the symbiotically competent *Nostoc punctiforme* (*Nostoc* PCC 73102), have been shown to contain uptake hydrogenases coded for by the *hupSL* genes (Tamagnini et al., 2002).

Establishment of the symbioses: Signal exchange, infection, and symbio-organ development

Although all four N_2-fixing root symbioses result in specialized root organs to house the microsymbionts, the establishment of these organs and their structure is quite different. While the root nodules of actinorhizal plants and the symbiotic coralloid roots of cycads are modified roots, the root nodules formed in the legume–rhizobia symbiosis arise from unique zones of cell division in the root cortex. In this section we will first discuss signal exchange between the partners in each symbiosis, and then address the actual infection and symbio-organ development.

Signal exchange

The establishment of the legume–rhizobia symbioses is initiated by the exudation of flavonoid and isoflavonoid compounds (e.g., genistein, naringenin, luteolin) from the host plant (Miklashevichs et al., 2001). These substances act as both chemoattractants to the rhizobia and inducers of the *nod* genes (i.e., the regulatory *nodD* genes and the structural *nodABC* genes) in the rhizobia (see

below). Other substances implicated in chemo-attraction and proliferation of rhizobia in legume rhizosphere include nutrients (amino acids, organic acids, and sugars; e.g., Pandya et al., 1999; Robinson and Bauer, 1993) and secondary metabolites such as betaines (Phillips et al., 1995). Likewise, the non-protein amino acid toxin, mimosine, produced by some tree and shrub legumes, enhances the abundance of specific rhizobial strains in the rhizosphere (Soedarjo and Borthakur, 1998).

There is no information on the excretion of signalling compound from *Parasponia* roots to its microsymbiont. However, given that the nodulation genes *nodABC* and *nodD* are highly conserved in rhizobia, including *Parasponia*–rhizobia (Marvel et al.; 1987; Scott et al., 1987), we can infer that flavonoid and/or isoflavonoid compounds are exuded by the plant to induce these *nod* genes (Bender et al., 1987a).

There is some evidence of chemo-attraction and proliferation of *Frankia* in the rhizospheres of *Betula pendula* (Smolander et al., 1990) and *Alphitonia neocaledonica* (Gauthier et al., 2000), non-nodulating species of the Betulaceae and Rhamnaceae, respectively, but the nature of the exuded substances is unknown. The actinorhizal species *Alnus glutinosa* exudes flavonols (e.g., quercetin and kaempferol) that can enhance the level of nodulation; however, their exact role in the process is unknown (Hughes et al., 1999). Likewise, the process of chemo-attraction of *Nostoc* to the cycad rhizosphere is not clear, although chemo-attractants are operative in other cyanobacterial–plant symbioses (Bergman et al., 1996; Knight and Adams, 1996). Symbiotically competent cyanobacteria are attracted at least to some non-cycad host plants, such as liverworts and *Gunnera*, but no signal/attractant compounds have been identified to date. A low-molecular-weight compound has been identified, but not chemically characterized; it induces the infectious form of the cyanobacteria, i.e. hormogonia (a highly motile form of the filamentous organism) in both liverworts and *Gunnera* (Adams, 2002; Bergman, 2002). Even roots of non-host plants, such as rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) attract cyanobacteria and release hormogonia-inducing factors (Bergman et al., 2004). Cycads are also well known for producing several secondary metabolites such as flavones, azoglucosides and neurotoxic non-protein amino acids (e.g., β-methylamino-L-alanine) (Brenner et al., 2003; Schneider et al., 2002). Whether these cycad root compounds function as chemo-attractants for cyanobacteria or only in preventing infection by other microorganisms (from incompetent cyanobacteria to bacteria and fungi) are open questions.

As referred to above, flavonoid/isoflavonoid compounds exuded by legume roots (and presumably *Parasponia*) induce the *nod* genes in rhizobia. The interaction of the rhizobial regulatory NodD protein with specific flavonoids is believed to be the first level of host-specific recognition in the symbiosis. The consequence of *nod* gene expression is the bacterial synthesis of the nodule-inducing, 'Nod factor' (see Miklashevichs et al., 2001). Nod factors are lipo-chito-oligosaccharides (LCOs) whose exact structure is a component of the second level of host-specific recognition in the symbiosis (i.e. only certain LCOs can initiate nodulation in certain legumes). Very recently, two putative Nod-factor receptor kinase gene (NFR1 and NFR5) have been identified in *Lotus japonicus* (Madsen et al., 2003; Radutoiu et al., 2003). Perception of Nod factor by the host legume results in numerous responses involved in infection and nodule formation, including root hair deformation, development of preinfection threads, cortical cell divisions, and induction of nodule-specific genes expressed early in nodule development (*ENOD* genes; Miklashevichs et al., 2001; Schultze and Kondorosi, 1998). Aside from Nod factor, there are a plethora of other rhizobial cell-surface and excreted compounds implicated in symbiosis-oriented signaling functions including bacterial polysaccharides (Price, 1999), the phytohormones, IAA (indole acetic acid) and cytokinins (Hirsch et al., 1997), and most recently low-molecular-weight proteins (nodulation outer proteins or Nops) (Marie et al., 2003).

Equivalents of rhizobial Nod factors have not been identified for *Frankia*. Root-hair deformation occurs in some actinorhizal plants in response to supernatants of *Frankia* cultures induced (Van Ghelue et al., 1997) or non-induced (McEwan et al., 1992) by root exudates, but also in response to substances produced by other soil bacteria (Knowlton et al., 1980). Attempts to purify *Frankia* Nod factors using the protocol developed for rhizobial Nod factors have failed, indicating chemical differences between both

types of molecules (Cérémonie et al., 1999). An *ENOD40* gene promoter, which is present in legumes and at least one actinorhizal species, *Casuarina glauca*, is induced during nodule induction in legumes by Nod factor, but not in actinorhizal plants (Santi et al., 2003b). Attempts to isolate *Frankia nod* genes by complementation of rhizobial mutants have failed as well, probably due to the fact that most *Frankia* promoters do not work in Gram-negative bacteria (Lavire and Cournoyer, 2003). It will be necessary to identify a promoter from an actinorhizal plant that is induced by bacterial signal factors in order to develop a reliable bioassay. There is no evidence at this time that the production of phytohormones by *Frankia* might be involved in the initiation of host-responses. Although *Frankia* produces cytokinins (Stevens and Berry, 1988) and pseudoactinorhiza (i.e., empty nodules) can be induced by cytokinins in some cases (Rodriguez-Barrueco and de Castro, 1973; C. Santi, C. Franche and E. Duhoux, personal communication), there is no evidence that cytokinins synthesized by *Frankia* are involved in nodule induction.

Homology to a few *nod* genes was detected in both compatible and non-compatible cyanobacteria as well as in cyanobacteria of the *Azolla* symbiosis (Plazinski et al., 1991; Rasmussen et al., 1996), however it was later found that an equivalent to *nodM* had no symbiotic relevance (Viterbo et al., 1999). Phenolics are also present in cycad symbiotic coralloid roots (Obukowicz et al., 1981), but it is unknown if these are involved in signalling to the microsymbiont. Extremely little is known about signals from the cyanobacterium microsymbiont which might influence development of its cycad host. Invading cyanobacteria trigger the development of a 'cyanobacterial zone' in a cell layer in the cortex or a layer underlying the root cap of coralloid roots (see below). These changes suggest that the invading cyanobacterium excretes a growth factor, which influences tissue development. There is some indirect evidence that this factor may be the phytohormone IAA (Sergeeva et al., 2002). Recent studies indicate that, like in rhizobia, surface-related or released proteins are up-regulated during hormogonium differentiation in a symbiotically competent *Nostoc*. These may be involved in aspects of symbiotic competence, such as in recognition and inter-organism signaling, in identification of target plant cells, or in camouflage to avoid plant defense responses (Klint et al., 2003).

Infection and symbio-organ development

The infection of the host by the microsymbiont, and the development of the root nodule/tissue to house the microsymbiont are very different among the four symbioses. Even within the legume–rhizobia symbiosis, there is considerable diversity of modes of infection and nodule development (Figure 2). Depending on the particular legume, the path of infection may be intercellular (Figure 2d and e) intracellular (Figure 2b, g, h) or a combination of the two (Figure 2c and f). Intracellular infection occurs via infection threads, tubular cell-wall-like structures constructed by the plant involving extensive cytoskeleton activity, the synthesis of which is initiated by bacterial Nod factor (Lhuissier et al., 2001).

The most intensively studied symbioses involve crop legumes (e.g., common bean, cowpea (*Vigna sinensis* [L.] Savi ex Hassk.), fababean (*Vicia faba* L.), pea (*Pisum sativum* L.), soybean) of the Papilionoideae subfamily with intracellular infections leading to either indeterminate (Figures 2g, 3b and 4b) or determinate root nodules (Figures 2h, 3a and 4a). Nodule primordia are initiated opposite to protoxylem poles. However, primordia of indeterminate nodules are initiated within the inner-root cortex, while determinate nodules are initiated in the outer-root cortex. Indeterminate nodules maintain a persistent apical meristem and continue to grow throughout the lifespan of the nodule, thus resulting in a developmental gradient within the nodule with a bacteroid differentiation zone, mature zone (where N_2 fixation occurs), and senescence zone (Figure 4b). Indeterminate nodules tend to be oblong in shape and, depending on the legume, may form singular or bifurcated nodules. Determinate nodules (Figures 3a and 4a) do not maintain an active meristem, are more spherically shaped, and have a defined lifespan (Vikman and Vessey, 1993). In both types of nodules, infection-thread development and initial cortical cell divisions of nodule primordia occur simultaneously. The bacteria move through the infection thread matrix and are 'released' into the infected cells of the developing nodule engulfed in a plant membrane (the peribacteroid membrane). Recently the *Sen1* gene, a plant gene involved in

the differentiation of bacteria into bacteroids, has been identified in *Lotus japonicus* (Suganuma et al., 2003). The structure containing the bacteroids, the symbiosome, is analogous to a plant organelle with the peribacteroid membrane overseeing the traffic into and out of the symbiosome (Day and Udvardi, 1993; Day et al., 2001). The symbiosome may contain one or several rhizobia that have undergone certain morphological changes associated with their new symbiotic lifestyle, and in this symbiotic state are referred to as bacteroids (Oke and Long, 1999). Again depending on the legume, a single nodule may contain several strains of rhizobia, but in the majority of cases a nodule hosts a single strain of rhizobia (e.g., Martinez-Romero, 2003). However, it is common that a single plant root will contain more than one strain of rhizobia among its nodules (e.g., Bromfield et al., 2001; Denison, 2000).

A plethora of nodule-specific plant genes are expressed during the development and functioning of legume nodules (Schultze and Kondorosi, 1998; Trevaskis et al., 2002). These genes have been characterized into two groups depending on whether the proteins they code for are synthesized early or late in nodule development (the early and late nodulins, respectively). Many of the early nodulin (*ENOD*) genes are associated with induction of root-hair deformations, cortical cell division, and cell-wall modifications (including *ENOD*s 2, 5, 10, 11, 12, and 40). The late nodulin genes (commonly classified *NOD* or simply *N* genes) are more closely associated with the mature N_2-fixing nodule. Genes coding for leghemoglobin are classical examples of late nodulins; however, some of their expression is as early in nodule development as some *ENOD* genes (Heidstra et al., 1997). Genes encoding proteins associated with carbon and nitrogen metabolism (e.g., Colebatch et al., 2002a; Silvente et al., 2003), and membrane transport (e.g., Kapranov et al., 2001; Szczyglowski et al., 1998) in the nodules are commonly studied late nodulins.

Like rhizobia, *Frankia* strains can enter the roots of their host plants either intracellularly, via root hairs, or intercellularly, depending on the host plant species (Miller and Baker, 1986; Racette and Torrey, 1989). Early work by Benson and Hanna (1983) using whole-cellular protein profiles indicated more than one genetically distinct *Frankia* within the same actinorhizal

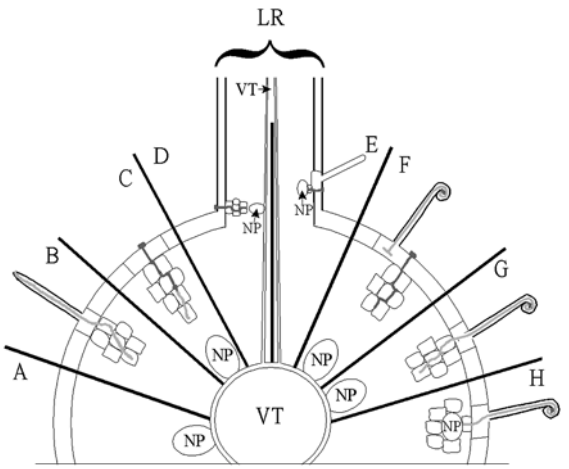

Figure 2. Diagrammatic illustration of examples of the diversity of infection and nodule-initiation patterns in legume–rhizobia symbioses. (a) Spontaneous nodulation in the absence of rhizobia as seen in some genotypes of *Medicago* sp. Nodule primordia (NP) are spontaneously initiated in the inner root cortex (adjacent the vascular tissue, VT). (b) Commonly in members of the Caesalpinioideae (the oldest legume subfamily) nodules do not form. The rhizobia enter the root through root hairs, and exist within intracellular infection threads (light grey tracing). (c) In some tree species of the subfamily Mimosoideae (e.g., *Mimosa scabrella*), infection occurs intercellularly (dark gray tracing) between intact epidermal cells and outer cortical cells, but an intracellular infection thread develops in the inner cortex. An NP, which will develop into an indeterminate-type nodule, is initiated in the cortex adjacent to the VT. (d) In some aquatic legumes of the Mimosoideae (e.g., *Neptunia natans*), infection occurs via crack entry through the epidermis at the junction of a lateral root (LR). As the rhizobia move toward nodule primordia initiated in the inner cortex of the lateral root, an intercellular infection thread develops. The nodule primordia give rise to indeterminate nodules. (e) In *Arachis hypogaea* (peanut; groundnut), a member of the subfamily Papilionoideae, infection occurs intercellularly at the base of an epidermal cell having a root hair. An infection thread does not develop. Rhizobia enter an NP initiated in the outer cortex of a LR which leads to a determinate-type nodule. (f) In *Chaemaecytisus proliferus*, tagasaste, a woody species of the Papilionoideae, initially infection occurs via 'shepherd's crook' root hairs, and an infection thread which initiates a NP in the inner root cortex. However, this infection thread aborts early after initial formation and the NP receives rhizobia via a secondary, intercellular (non-infection thread) infection. The NP develops into an indeterminate-type nodule. (g) In a large number of genera used in agriculture (e.g., *Lens*, *Medicago*, *Pisum*, *Trifolium*, *Vicia*) of the Papilionoideae, rhizobia enter shepherd's crook root hairs via an intracellular infection thread. The infection threads deliver the rhizobia to NP initiated in the inner cortex which gives rise to indeterminate-type nodules. (h) In another large group of crop genera (e.g., *Glycine*, *Lotus*, *Phaseolus*, *Vigna*) of the Papilionoideae, rhizobia also enter shepherd's crook root hairs via an intracellular infection thread. However, the infection threads deliver the rhizobia to NP initiated in the outer root cortex, which gives rise to determinate-type nodules. Adapted from Guinel and Geil (2002).

nodule. However, *in situ* hybridization based on a 23S rRNA insertion target which allowed for the analysis of *Frankia* populations in nodules of various *Alnus* species revealed the presence of only one *Frankia* population in every nodule homogenate (Zepp et al., 1997). However, in *Ceanothus caeruleus*, a host that *Frankia* infects intercellularly (in contrast to *Alnus*, which is infected intracellularly via root hairs), nodules also contained a non-fixing, non-nodulating actinomycete strain (Ramirez-Saad et al., 1998).

Intracellular infection takes place in the Betulaceae, Casuarinaceae and Myricaceae. When a *Frankia* hypha is trapped in a root hair curl, an infection thread develops by which the hypha enters the plant root (Berg, 1999; Berry and Torrey, 1983; Callaham et al., 1979). No equivalent of the infection thread matrix (i.e., a space encompassed by the infection thread walls) of legumes exists in actinorhizal symbioses. Instead, *Frankia* hyphae are embedded within the cell-wall-like material or 'interfacial matrix' (Berg, 1999), which is the equivalent of the infection thread wall in legume nodules. As in the legume–rhizobia symbiosis, concomitant with root-hair infection, cell divisions are induced in the root cortex. The infection thread grows to the dividing cortical cells, and infects some of them by intense branching within the cells, filling them with *Frankia* hyphae from the inside outward (Burgess and Peterson, 1987; Schwintzer et al., 1982). This cortex-based structure is called the prenodule. While the prenodule develops, the formation of a nodule primordium is induced in the pericycle of the root, like in the case of lateral root primordia (Callaham and Torrey, 1977). Note that this is in contrast to legume–nodule primordia which are initiated in the inner or outer root cortex (Figure 2), not the root pericycle. However, like lateral roots and legume–nodule primordia, actinorhizal nodule primordia are usually located opposite protoxylem poles. Hyphae in the infection threads grow from the prenodule to the nodule primordium, again by cell-to-cell passage, and infect primordium cells. The nodule primordium develops into a nodule lobe. Depending on the host plant species, more than one nodule primordium can be formed per prenodule (Torrey and Callaham, 1979).

During intercellular infection, *Frankia* hyphae enter the root between epidermal cells, and colonize the root cortex intercellularly (Miller and Baker, 1985; Racette and Torrey, 1989). In contrast

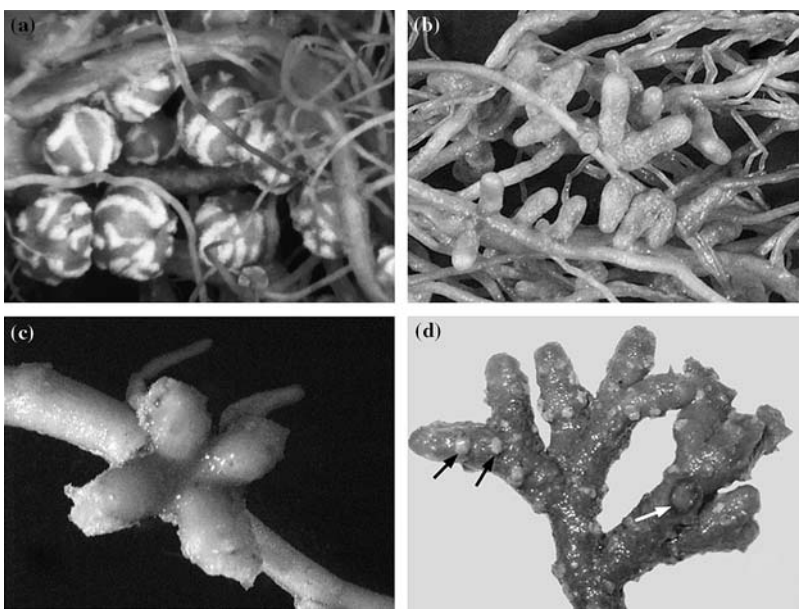

Figure 3. Examples of (a) determinate legume nodules (*Glycine max*), (b) indeterminate legume nodules (*Pisum sativum*), (c) an actinorhizal nodule (*Datisca glomerata*), and (d) a portion of a symbiotic coralloid root cluster (*Cycas revoluta*). In the coralloid root cluster (d), the black arrows indicate lenticels and the white arrow indicates where a root lobe has been cross sectioned to reveal the cyanobacterial infected zone as a darker ring.

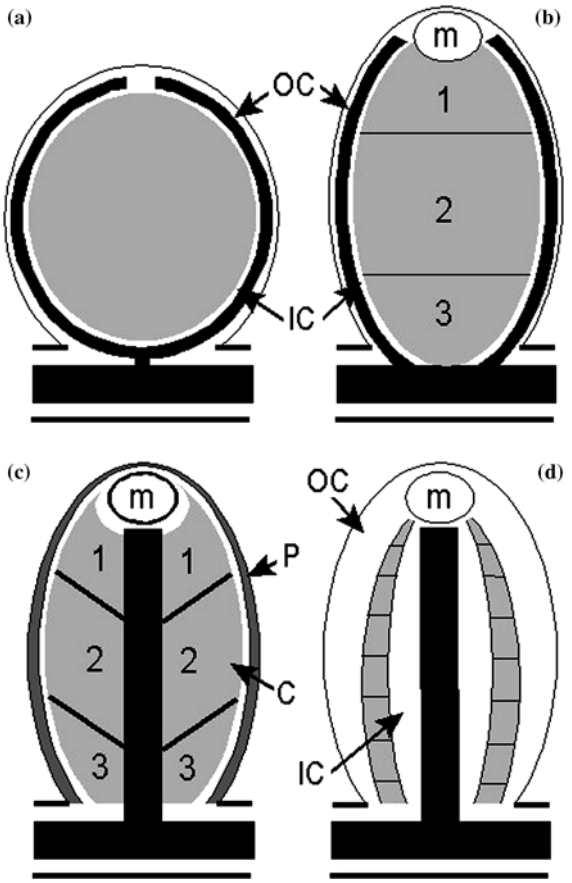

Figure 4. Diagrammatic representation of longitudinal sections through (a) a determinate legume nodule, (b) an indeterminate legume nodule, (c) an actinorhizal nodule, and (d) a lobe of a symbiotic coralloid root cluster. The grey colored regions represent the infected zones. The dark, thick lines represent vascular tissues. Cortical tissue (C), outer cortical tissue (OC), inner cortical tissue (IC) and meristems (m) and periderm tissue (P) are indicated in the various symbio-organs. In the indeterminate legume nodule (b) and the actinorhizal nodule (c), the zones of infection/differentiation (1), N_2-fixation (2) and senescence (3) are indicated.

to rhizobia, *Frankia* does not depend on gaps in the root epidermis for entering the root. During the colonization of the cortex, the root cortical cells secrete an electron-dense pectin- and protein-rich material into the intercellular spaces, and the formation of a nodule primordium is induced in the root pericycle (Liu and Berry, 1991; Valverde and Wall, 1999). *Frankia* hyphae infect primordium cells from the apoplast by intense branching of hyphae, concomitant with continuous invagination of the plant plasma membrane. Intercellular infection takes place in host plants of the Rhamnaceae, Elaeagnaceae and Rosaceae families. In host plants of the actinorhizal Cucurbitales (*Datisca* and *Coriaria*), the infection mechanism has not been examined yet, but since no prenodules or infection threads are found in these plants, infection is assumed to follow the intercellular pathway. In contrast with all other host plants examined, infected cells of *Datisca* and *Coriaria* nodules are filled with branching *Frankia* hyphae from the periphery inward, instead of from the center outward, and they retain large central vacuoles (Hafeez et al., 1984; Newcomb and Pankhurst, 1982). Another unique feature of actinorhizal Cucurbitales is that their nodule cortical cells become multinucleate prior to infection.

Frankia induce the formation of multiple 'lobed' root nodules composed of modified lateral roots without root caps, a superficial periderm, a central vascular system (in contrast to the peripheral vasculature of legume nodules), and infected cells in the expanded cortex (Figures 3c and 4c). Due to the activity of the apical meristem, the infected cells in the expanded cortex are arranged in a developmental gradient in a similar fashion to indeterminate nodules of legumes. In the infection zone, infected calls gradually fill with branching *Frankia* hyphae. Once an infected cell is filled with branched hyphae, vesicles develop and N_2 fixation starts (Huss-Danell and Bergman, 1990). In the N_2-fixation zone, *Frankia* vesicles develop and bacterial N_2-fixation takes place. In the zone of senescence, bacterial material is degraded by the plant. In most actinorhizal nodules, infected and uninfected cells are interspersed in the cortex of the nodule lobe. In the actinorhizal Cucurbitales, however, the infected cells form a continuous patch, kidney-shaped in cross-section, at one side of the acentric stele (Hafeez et al., 1984; Newcomb and Pankhurst, 1982).

As in legume nodules, the search for, and characterization of, genes and their encoded proteins has been a focus of research in actinorhizal plants. In general, genes expressed at significantly higher levels in nodules than in roots encode products involved either in (a) nodule metabolism (e.g., symbiotic hemoglobin; Gherbi et al., 1997; Jacobson-Lyon et al., 1995), (b) the internalization of the microsymbiont (e.g., cell-wall proteins specific to infected cells; Pawlowski et al., 1997), or (c) nodule-specific development (e.g., subtilases,

possibly involved in inducing infected-cell differentiation within nodules; Berger and Altmann, 2000; Svistoonoff et al., 2003). Interestingly, only one legume *Enod* gene homolog has been discovered so far in an actinorhizal plant, namely the *dg93* mRNA sequence common to soybean nodules and *Datisca glomerata* (Okubara et al., 2000).

Parasponia nodule development and structure in some ways can be seen as an intermediate between legume and actinorhizal nodules, but also has unique features. Despite early observations that inoculation effected root hair growth and morphology (Lancelle and Torrey, 1984), it is now clear that *Parasponia* rhizobia do not infect the plant through root hairs (Becking, 1992). In *P. andersonii* (Bender et al., 1987b) and *P. parviflora* (Becking, 1992), rhizobia erode the root epidermis below an area of bacterial colonization on the root surface. Bender et al. (1987a,b) referred to subsurface swelling zone as a prenodule, comparable with the prenodule development that occurs in actinorhizal plants. However, Becking (1992) argues that these swellings are not always observed and are not morphogenetically comparable to prenodules of actinorhizal plants. Others have observed 'crack entry' of rhizobia into *Parasponia* roots (Webster et al., 1995a), similar to that seen in the infection of some legumes (Figures 2c, 2d and 2e). Thick-walled infection threads of plant origin in the intercellular spaces act as a conduit for the rhizobia to nodule primordia developing from the root pericycle. In this way, *Parasponia* nodules are more similar to actinorhizal nodules than legumes, being modified lateral roots and the mature nodule having a centralized vascular system (similar to Figure 4c). The mature nodule is also actinorhizal in appearance being multi-lobed and indeterminate. However, the rhizobia within the infected cells of the nodule are contained in thin-walled 'fixation threads' (Price et al., 1984) which Becking (1992) contends are actually filamentous 'peribacteroid sacs' associated with, or extensions of the infected plant cell's membrane. Although cell-wall-like deposits are associated with this peribacteroid membrane (Smith et al., 1986), it is not cell wall *per se*.

In the cycad–cyanobacteria symbiosis, the cyanobacterial microsymbiont, or cyanobiont, invades a particular root type, referred to as coralloid roots due to their 'coral-like' appearance. It has been speculated that the coralloid roots may have initially evolved as pneumatophores (apogeotropic roots facilitating an efficient gas exchange) that only later became cyanobacterial host-organs (Grobbelaar et al., 1986; Wittman et al., 1965). However, fossil evidence of symbiotic tissues invaded by cyanobacteria is still missing. As there are no other reports of plant pneumatophores infected by cyanobacteria, additional components only held by the cycad roots must be involved in acquiring cyanobacteria as symbionts. Root hairs, which are necessary for the infection of many legume and actinorhizal plants, are absent in cycad roots.

Coralloid roots develop from 'precoralloid' roots arising either as adventitious roots from the hypocotyl, or as secondary roots from the upper regions of the tap root. Precoralloid roots are easily recognizable by their swollen appearance (compared with normal roots), their papillose sheath, and their apogeotropic growth. As the precoralloid root matures, lenticels replace the papillose sheath at the base of the root and repeated bifurcations give rise to the extensively branched coralloid root cluster (Figure 3d). Such individual coralloid clusters can reach up to 10 cm in diameter and weigh up to 500 g, and may be found down to about 50 cm below ground. Coralloid roots are formed in the absence of cyanobacteria as has been shown using axenic cultures of *Zamia* sp. (Ow et al., 1999); however, irreversible morphological changes occur upon colonization by cyanobacteria (Ahern and Staff, 1994; Costa and Lindblad, 2002; Wittman et al., 1965).

Processes involved in the invasion of the coralloid roots by cyanobacteria are still largely unclear, and very few cycad genera have been examined. Nonetheless, the apogeotropic growth of the precoralloid roots brings the root caps close to the soil surface, the habitat where the photoautotrophic cyanobacterial microsymbiont would be expected. Some studies suggest that colonization takes place either at the apex, at the base, or in an intermediate position of the symbiotic coralloid root, and lenticels have often been proposed as the cyanobacterial root entry point (Figure 3d). For example, Ahern and Staff (1994) found that 95% of the infected roots of 60 *Macrozamia* seedlings had prominent apical lenticels. Other possible modes of cyanobacterial entry are through the papillose sheath, through breaks in the dermal layer, or through channels leading from the root surface to the cyanobacterial zone.

In contrast to both legume and actinorhizal symbioses, there is no evidence of infection thread development in the infection process in cycads.

Upon colonization by the cyanobiont, developmental and morphological changes are initiated in symbiotic coralloid roots (Ahern and Staff, 1994; Costa and Lindblad, 2002; Lindblad et al., 1990). The production of the papillose sheath ceases, the roots become wider, and the strict apogeotropic growth of the roots ceases. The widening is due to the development of a root 'zone' which the cyanobacteria invade. This zone may be the result of the development of a persistent root cap which forms a secondary (outer) cortex overlying the original root epidermis (equivalent to the inner cortex) of the non-infected root. Another possibility is that the cyanobacterial invaded zone is the result of the development of a specialized secondary (outer) cortex overlying the original (inner) cortex. Irrespective of the origin, the cyanobacterial invaded zone develops between these two root layers (Figures 3d and 4d). The widening of the cyanobacterial zone is due to considerable host-cell elongation, radially to the long axis of the roots, and a partial separation of the host cells located between these layers.

The outcome of the developmental process in symbiotic coralloid roots is the creation of a mucilaginous extracellular space, densely filled with cyanobacteria and traversed by numerous elongated host cells (Figure 4d). These elongated cells interconnect the inner (original) and outer ('new') cortex. The cyanobacterial zone may vary in prominence, from 230 to 365 μm in diameter depending on the cycad genus (Chaudhuri and Akhtar, 1931). However, in contrast to the symbioses of legumes and actinorhizal plants, the cyanobionts always remain extracellular, although the same cyanobacterium is capable of invading plant cells in the angiosperm *Gunnera* (Johansson and Bergman, 1992). The elongated host-root cells that cross the zone filled with cyanobacteria show transfer-cell morphology (cell-wall invaginations, amyloplasts and numerous mitochondria), suggesting a role in nutrient exchange (see below) between the partners (Lindblad et al., 1985a; Obukowicz et al., 1981). The zone infected with cyanobacteria is clearly visible by the naked eye as an intensely blue-green pigmented band midway between the stele and the root surface on sectioning of the root (Figures 3d and 4d). The retention of pigments (chl *a* and phycobiliproteins) in cyanobacteria living in darkness in plant roots is due to pigment synthesis in cyanobacteria being a light-independent process, as in some lower plants. The cyanobacterial root zone is absent at the root apex, but otherwise it forms a continuous cylinder all the way down the symbiotic coralloid root, except below lenticels.

To date, no symbiosis-related genes have been identified (or searched for) in cycads, although it is obvious that the cyanobacterium influences cycad root development as evidenced by microscopic studies (see above). The search for differences in gene expression patterns of non-infected and infected coralloid roots is now desirable. More is known regarding symbiosis-related gene expression of the cyanobiont. For instance, within the host tissue, cyanobacterial cell division is slowed down to synchronize development, cell-volume increases, cell-surface structures are altered, and subcellular rearrangements are apparent (Rai et al., 2000, 2002). Host plants are also capable of dictating cell differentiation processes (that of hormogonia and heterocysts) in the cyanobacterium. For example, it has been shown in other cyanobacterial–plant symbioses that host plants may elicit or prevent the differentiation of hormogonia via the release of a hormogonia-inducing factor (HIF) or a hormogonia-repressing factor (HRF). Interestingly, a mutation in *hrmA*, a gene induced by HRF, increases the cyanobacterial infectivity several-fold, probably by prolonging the hormogonial stage (Cohen and Meeks, 1997; Meeks et al., 1999). Infectivity is also enhanced by mutations in the expression of *sigH*, which is induced by HIF. This is interesting, as it points to the possibility of obtaining 'super-infectious' cyanobionts. Such mutants may perhaps also be used to infect roots of non-host plants of great commercial interest, such as wheat and rice, which readily associate with some cyanobacteria (Gantar and Elhai, 1999; Nilsson et al., 2002).

Once inside the root, differentiation of the N_2-fixing heterocysts is stimulated, and soon reaches frequencies never seen in their free-living state (Lindblad et al., 1985b). This suggests overexpression of *hetR*, the heterocyst regulatory gene highly expressed in free-living cyanobacteria upon deprivation of combined N (Buikema and Haselkorn, 1993). Since this gene is under the control of the nitrogen-dependent global transcription factor NtcA, it has been proposed that the plant may

bypass this control. However, recent data indicate that both *ntcA* and *hetR* are over-expressed *in planta* (Wang et al., 2004). Moreover, *hetR* mutants still infect liverworts while *ntcA* mutants fail to do so (Wong and Meeks, 1994). Although these studies have been performed on cyanobionts in the *Gunnera* and bryophyte symbioses, respectively, they are probably relevant also for cycads.

Supplying the microsymbiont with energy

An integral aspect of symbioses between host plants and their N_2-fixing microsymbionts is the supply of reduced carbon substrates from the host to the microsymbiont. Evolutionarily, it is believed that this supply of energy has played a large role in 'drawing' the microsymbionts into the symbioses (Simms and Taylor, 2002). In all four symbioses examined here, the carbon supplied to the microsymbionts ultimately derives from photosynthesis by the host. However, a great difference among the symbioses is that free-living (i.e., non-symbiotic) rhizobia and *Frankia* in the soil are carbon heterotrophs, but free-living cyanobacteria are photoautotrophic. Hence, for host-supplied carbon to play a *major* role in initiating and maintaining the cycad–cyanobacteria symbiosis through evolution, the carbon supply from the host must be superior in some fashion to the cyanobacterium supplying its own carbon from photosynthesis.

Carbon provision and assimilation to bacteroids in legume nodules has been reviewed recently by Lodwig and Poole (2003). In summary, photosynthate is supplied to nodules as sucrose in the phloem. Sucrose synthase activity in the nodule is primarily responsible for the hydrolysis of the sucrose. If carbon supply to the nodule is more than sufficient, some of the released monosaccharides can be stored as starch within the nodule. Sugars destined for utilization by the bacteroids pass through glycolysis and enzymes of the tricarboxylic acid cycle, to form malate, fumarate and succinate, the three dicarboxylic organic acids that are the primary carbon sources for bacteroids in the nodules. The transport of these dicarboxylic acids across the peribacteroid membrane and their utilization by bacteroids have been studied extensively (Day and Udvardi, 1993; Day et al., 2001; Lodwig and Poole, 2003).

Very little is known about the carbon supply to rhizobia in *Parasponia*. Mohapatra and Gresshoff (1984) found that *Parasponia–Rhizobium* strain ANU289 grew well *in vitro* on a wide range of carbon sources, but that succinate, fumarate, or pyruvate was necessary to support good levels of nitrogenase activity. It is interesting that nitrogenase activity was enhanced by more than threefold if mannitol or sorbitol was added to succinate in the medium compared to succinate alone. The forms of carbon transported in the phloem of *Parasponia* are unknown. Micrographs of bacteroids (Becking, 1992; Trinick, 1979) within *Parasponia* nodules commonly depict abundant poly-β-hydroxybutyrate globules which Becking (1992) concluded indicated that carbon was not a limiting factor to the rhizobia. Newly infected cells contain starch-filled plastids (Lancelle and Torrey, 1985; Trinick, 1979) but the plastids of mature cells plastids contained little to no starch. It is not clear from these micrographs and others (Becking, 1979, 1992; Lancelle and Torrey, 1984, 1985; Trinick, 1979; Webster et al., 1995b) if starch accumulates in uninfected cells in the infected zone as is common in legume nodules. However, in *P. andersonii* nodules, one of the authors (K. Pawlowski, unpublished data) has observed large amyloplasts in uninfected cells in the region between the vascular tissue and the infected zone.

There are many similarities between the scenario for supplying carbon to legume bacteroids and the supply of energy to *Frankia* from its actinorhizal host. As in legumes, sucrose is believed to be the major sugar delivered to the nodules of most actinorhizal plants. Sucrose synthase expression levels are high in the nodules of *Alnus glutinosa* (Van Ghelue et al., 1996). However, sorbitol is a major phloem-transported carbohydrate in the Rosaceae (Brown and Hu, 1996), but it is not known if actinorhizal genera of this family might utilize this sugar alcohol as their major carbon supply to nodules. All actinorhizal nodules, except for those formed by *Casuarina* spp., accumulate starch, though not in infected cells (E. Duhoux, unpublished observation). It is unclear whether nodule starch plays a role in carbon storage for particular growth situations; at any rate it is not used during spring flush growth (Wheeler et al., 1983). Nodules are strong carbon sinks, and starch biosynthesis may simply represent a

metabolic 'safety valve' when carbon is received in excess of demand, as appears to be the case in legumes (Walsh et al., 1987).

In the free-living state, *Frankia* strains grow on short-chain fatty acids (acetate, propionate), variably on succinate, malate or pyruvate, and (except for some living symbiotically with members of the Elaeagnaceae and Casuarinaceae) poorly or not at all on a range of sugars (Benson and Silvester, 1993). However, the *Frankia* microsymbionts of many actinorhizal plants (e.g., *Ceuthostoma* sp., *Kentrothamnus* sp., and *Chamaebatia* sp.) have not been isolated yet, so their carbon source preferences have yet to be analyzed. Studies on the metabolism of symbiotic *Frankia* have been performed using so-called vesicle clusters, consisting of symbiotic vesicles together with a part of their subtending hyphae isolated from nodules (Van Straten et al., 1977; Vikman and Huss-Danell, 1987b). Vesicle clusters from *Alnus* spp. have an aerobic metabolism (Vikman and Huss-Danell, 1987a). In agreement with the hypothesis that *Frankia* is fed dicarboxylates *in planta*, succinate, as well as a combination of malate, glutamate and NAD, were found to stimulate respiration (Akkermans et al., 1981; Vikman and Huss-Danell, 1991). Evidence for CO_2 fixation in actinorhizal nodules was found, although this might be due to reactions associated with ammonia assimilation, instead of metabolism of dicarboxylic acids (Huss-Danell, 1990; McClure et al., 1983).

An alternative hypothesis is that symbiotic *Frankia* are supplied with hexoses by the host plant. Hexoses are not the ideal carbon sources for free-living *Frankia*, but it is possible that *Frankia*'s carbon preferences in nodules do not reflect those in free culture. Sucrose, trehalose, maltose, glucose and fructose stimulated respiration in vesicle clusters from *Alnus rubra* (Lopez et al., 1986). On the other hand, these observations might simply reflect the ability of *Frankia* to metabolize its own storage carbohydrates, glycogen and trehalose (Lopez et al., 1984). Furthermore, given the dissimilarities between actinorhizal nodules formed by plants from different families, differences in the carbon source for the microsymbionts might exist amongst Fagales, Cucurbitales and Rosales. In summary, the carbon sources delivered to symbiotic *Frankia* are still not clearly known.

Compared with the legume and actinorhizal symbioses, relatively little is known about the carbon supply to cyanobionts from their cycad hosts. Free-living cyanobacteria are capable of higher-plant-type oxygenic photosynthesis; however, in cycad symbiotic coralloid roots, where the availability of light would be negligible, the normally photoautotrophic cyanobacteria would become functionally non-photosynthetic. A heterotrophic life style and a dependence on carbohydrates supported by the host plant are therefore expected. *Nostoc*, as opposed to many other cyanobacteria, can easily switch from growing photoautotrophically to growing heterotrophically (Rippka et al., 1979) which probably is a very important part of their symbiotic competence. Evidence of the heterotrophic nature of cycad cyanobionts includes the fact that externally added fructose and glucose to cycad isolates grown in darkness stimulated their nitrogenase activity (Lindblad, 1992; Martel et al., 1993). The carbon supplied by the host is still unknown, and whether the mucilage filling the extracellular space of the symbiotic coralloid roots has a role in the carbon supply to the cyanobiont, or whether it is of cyanobacterial origin, is also an open question. However, a plant-derived mucilage is apparent in organs infected by cyanobacteria of other plant symbioses (e.g., *Gunnera*) (Bergman, 2000). Amyloplasts are common in the elongated cells of the cyanobacterial zone which show transfer cell morphology. The plant carbohydrates filling the cyanobacterial zone may be made available via, or stored in, these plastids (see Rai et al., 2002).

Regulation of O_2 supply to the microsymbiont

Three types of nitrogenase (the molybdenum–iron, the vanadium–iron, and the iron–iron complexes) exist in the Eubacteria (Burris, 1991), and more recently a molybdenum–carbon monoxide nitrogenase complex was discovered in *Streptomyces thermoautotrophicus* of the Archaea (Ribbe et al., 1997). The legume–rhizobia, *Parasponia*–rhizobia, actinorhizal–*Frankia*, and cycad–cyanobacteria symbioses all contain the Mo–Fe nitrogenase complex, by far, the most abundant form of nitrogenase in the biosphere (Burris, 1991). This nitrogenase is sensitive to inhibition by molecular oxygen. The exact mechanism of the inhibition is

unknown (Gallon, 1992), but it may be that O_2 itself is a substrate of nitrogenase, and its reduction leads to highly reactive oxygen species (e.g., O_2^-) which result in the denaturation of the nitrogenase complex. Regardless of the mechanism, all diazotrophs containing the Mo–Fe nitrogenase complex must protect nitrogenase from O_2. In aerobic bacteria such as rhizobia, *Frankia*, and cyanobacteria, this is a particular dilemma because the organisms require an adequate O_2 flux for oxidative phosphorylation to provide the energy required for nitrogenase activity, but not so high a flux that it will result in inhibition of nitrogenase. In fact, despite existing in an aerobic milieu, it is necessary that the environment immediately surrounding the nitrogenase complex be microaerobic.

Solving this 'O_2 dilemma' in cyanobacteria is particularly interesting because in a free-living (non-symbiotic) state the bacteria can fix N_2, but also carry out photosynthesis, which generates O_2. Protection of nitrogenase from O_2 in *Nostoc* in a free-living state is accomplished by isolating nitrogenase in thick-walled, non-O_2 producing (i.e., non-photosynthetic cells) heterocysts in the filamentous organism (Gallon, 1992, 2001; Meeks et al., 1999). The double envelope surrounding the outer membrane of the heterocyst decreases the diffusion of O_2 into the cell. Likewise, O_2 consumption by respiration has been implicated in helping to maintain microaerobic conditions inside the heterocyst. In the heterocystous *Anabaena* PCC 7120, the *coxA* gene which codes for a subunit of cytochrome oxidase was stimulated during heterocyst differentiation, but not in a *coxA* mutant, which could only fix N_2 under anaerobic conditions (Haselkorn et al., 1997).

Compared with free-living, non-symbiotic forms of the cyanobacteria, it may be easier for a cyanobiont to cope with O_2. The lack of light in the symbiotic coralloid root means little or no O_2 production from cyanobacterial photosynthesis (Bergman et al., 1986). In addition, it also appears that the partial pressure of O_2 (pO_2) in the cyanobacterial zone of coralloid roots, as in other cyanobacteria-infected plant organs, is lower than levels free-living cyanobacteria may experience. Heterocyst abundance is much higher in the cyanobiont than in the free-living state (Bergman et al., 1986), but in freshly isolated cycad cyanobionts nitrogenase activity is inhibited on exposure to pO_2 above 1 kPa (Lindblad et al., 1991). This implies the maintenance of a low pO_2 in the cyanobacterial zone, that the heterocyst envelope may be impaired, or that isolation of the cyanobiont from the plant disrupts the protective conditions or prevents important interactions between the organisms (Costa and Lindblad, 2002). How the plant manages to lower the pO_2 in the symbiotic tissues is an intriguing question. However, the abundance of mucilage in the extracellular spaces of the cyanobacterial zone may play a role. Protection of nitrogenase activity from excessive O_2 flux by mucilage has been implicated in other plant-associated diazotrophs (Vessey and Pan, 2003; Zhulin et al., 1996).

In contrast to cyanobacteria, rhizobia in their free-living state in soil are incapable of N_2 fixation. Nitrogenase activity can be induced in rhizobial cultures or isolated bacteroids, but only when the pO_2 is maintained at very low levels (e.g., 0.5–1.5 kPa O_2) (Allen and Elkan, 1990; Karr et al., 2003). In its symbiotic state, the legume nodule creates a microaerobic environment to protect nitrogenase from O_2, but a relatively high flux of O_2 is maintained to the bacteroids for respiration. These seemingly contradictory functions (low O_2 concentration/high O_2 flux) are accomplished via an O_2-diffusion barrier in the cortex of the nodules, and facilitated diffusion of O_2 bound to the transporting hemoprotein, leghemoglobin.

The subject of the regulation of O_2 flux to bacteroids within legume nodules has been well reviewed (see Hunt and Layzell, 1993; Lodwig and Poole, 2003). In brief, an O_2-diffusion barrier exists in a region of densely packed cells in the inner cortex of legume nodules. There is some controversy whether expression of *Enod2* is related to the development of this diffusion barrier (Wycoff et al., 1998). The exact nature of this diffusion barrier is unknown, but there is evidence that the path length of intercellular water (Denison, 1992) or the abundance of intercellular glycoprotein (James et al., 2000) may play roles in establishing the diffusion resistance to O_2. A very important feature of this diffusion barrier is that it can quickly change (i.e., in seconds to minutes) its resistance to O_2 diffusion when either the external concentration of O_2 or the internal demand for O_2 changes. In fact, it appears that a number of stresses (drought, temperature, supplemental mineral N, carbohydrate

limitations) decrease nitrogenase activity indirectly by decreasing O_2 diffusion into the infected zone of the nodule (Kuzma and Layzell, 1994; Serraj et al., 1999; Vessey et al., 1988). In the infected zone of the nodule, leghemoglobin acts as a shuttle, binding O_2 from the intercellular spaces within the infected zone, diffusing down the oxyleghemoglobin concentration gradient, and delivering O_2 to the sites of respiration (cytochrome oxidase) in the bacteroids (Becana and Klucas, 1992; Bergersen, 1996; Denison and Okano, 2003). Leghemoglobin represents approximately 5% of the total protein of a mature nodule and is coded for by at least four *lb* genes, of which *lbc3* is the most intensively studied (e.g., Cvitanich et al., 2000).

In terms of regulating O_2 flux within symbioorgans, the actinorhizal–*Frankia* symbiosis can be seen as an intermediate between the cycad and legume symbioses. In contrast to rhizobia, *Frankia* can fix N_2 in the free-living state at ambient pO_2. The vesicles of *Frankia* have similarities in form and function to cyanobacterial heterocysts, but in some actinorhizal plants hemoglobins also appear to have a role in O_2 flux as in legumes. Hemoglobins have not been found in cyanobacterial symbioses; however, some cycad symbiotic coralloid roots are pink. Whether this color is due to hemoglobins or to the red water-soluble pigment phycoerythrin, abundant in symbiotic *Nostoc* (Poza-Carrion et al., 2001), has yet to be resolved.

When *Frankia* are cultured with limiting levels of mineral N and ambient pO_2, they form specialized vesicles at the end of normal hyphae or at the ends of short side hyphae (Silvester et al., 1990). Within the vesicles, nitrogenase is protected from O_2 and N_2 fixation can take place (Meesters, 1987; Parsons et al., 1987). The vesicles are surrounded by envelopes consisting of multiple layers of hopanoids, bacterial steroid lipids (Berry et al., 1993; Huss-Danell, 1997). The number of layers depends on the pO_2 (Parsons et al., 1987). While vesicles formed in the free-living state are always round and non-septate, the shape and cellular location of vesicles formed *in planta* depends on the host plant genus (Baker and Mullin, 1992), indicating that here, vesicles represent a symbiosis-specific differentiation comparable to bacteroids in legume nodules. In addition to diffusion resistance, the fast respiration rate of *Frankia* vesicles suggests that metabolic consumption of O_2 also plays a role in O_2-protection of nitrogenase (Vikman, 1992). In nodules of *Datisca*, *Frankia* forms lanceolate vesicles in radial orientation that form a ring around the central vacuole. Multiple mitochondria accumulate at the vesicle base and probably play a role in the metabolic removal of O_2 (Silvester et al., 1999). In contrast, the respiratory protection of nitrogenase at work in some actinorhizal symbioses appears to have little role in the protection of nitrogenase in legume nodules (Weisz and Sinclair, 1987).

Actinorhizal nodule lobes are surrounded by a superficial periderm that may be impermeable to O_2. To provide the infected zone with O_2, the nodule periderm can be disrupted by lenticels (*Betulaceae*, *Datisca*, *Coriaria*), or nodule roots can be formed (*Casuarinaceae*, *Myricaceae*, *Datisca*) (Huss-Danell, 1997). Nodule roots are agravitropically growing roots with large air spaces in the cortex that are formed at the tip of nodule lobes, i.e. by a change in the activity of the nodule lobe meristem (Bond, 1952). Subsequently, new nodule lobe meristems can be induced next to the origin of the nodule root. Nodule roots provide access to O_2 for nodules formed on roots submerged in water, and their length depends on the pO_2 level (Silvester et al., 1988; Sprent and Scott, 1979). Diverse O_2 protection systems for bacterial N_2 fixation have evolved in different actinorhizal plant genera (reviewed by Silvester et al., 1990). The lack of a region of densely packed cells encompassing infected zones (i.e., an O_2-diffusion barrier) in the actinorhizal nodule lobes is inconsistent with a legume–nodule-like O_2 diffusion control system. Only *Coriaria* nodules have developed a comparable system, in that these contain a long lenticel at the non-infected side of the nodule lobe with a variable O_2-diffusion control system in the cell layers between stele and periderm that enables control of O_2 flux to the infected cells (Silvester and Harris, 1989).

In some cases, in symbioses with *Casuarina* spp. for example, only the plant seems to be responsible for O_2 protection of nitrogenase, more comparable to the situation in legume nodules. In these nodules, *Frankia* do not form vesicles (Berg and McDowell, 1987a), the walls of infected cells are lignified to prevent O_2 access (Berg and McDowell, 1987b), and large amounts of a nodule-specific hemoglobin are present in the cytosol of infected

cells (Jacobson-Lyon et al., 1995). Nodules of *Myrica* spp. also contain large amounts of hemoglobin and the walls of infected cells are heavily lignified (Pathirana and Tjepkema, 1995; Zeng and Tjepkema, 1994), although in this genus, *Frankia* forms vesicles (Baker and Mullin, 1992). Even if hemoglobin is clearly plant-based in some cases (Christensen et al., 1991), recently Beckwith et al. (2002) have found production of a hemoglobin in genetically diverse strains of *Frankia*. The combined role of plant-based and *Frankia*-based hemoglobins in the regulation of O_2 flux in actinorhizal nodules has yet to be elucidated.

Although *Parasponia* nodules are morphologically similar to actinorhizal nodules, in terms of regulation of O_2 flux to bacteroids, they are more similar to legume nodules. *Parasponia* nodules have a zone of tightly packed cells surrounding the infected zone which is presumed to function similarly to the O_2-diffusion barrier of legume nodules (James et al., 1994; Tjepkema and Cartica, 1982). Hemoglobin is probably the most intensively studied aspect of the *Parasponia*–rhizobia symbiosis (e.g., Appleby et al., 1983; Bogusz et al., 1988; Hunt et al., 2002). In a recent comparison of hemoglobin gene sequences from a wide range of symbiotic and non-symbiotic plants, it was concluded that the symbiotic hemoglobins from legumes and actinorhizal plants are related to the class 2 non-symbiotic hemoglobin genes assumed to be common to all dicots (Hunt et al., 2001). Interestingly, hemoglobin sequences from *Parasponia* nodules are more closely related to class 1 non-hemoglobin genes assumed common to both monocots and dicots. These observations lead Hunt et al. (2001) to conclude that symbiotic hemoglobin of *Parasponia* was probably recruited from non-symbiotic hemoglobins independent from the symbiotic hemoglobin in extant legume and actinorhizal plants.

Transfer of the fixed N from the microsymbiont to the host

The host's benefit from a N_2-fixing symbiosis is only realized when it receives the fixed N from the microsymbiont. The product of nitrogenase activity in the microsymbiont is NH_3; dependent upon pH, a proportion of the NH_3 will form NH_4^+ ($pK_a = 9.25$). In legume nodules, the NH_3 quickly diffuses out of the bacteroid's alkaline protoplasm into the symbiosome's acidic peribacteroid space where it is protonated to NH_4^+. An ammonium-transporting system (Amt) present in free-living rhizobia is suppressed in bacteroids (Udvardi and Day, 1990), thereby stopping the potential for cycling the NH_4^+ back into the bacteroid. An ion channel, specific for monovalent cations, facilitates the transport of the NH_4^+ across the peribacteroid membrane and into the infected cells' cytoplasm (Tyerman et al., 1995). Once in the plant cytosol, the glutamine synthetase (GS) and glutamate-oxoglutarate aminotransferase (GOGAT) enzyme systems are the main conduits of NH_4^+ assimilation into amino acids (Cullimore and Bennett, 1992). However, additional aminotrasferase activity is required to form the main N compounds exported from the nodules, primarily amides (glutamine and aspargine) in legumes of temperate origin and ureides (allantoin and allantoic acid) in legumes of tropical origin (Parsons and Sunley, 2001; Pate, 1989).

The above scenario is the most widely accepted model for the movement of NH_3 from the sites of N_2 fixation to the cytosol of infected legume cells. However, there are alternative hypotheses. There is some evidence that alanine may be the export form of the fixed N from bacteroids (Allaway et al., 2000; Waters et al., 1998). It is still being debated as to how important this alanine may be to the overall export of fixed N from bacteroids (Li et al., 2002).

The form of N exported from *Parasponia*–rhizobia to the host cells is unknown, but there is little reason to believe it should be exceptional as compared to legume rhizobia (e.g., Udvardi et al., 1992). Early work (Becking, 1983a) on the amino acid content of *P. parviflora* nodules indicated higher levels of the glutamate, aspartate and amides in nodules with greater N_2 fixation. This would suggest that *Parasponia* nodules are amide exporters similar to legumes of temperate origin (Webster et al., 1995a) and many actinorhizal plants [see below; note that *P. parviflora* nodules did not contain citrulline (Becking, 1983a), an N-export product of *Alnus* sp. and *Casuarina* sp.]. Synthesis and transport of these amino acids in nodules of *P. andersonii* were confirmed by Baker et al. (1996), however they also demonstrated the incorporation of significant quantities of $^{15}N_2$ into 4-methylglutamate in nodules, demonstrating the *de novo* synthesis of

this non-protein amino acid and suggesting a role in xylem transport of fixed N as well.

The form of fixed N exported from *Frankia* is unknown. However, similar to legume nodules, high expression levels of GS have been found in infected cells of actinorhizal nodules of *Alnus glutinosa* (Guan et al., 1996) and *Casuarina glauca* (L. Laplaze and K. Pawlowski, unpublished), supporting the proposal of NH_4^+ export by *Frankia*. However, in *Datisca glomerata* nodules, plant GS expression was restricted to the uninfected cells surrounding the infected cells (Berry et al., 2004; Pawlowski et al., 2003). Since NH_4^+ is relatively toxic, and thus unlikely to be allowed to diffuse through several plant cell layers prior to assimilation, these results suggest that within the infected cells, an assimilated form of N is exported by *Frankia* that is degraded in the uninfected cells, followed by the re-assimilation of NH_4^+ in the GS/GOGAT pathway (Berry et al. 2004). In contrast, Valverde and Wall (2003) recently demonstrated the expression of most of the enzymes involved in the synthesis of aspargine in nodules of *Discaria trinervis*, but not in roots. They hypothesized that the assimilation of fixed N, exported as NH_4^+ from the microsymbiont, followed a similar pattern to that seen in amide-exporting legume nodules (e.g., alfalfa). The above models describe amide synthesis in actinorhizal nodules, and most actinorhizal plants transport amides in their xylem. However, *Alnus* spp. and *Casuarina equisetifolia* transport citrulline (Schubert, 1986; Sellstedt and Atkins, 1991). Results on the cell-specific localization of an enzyme in the citrulline biosynthetic pathway, acetylornithin aminotransferase, have led to the conclusion that citrulline biosynthesis takes place in the infected cells (Guan et al., 1996).

As opposed to other cyanobacterial–plant symbioses (i.e. *Gunnera*; Silvester et al., 1996) and the plant–microsymbiont systems described above, NH_4^+ may not be the fixed N transported from the cyanobiont to the cycad. This is indicated by the fact that activities, protein levels and cellular location of glutamine synthetase in cyanobionts of cycads (*Cycas*, *Ceratozamia* and *Zamia*) resemble that of free-living cyanobacteria (Lindblad and Bergman, 1986). Moreover, $^{15}N_2$ analyses of cycad symbiotic coralloid roots suggest that either glutamine and citrulline (Zamiaceae) or only glutamine (Cycadaceae and Stangeriaceae) are the N compounds translocated to the cycad (Costa and Lindblad, 2002; Pate et al., 1988). This implies that, although all other non-cycad cyanobionts tested so far release the fixed N as NH_4^+, by either lowering the cyanobiont GS activity or GS protein levels (Bergman et al., 2004; Rai et al., 2000), the cycad–cyanobacterial symbioses have apparently solved this crucial symbiotic issue using other mechanisms (Pate, 1989; Pate et al., 1988).

Conclusions/summary

Despite the evolutionary, phylogenetic, and ecological diversity among the four root symbioses, there are numerous similarities in the means by which symbiotic partners in legumes, actinorhizal plants, and cycads facilitate the developmental and physiological imperatives that enable the symbioses to function. However, although all four symbioses meet the same symbiotic imperatives (e.g., infection, colonization, control of O_2 flux, exchange of C and N), they have not developed the same physiological and anatomical mechanisms to achieve these ends.

While there is evidence of a relatively close phylogenetic relationship between legumes, *Parasponia* and actinorhizal plants (i.e. the N_2-fixation clade) which may point to a common ancestry of these groups, the cycads, being a much more ancient order, are not closely related to the others. The three groups of microsymbionts (rhizobia, *Frankia* and cyanobacteria) are all Eubacteria, but are phylogenetically quite distant. Nonetheless, the highly conserved nature of many of the genes coding for the Mo–Fe form of nitrogenase shared by the four symbioses indicates some degree of commonality, and possibly lateral gene transfer. Ecologically, the four symbioses are dispersed across many geographical and climatic regions; however, not surprisingly, there is a propensity for distribution among low-fertility habitats.

In the infection processes there is evidence for roles of chemo-attractants, and flavonoid-type substances have been implicated in all four symbioses. There are many similarities between legumes and actinorhizal plants in the infection process, with both intercellular and intracellular infection pathways, however, the actual nature of the infection threads are quite different, and the equivalent of nod factor has not yet been

identified in the actinorhizal symbiosis. *Parasponia* infection is unique as it seems to involve a degradation of the root epidermis, and two sorts of symbiotic 'threads'; a thick-walled intercellular infection thread and a 'fixation thread' that is more comparable to legume symbiosome than an infection thread in the other symbioses. Coralloid root infection is most comparable to crack-infection processes seen in some members of the other three symbioses. Little is known regarding signal exchange in the cycad–cyanobacteria symbiosis. While the symbio-organs of cycads, actinorhizal plants and *Parasponia* are modified roots, legume nodules arise from distinctly different zones of cell divisions than those that give rise to lateral roots.

In terms of energy supply to the microsymbionts, organic acids are the primary carbon supply to rhizobia and are implicated in supplying *Frankia*. Likewise, simple sugars have been proposed as substrates for *Frankia* and cyanobacteria in their respective symbioses. In legumes, most actinorhizal plants, cycads and possibly *Parasponia*, starch appears to be a common form of stored carbohydrate when supplied to the symbio-organ in surplus of the microsymbiont's requirements.

Rhizobia in both legume and *Parasponia* symbioses are extremely dependent upon the plant nodule with its O_2 diffusion barrier and leghemoglobin to provide the O_2 flux required for respiration, but without diminishing nitrogenase activity. By thickening the walls of the cells (compartments) where nitrogenase is located, *Frankia* and cyanobacteria are more independent in their regulation of O_2 flux within their respective symbioses, but still some level of control is enacted by the host tissue (e.g., hemoglobin and/or mucilage, respectively).

In terms of the means of transferring the fixed N from the microsymbiont to the host, some actinorhizal plants, legumes and possibly *Parasponia* have many similarities with NH_4^+ appearing to be the main form of N export from the bacteria, processing by GS/GOGAT, and the synthesis of amides as a form of N-transport compound out of the nodules. Less is known about the N transfer process in the cycad–cyanobacteria symbiosis, but it may be unique from the other two, with an organic-N compound (amino acid) being the potential export product from the heterocyst.

As stated at the outset of this chapter, a great challenge in comparing the four symbioses is the unbalanced amount of knowledge we have of the given partnerships. It is not surprising that the amount of knowledge and research on each of the four symbioses is relative to their recognized economic importance. Agronomically important legume–rhizobia symbioses have been, and continue to be, well examined. The use of the 'model' legumes, *Lotus japonicus* and *Medicago truncatulata* (Colebatch et al., 2002b), and the complete sequencing of the genomes of a several of the rhizobial microsymbionts (Galibert et al., 2001; Kaneko et al., 2000, 2002) will further help to accelerate the investigation of these symbioses. The actinorhizal–*Frankia* symbioses are not as well characterized, despite their importance in forest succession and revegetation (Benoit and Berry, 1990). Some challenges to the rate of progress in this field is that *Frankia* cannot yet be transformed (John et al., 2003), and the proportion of the genome which has been sequenced is relatively small. Nonetheless, progress on *Frankia* genetics is being made, particularly in specific areas such as the *nif* genes (e.g., Lavire and Cournoyer, 2003; Oh et al., 2003). Transformation of actinorhizal plants is possible with *Agrobacterium rhizogenes* and *A. tumefaciens*, and progress is being made in the host's genetics, particularly in the Casuarinaceae compared with the other seven actinorhizal plant families (Franche et al., 1998; Santi et al., 2003a). Despite the fact that *Parasponia* represent only five species native to a well-defined geographical region, they have received quite a bit of attention due to the fact that they are the only non-legume plant to establish an effective symbiosis with rhizobia. Some aspects of the symbiosis have been quite well studied (e.g., hemoglobin), but there is still an absence of much basic information on the development and maintenance of the symbiosis. Our knowledge of cycads, and in particular of their peculiar cyanobacteria root symbiosis, is still highly inadequate and fragmentary, and many questions remain, although this division dominated the Earth's flora some hundred million years ago. For example, why are cycads the only extant gymnosperms to form a symbiosis with a N_2-fixing organism, given the competitive advantages of such a trait? Likewise, why do cycads, but no other plants, accept cyanobacteria in their roots, although root colonization is dominating among

symbiotic angiosperms? Cyanobacteria do colonize cells of one angiosperm family (Gunneraceae), but exclusively cells in stem glands, not its roots. Why the cycad roots are never colonized by rhizobia or *Frankia* although these N_2-fixing bacteria form distinct root symbioses with angiosperms is another open question. However, the fact that (almost) the complete genome of *Nostoc* ATCC 29133, isolated from a cycad, is known will open new research avenues.

Regardless of the genetic, evolutionary, and functional diversity of the respective hosts and microsymbionts, the outcome of all the root symbioses is equally elegant: a microorganism finds shelter and sustenance within the host plant and compensates its host with a constant and renewable source of N.

Acknowledgements

The authors thank B. Luit and W. Zheng for the photos of nodules and symbiotic coralloid roots. The authors' research programs are largely funded by the Natural Sciences and Engineering Research Council of Canada (JKV), the Swedish Research Council and the Swedish Research Council for Environment, Agricultural Sciences (BB), and the German Research Foundation (KP).

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Plant and Soil (2005) 274:79–100
DOI 10.1007/s11104-004-1419-5

Mycorrhizas: Gene to function

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Received 8 March 2004. Accepted in revised form 15 July 2004

Key words: arbuscular mycorrhizal fungi, ecophysiology, ectomycorrhizal fungi, evolution, gene expression, genetic diversity, integration of scales, nutrient transport

Abstract

Substantial progress has been made toward development of molecular tools for identification and quantification of mycorrhizal fungi in roots and evaluation of the diversity of ectomycorrhizal (ECM) fungi and the phylogeny and genetic structure of arbuscular mycorrhizal (AM) fungi. rDNA analysis confirms high diversity of ECM fungi on their hosts, and for AM fungi has revealed considerable genetic variation within and among morphologically similar AM fungal species. The fungal and plant genes, regulation of their expression, and biochemical pathways for nutrient exchange between symbiotic partners are now coming under intense study and will eventually be used to define the ecological nutritional role of the fungi. While molecular biological approaches have increased understanding of the mycorrhizal symbiosis, such knowledge about these lower-scale processes has yet to influence our understanding of larger-scale responses to any great extent.

Abbreviations: AM – arbuscular mycorrhizas; CHO – carbohydrate; ECM – ectomycorrhizas; ERH – extraradical hyphae; IRH – intraradical hyphae.

Introduction

The structure and function of terrestrial plant communities are strongly influenced by interactions of microorganisms with roots (Bever, 2002; Reynolds et al., 2003). An estimated 90% of terrestrial plants exist in a symbiotic association with soil fungi forming mycorrhizal associations (Smith and Read, 1997). Mycorrhizas comprise several distinctive groups that are recognized by a taxonomic classification of the plant and fungus along with the structures that form on root systems. This review features two of the most common types: arbuscular mycorrhizas (AM) and ectomycorhizas (ECM). Details about other types of mycorrhizas are to be found in the excellent book by Smith and Read (1997).

The function for both AM and ECM associations is largely based upon the transfer of carbon (C) from plant to fungus, and mineral nutrients (mainly phosphorus [P] and/or nitrogen [N]) from fungus to host plant. Mycorrhizas may confer other benefits to plants including improved soil structure, plant water relations and resistance to plant pathogens under certain conditions (Daniell et al., 1999; Miller and Jastrow, 2000; Smith and Read, 1997). While the general benefits of the different mycorrhizal associations are similar, their molecular characterization has enabled understanding of distinctly different processes from the gene to function for each mycorrhizal symbiosis. For ECM, the availability of large databases of fungal sequences has enabled

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studies of diversity of the host-fungus interaction for predicting their ecosystem behavior. Diversity of AM associations has been far more difficult to assess due to rudimentary understanding of the genetics of AM fungi (Kuhn et al., 2001; Hijri and Sanders, 2003). By contrast, details of molecular mechanisms for carbon and P exchange between AM symbionts have emerged rapidly from studies of genes and their expression for nutrient transport and metabolic pathways at the cellular level (Harrison, 1999). On the other hand, dissection of host-fungal interaction for ECM fungi has been problematic due to their structural complexity and diversity (Martin et al., 2001).

AM structure, function and host specificity

AM symbiosis is initiated when fungal hyphae, arising from spores in the soil or adjacent colonized roots, contact the root surface and form appressoria. From root penetration points intraradical hyphae (IRH) grow between epidermal cells, through passage cells of the hypodermis, and colonize the intercellular space of root cortex. At the inner cortex, IRH penetrate individual cortical cells and differentiate to form highly branched structures known as arbuscules. Although the arbuscule develops within a cell, the fungus remains essentially apoplastic as the plant plasma membrane remains intact. In these cells, an extensive intracellular interface develops between the two symbionts across which nutrient exchange is thought to occur (Gianinazzi-Pearson, 1996; Smith and Smith, 1990). Two morphological types of colonization have been described, Arum and Paris (Smith and Read, 1997). The Arum type is defined on the basis of an extensive intercellular phase of hyphal growth in the root cortex and the development of terminal arbuscules on intracellular hyphal branches. The Paris type is defined by the absence of the intercellular phase and the presence of intracellular hyphal coils (Smith and Smith, 1996). Much of what is known about the physiology and molecular biology of the AM symbiosis has been investigated for the Arum type of colonization. However, recent comparative study of the Paris type has shown that coils have functional homology with arbuscules (e.g., Cavagnaro et al., 2001, 2003) and that Paris type coils have as much area of interface as the Arum type (Dickson and Kolesik, 1999).

Following colonization of the root cortex, the fungus develops an extensive network of extraradical hyphae (ERH) in the soil. ERH have several functions important to the symbiosis, including uptake of Pi and other mineral nutrients from the soil and their subsequent translocation to the plant, colonization of additional roots, and in many AM fungi, the production of spores. Acquisition of Pi from the soil is particularly significant because the ERH extend beyond the Pi depletion zone of the host root to access sources unavailable to the plant (Zhu et al., 2001). However, the contribution of the AM to Pi uptake varies with fungus and plant species, indicating that a functional compatibility exists in the symbiosis that is not apparent at the level of internal colonization (Burleigh et al., 2002; Pearson and Jakobsen, 1993; Ravnskov and Jakobsen, 1995). Carbohydrates (CHO) and mineral nutrients are transferred across interfaces that are bordered by plant and fungal plasmalemmas (Smith and Smith, 1990). Although the molecular mechanisms are not clearly understood, such bi-directional transfer is generally recognized as passive efflux into the symbiotic interfaces and then active uptake by the receiver organism (Smith and Read, 1997; Woolhouse, 1975; Figure 1). A major obstacle to study of such exchanges is inability to culture the fungus in the absence of the host or to isolate the intact arbuscules within the root cortex cells.

AM associations are the most widespread symbiosis occurring in the roots of most angiosperms and pteridophytes, along with certain gymnosperms and gametophytes of some lower plants. The fungi that form AM associations are exclusively glomeromycotan fungi in the order Glomales. In contrast to the diversity of the plants that form AM, there are only about 200 species of AM fungi described thus far (IN-VAM website: http://invam.caf.wvu.edu/). The fitness of these fungi is wholly dependent on carbon supplied from the plant. The association is facultative for at least some plants, especially those growing in nutrient-rich environments.

Fossil evidence indicates that glomalean-like fungi were present in roots of land plants during the early Devonian, some 400 mya (Remy et al., 1994; Taylor et al., 1995). rDNA sequences of

modern Glomales support the hypothesis of the origin of AM fungi date from 353 to 462 mya (Redecker et al., 2000a). Concurrent with the colonization of land by mycorrhizal plants, there was the occurrence of phylogenetic radiation (Redecker et al., 2000b). From an ancestral AM condition, current plant taxa have either lost the mycorrhizal habit or have gained the ability to form another type of mycorrhiza (Fitter and Moyersoen, 1996). The non-mycorrhizal condition is common in plant taxa that colonize disturbed sites and wet habitats where lack of competition for mineral nutrients and greater nutrient mobility exist (Peat and Fitter, 1993). It is also very common on severely Pi-depleted soils in Australia and South Africa, where many species have cluster roots (Shane and Lambers, 2004 – another chapter in the Special Issue). These habitat conditions may have selected against mycorrhizal associations. Also, in some circumstances, prevalence of root pathogens may have selected for generalized resistance to root-infecting fungi at the expense of AM fungi (Peat and Fitter, 1993).

AM symbiosis may be maintained through stabilizing selection, perhaps because AM fungi do not provoke strong defense reactions in the host, yet provide sufficient benefits to be conserved (Cairney, 2000). Host specificity coupled with the reaction of the host via a common set of gene interactions, suggests gene for gene mechanisms are less likely in AM associations (Ruiz-Lozano et al., 1999). However, symbiosis receptor-like genes from Lotus and pea are required for both fungal and bacterial recognition (Stracke et al., 2002). Thus, at least some recognition genes existing for AM have been recruited during the evolution of root nodule symbioses (Stracke et al., 2002). Although AM fungus genetics are poorly understood, there may be as yet undiscovered genetic diversity that confers the principal benefits for different hosts (Sanders, 2003).

One of the great challenges is placing the context of the AM symbiotic function in a community and ecosystem perspective. The presence of AM fungi has been shown to be an important determinant of plant community structure (Klironomos et al., 2000; Van der Heijden et al., 1998a,b). Different AM fungal isolates have varying effects on the growth of host plants; hence the negative and positive interactions between fungal and plant species appear to directly influence the resultant community composition (Bever, 2002; Hartnett and Wilson, 2002; Van der Heijden and Sanders, 2002).

ECM structure, function and host specificity

ECM are much more complex phenotypes than AM that involve several plant and fungal physiological responses and environmental interactions (Martin et al., 2001). ECM are characterized structurally by the presence of a dense mass of fungal hyphae forming a tissue sheathing the root, called the mantle. This mantle is connected to the Hartig net of intercellular hyphae and a network of ERH proliferating into the soil (Kottke and Oberwinkler, 1987). The mantle of fungal tissue surrounding the host lateral roots varies from pseudoparenchymatous tissue to a weft arrangement of hyphae on the root surface. Development of the mantle proceeds through a programmed series of events (Martin et al., 1997). Fungal hyphae, originating from a soil propagule or an existing mycorrhizal root tip, penetrate into the root cap cells and grow between them. The root cap tissue is progressively transformed by intercellular colonization into the inner layers of the mantle. A high level of structural and physiological heterogeneity exists within the mantle and between the mantle and the fungal networks (Cairney and Burke, 1996). The ERH, the mantle and the network of IRH the structures for reciprocal supply of nutrient resources, principally P and N, to the host plant and CHO to the fungus.

ECM occur on woody trees and shrubs and also in a small number of herbaceous taxa and ECM fungal partners are mostly basidiomycetes and ascomycetes with a few members of the zygomycete genus Endogone (Smith and Read, 1997). An estimated 5000 to 6000 fungal species form ECM, but this estimate is probably conservative (Massicotte et al., 1999; Molina et al., 1992). ECM fungi are well known to facilitate the uptake of mineral forms of P and N and complexed organic forms of N, not available for uptake by roots (Cairney and Burke, 1994).

The evolutionary history of ECM is not as well documented in the fossil record as it is for

AM (LePage et al., 1997). Basidiomycete and ascomycete fungi are thought to have diverged as separate lineages about 500 mya, with ECM symbiosis arising in the Pinaceae in the late Triassic, ca. 200 mya, before the appearance of mushroom fungi (Berbee and Taylor, 1993), Many ECM fungi are resupinate, i.e., they produce basidia on the surface of litter or soil particles, a spore-bearing structure presumed to be an evolutionarily less advanced structure than possessed by mushrooms. The ability to form ECM appears to be associated with the evolution of certain angiosperm woody plants, e.g., members of the Fagaceae and Dipterocarpaceae (Smith and Read, 1997).

Phylogenetic studies reveal that ability to form ECM evolved several times from saprophytic ancestors (Kretzer and Bruns, 1999). Furthermore, many ECM basidiomycetes have retained some of the saprophytic abilities of their decomposer relatives and, thereby, have the potential to access organic sources of N and P from the soil and/or to partially degrade some lignocellulose fractions of dead plant material (Cairney and Burke, 1994; Leake and Read, 1997; Read and Perez-Moreno, 2003).

ECM hosts may support hundreds of mycorrhizal fungal associates (Smith and Read, 1997); thus the observed specificity may reflect the inability of the fungus to form a symbiotic association with a given host. ECM host specificity may represent co-adaptation of the fungus to certain soil and environmental conditions (Taylor, 2000). These co-adaptations have occurred on both widespread and local scales that define the patterns of mycorrhizal diversity and specificity in ECM systems (Cairney, 2000).

Ecophysiological characteristics of the mycorrhizal symbioses

Microcosm experiments have repeatedly demonstrated that ECM and AM fungi influence the ecophysiological dynamics of small consortia of mycorrhizal fungi and hosts; but quantifying the dynamics of host-fungus interactions in complex natural settings has been difficult (Read, 2002). Mycorrhizas play a crucial role in mineral nutrient and carbon cycling, altering plant tissue nutrient content, especially that of P (Bolan, 1991; Simard et al., 2002; Smith and Read, 1997). AM and ECM plants frequently have higher photosynthetic rates, even at equivalent nutrient supply to non-mycorrhizal plants (Graham, 2000; Jakobsen et al., 2002; Simard et al., 2002). Such fungus-mediated increases in photosynthesis can result in altered carbon allocation, even if no host biomass response is evident (Jifon et al., 2002; Miller et al., 2002; Staddon et al., 1999; Wright et al., 1998b). The carbon supply response seems to be driven by upregulation of photosynthesis due to the CHO demand by the mycorrhizal fungus (Wright et al., 1998a).

In contrast to mycorrhizal influences on photosynthesis, AM fungi down-regulate root Pi uptake from soil solution once roots become mycorrhizal (Smith et al., 2003, 2004). AM-specific Pi transporters can dominate the plant's uptake process replacing the root hair and epidermal cell surface as the main site of Pi uptake. In the case of ECM, down-regulation of direct root uptake, *per se*, might not occur; but the plants rely heavily on the fungus for Pi and N uptake because the mycorrhiza may be able to take up complex forms of these nutrients that the root cannot absorb (Read and Perez-Moreno, 2003).

Mycorrhizal effects go beyond the individual and determine vegetation responses at the community and ecosystem levels (Read, 1991; Read and Perez-Moreno, 2003; Woodward and Kelly, 1997). The type of mycorrhiza that dominates an ecosystem appears to be driven by climate and such factors as litter accumulation, belowground litter quality and decomposibility, soil nutrient supply and the plant's photosynthetic capacity (Langley and Hungate, 2003; Read and Perez-Moreno, 2003; Woodward and Kelly, 1997). Ecosystems with low organic N availability and high rates of decomposition are dominated by AM associations or plants that are non-mycorrhizal. In contrast, plants growing in the highly organic soils of boreal forests or tundra exhibit slow rates of N uptake. In these ecosystems, the host plants are incapable of absorbing N from complex organic forms, and hence uptake of N may be obligately dependent upon ECM (Read, 1991). The association between plant N uptake and photosynthesis appears to also be related to a plant's mycorrhizal association wherein those plants with the highest maximum assimilation

rates are non-mycorrhizal, followed by AM and ECM hosts (Woodward and Kelly, 1997).

The physiological phenotype depends on a balance between the demands of the mycorrhizal fungus for energy (i.e., the fungus' need for CHO) and the plant's need for limiting nutrients (primarily P and N). Mycorrhizal plants growing in soils deficient in essential nutrients (P and N) increase their allocation of CHO from leaves to roots (Graham et al., 1997). Furthermore, the CHO drain on hosts due to mycorrhizas can be significant, being 4 to 26% of photoassimilates (e.g., Jakobsen and Rosendahl, 1990; Peng et al., 1993; Simard et al., 2002).

Understanding CHO partitioning alone, aside from fungal contributions to assimilation of mineral nutrients, gives an unsatisfactory view of symbioses. ECM fungi are adapted for mobilizing the sparse heterogeneous resources in the litter layer of forest ecosystems. Discrete patches of organic nutrients are accessed through exploration and colonization by ERH and rhizomorphs (e.g., Aerts, 1999; Read and Perez-Moreno, 2003; Tibbett and Sanders, 2002). Some evidence suggests that AM can exploit nutrient-rich patches, although most likely on a limited scale (Hodge et al., 2001). One of the unique features of ECM-dominated plant communities is high structural diversity of ERH and IRH networks. Root tips of a host may be colonized by hundreds of ECM fungal species, in contrast to fewer fungal species on roots of AM hosts (Bever et al., 1996; Smith and Read, 1997). Thus, acquisition of complex organic N compounds is of considerable adaptive significance in nutrient-poor environments, because ECM plants access are sources of N that is probably not available to neighboring AM and non-mycorrhizal plants (Aerts, 1999, 2002; Chapin et al., 1993; Read and Perez-Moreno, 2003).

Introductory consideration of scales for integrating genetics and physiology

Ecological research requires comprehensive understanding of whole-organism ecophysiology, species interactions, community organization and ecosystem function (Jackson et al., 2002). Considering the diverse disciplines in mycorrhizal research, development of a coherent picture of the mycorrhizal symbiosis has been a complex undertaking. Rapid advancements during the last decade in the various disciplines of mycorrhizal research have been made through study of discrete scales of size and organization that are suitable for each respective discipline (Miller and Kling, 2000). Function at the molecular level should account for the temporal and spatial interactions for each genotype, phenotype and environment. Identification of candidate genes underlying ecologically relevant traits, such as N and P acquisition and C allocation is expected to provide new tools for addressing ecological questions.

So far, slow incorporation of new tools has limited understanding of ecologically relevant traits of AM and ECM (e.g., Miller and Kling, 2000). Prediction of the responses to environmental change of genetically and functionally diverse populations and communities of plants and their mycorrhizal fungi is challenging. But characterization of the mechanisms for these changes, as well as their consequences for ecosystem functions such as primary production, nutrient cycling and carbon sequestration, is critical for understanding ecosystem adaptation to global change (Fitter et al., 2000; Rillig et al., 2002). The difficulty in this quest is the need for development of new and creative approaches for integrating fungus and host responses in a manner that accounts for the temporal and spatial variability of their interaction. Yet, as we scale-up from sequence data to whole plant and fungal functions, the databases needed for such steps are limiting. Unlike the reductionist approach of molecular biology, where individual action of genes allow for metabolism to be investigated in a methodical step-by-step approach, scaling-up requires a genomics based approach that uses robust sequence data as well as utilizing a systems approach to study information flow. Although programs like the mycorrhizal sequencing project associated with the Poplar Genome Project should alleviate many of these deficiencies (Martin et al., 2004), scaling is still an important issue that needs to be addressed.

Molecular genetic approaches for study of the symbiosis and its partners

Advances in genome sequencing and associated functional genomics provide an unprecedented

opportunity to study the key components of biodiversity and organism-environment interactions. What follows are examples of the most advanced applications of molecular approaches for assessment of genetical and functional diversity in mycorrhizal symbioses.

Fungal ITS-RFLP for studying ECM fungal diversity Application of the polymerase chain reaction (PCR) for fungal identification has been an outstanding methodological advance in the study of ECM communities (Lanfranco et al., 1998). Most of the molecular ecology on ECM fungi has involved restriction analyses of the ITS region which lies between the genes encoding small subunit (SSU) and the large subunit (LSU) ribosomal (rRNA) and contains two non-coding spacer regions separated by the 5.8S rRNA gene (Gardes and Bruns, 1993). Restriction fragment length polymorphism analysis of the ITS region (ITS-RFLP) has been widely applied to efficiently separate many species with minimal technical requirements (Horton and Bruns, 2001). The number of unidentified types is high, even at locations where extensive ITS-RFLP databases are expanding rapidly (Horton, 2002; Kårén et al., 1997). A problem is that the ITS-RFLP databases are over-represented by species that make large or obvious sporocarps and these species are not the most common ones encountered on colonized roots (Horton and Bruns, 1998). Exact matches in database searches are uncommon, because intraspecific variation exists across large geographical scales (Kårén et al., 1997). RFLP databases are most effectively applied when the fungal diversity is low enough to minimize intraspecific variation in ITS sequences, thereby increasing the chances of matches and reducing the potential for overestimating diversity (Horton, 2002).

Molecular phylogeny and identification of AM fungi rDNA sequences also have been applied to identify phylotypes and assess the phylogeny of glomalean fungi (Redecker, 2002). An unexpected outcome of rDNA analysis is that spores of *Acaulospora gerdmannii* and *Glomus leptotichum* on the same hyphae (Morton et al., 1997) are members of neither genus (Sawaki et al., 1998). Instead this dimorphic fungus belongs to one of two ancient lineages described as new genera, *Archaeospora* and *Paraglomus*, each in its own family (Morton and Redecker, 2001; Redecker et al., 2000b). The glomoid spores of *Archaeospora* do not have morphological characters that distinguish them from distant relatives in *Glomus*. The same is true for *Glomus occultum* and *Glomus brasilianum*, members of the other new genus, *Paraglomus*. These new families are defined by unique fatty acid composition and mycorrhizal morphology (Redecker, 2002). rDNA sequencing has also brought attention to the phylogeny of glomeromycotan fungi with the discovery that *Geosiphon pyriforme*, which forms endocytobiosis with the cyanobacterium *Nostoc*, is a close relative to AM fungi (Gehrig et al., 1996). The fungus produces spores that resemble those of *Glomus* even in spore-wall structure (Schüßler et al., 1994). Although *Paraglomus* seems to be the most ancient lineage, *Geosiphon* may be closely related to the basal groups within the Glomales (Redecker, 2002; Schüßler, 2002). Perhaps, the definition of Glomales will now have to be changed to accommodate fungi with non-mycorrhizal nutritional modes.

As more rDNA sequences become available, systems for molecular identification of the Glomales from spores and roots are applied more routinely (Redecker, 2002). Most importantly, these detection methods permit study of populations of AM fungi that cannot be easily identified within roots. Spores present or trapped from soils have been criticized as unrepresentative of which species are present and active (Morton, 1988). Sporulation is highly dependent on root physiological parameters and often not correlated with root colonization. Identification of AM fungi has been conducted with the primers based on 18S subunit sequences and single-strand conformation polymorphisms (SSCP) with mixed results (Clapp et al., 1995, 1999; Simon et al., 1993). Others primer designs have targeted the LSU or the ITS of rDNA with nested PCR to circumvent the problem of inhibitors in roots. This approach detects colonization patterns in roots by more than one fungus (Van Tuinen et al., 1998). Colonization of two species in the genus *Gigaspora* is enhanced by the presence of certain other species, suggesting synergistic interactions among the AM fungi. Without molecular identification tools, this complex pattern of colonization could not be detected. A specific primer

from a part of the LSU for members of the *Glomus* A group (Redecker, 2002) and SSCP are used to screen PCR products with sequence differences (Kjøller and Rosendahl, 2000). Isolates of *G. mosseae* and *G. caledonium* can be differentiated from one another side-by-side in roots and distinguished from *G. geosporum*. In this event, some types undetected in trap cultures are found in roots, and *G. mosseae* not detected in roots is found in trap cultures (Kjøller and Rosendahl, 2001).

Sequences from the 18S, ITS and 5.8S detect all the groups of AM fungi including those from *Archeaespora* and *Paraglomus* that do not stain in roots (Redecker, 2000). Several studies of the phylotype diversity utilizing the SSU of the 18S in a single step with a primer AMI show *G. mosseae* is the most abundant type in an arable soil, but is absent from an adjacent woodland (Helgason et al., 1998). The differences in AM fungal species composition are attributed to recurring disturbance by cultivation of the field sites (Helgason et al., 1999,2002).

The power of molecular methods to identify the species composition of the AM fungal community is widely appreciated but, thus far, additional markers for glomalean identity have not been established. For rDNA sequences available in the databases, there are a growing number of misidentified sequences. The sequences within spores and morpho-species vary because each spore may harbor different sequence types (Hosny et al., 1999; Redecker et al., 1999; Sanders et al., 1995). The phylogenetic definition of a species for AM fungi is under intensive study (Kuhn et al., 2001; Sanders, 2002). Recent evidence for one taxon, *G. intraradices*, is that the genome is haploid and the genome size at the lower limit of eukaryotes (Hijri and Sanders, 2003). However, until more knowledge of the evolutionary genetics of additional phylotypes is obtained, taxa remain in groups that share some ecological and physiological characteristics (Redecker, 2002).

Identification and function of symbiosis genes

ECM formation

The discovery of symbiosis-related (SR) genes expressed during the interaction between ECM fungi and roots has proceeded at an extremely rapid pace (Voiblet et al., 2001). Communication genes, such as heterotrimeric GTPases, ras, the Ca^{2+}/calmodulin-dependent phosphoprotein phosphatases (calcineurin), and serine/threonine kinases are expressed in 4-day-old Pisolithus/Eucalyptus ECM; these represent about 13% of the cloned genes in this symbiosis (Martin et al., 2001; Table 1). SR genes display similarity to genes involved in cell wall and membrane synthesis, defense response, protein degradation (in plant cells) and protein synthesis (in hyphae). So far, early symbiosis events do not appear to induce the expression of ECM-specific genes but, rather, a marked change in gene expression in the partners (Martin et al., 2001). A similarly intensive effort is also underway to discover genes that are expressed in the ECM fungi during symbiosis development. For *Laccaria bicolor* and *Pisolithus microcarpus*, 1519 and 1681 expressed sequence tags (ESTs) have been sequenced from respective cDNA libraries (Kim et al., 1999; Peter et al., 2003; Podila et al., 2002; see EctomycorrhizaDB at: http://mycor.nancy.inra.fr/ectomycorrhizadb/index.html).

Genomic models

The genome sequencing of the model tree *Populus trichocarpa*, has provided the impetus for sequencing of its AM and ECM associates, *G. intraradices* and *Laccaria bicolor* (Martin et al., 2004). *Populus* has been advanced as a model woody plant because of its relatively small genome size, extensive existing genetic resources, ease of clonal propagation and transformation (Bradshaw et al., 2000; Wullschleger et al., 2002). Also, details of the host genetics of ECM formation have been studied in *Populus* (Tagu et al., 2001, 2002).

G. intraradices together with *L. bicolor* are the first mycorrhizal fungi to be sequenced. Comparative genomics between *L. bicolor* and *G. intraradices* and other free-living and pathogenic fungi will provide evolutionary and functional genetic clues about mycorrhizal habits. *G. intraradices* has been chosen for several reasons (Martin et al., 2004). The morphotype is cosmopolitan, being found in many different ecosystems, including the tropics. As a symbiont, this species has been studied extensively and is highly effective in uptake and transfer of mineral nutrients. The fungus readily colonizes many agriculturally

Table 1. Differential expression of genes of signalling pathways in ectomycorrhiza vs free-living partners. The columns represent: (1) the expressed sequence tag (EST) clone ID, (2) the genomic origin (fungus or plant), (3) the ratio for the normalized hybridization values of transcripts expressed in the symbiotic tissues and in the free-living partners and (4) the best database match (and corresponding species) (after Martin et al., 2001)

Clone ID	Organism	Ratio	Best database match (species)
Ud283	Fungus	4.0	PWP2/Transducin (*Saccharomyces cerevisiae*)
9B9	Plant	3.4	Calmodulin (*Daucus carota*)
8A7	Plant	2.7	ADP-ribosylation factor (*Oryza sativa*)
8D10	Fungus	2.5	SHP1 protein phosphatase (*Schizosaccharomyces pombe*)
11A6	Fungus	2.1	$^{\alpha}$GTP-binding protein, subunit (*Coprinus congregatus*)
St92	Fungus	1.9	Putative histidine kinase (*Arabidopsis thaliana*)
1D4	Fungus	1.8	GTP-binding protein YPTl (*Neurospora crassa*)
St114	Fungus	1.8	Serine/Threonine protein kinase (*S. cerevisiae*)
5E9	Fungus	1.7	Rab11D/ras-related protein (*Lotus japonicus*)
8D4	Fungus	1.5	Lectin receptor-like protein (*A. thaliana*)
6C8	Fungus	1.5	PtCPC2, G^{β}/RACK-like protein (*Pisolithus tinctorius*)
EgPtdB24	Fungus	1.3	GTP-binding protein GTB1 (*Mus musculus*)
7B5	Fungus	1.2	Ras1p (*Suillus bovinus*)
Stl8	Fungus	1.2	SNF1 (carbon catabolite derepressing) (S. *cerevisiae*)
8B8	Plant	1.0	ADP-ribosylation factor (*Vigna unguiculata*)
8A1	Fungus	0.9	Serine/Threonine protein kinase (*A. thaliana*)
11C5	Fungus	0.9	Serine/Threonine protein kinase (*Caenorhabditis elegans*)
11D1	Fungus	0.9	Calcineurine $^{\beta}$subunit (*N. crassa*)

important crops such as maize, wheat, alfalfa, rice and key model plants such as *Medicago truncatula, Lotus japonicum*, as well as *P. trichocarpa*. As a model, *G. intraradices* can be grown in vitro in dual culture with transformed carrot roots for development of a split plate system for compartmentation of the fungal tissues (St.-Arnaud et al., 1996). *G. intraradices* is available commercially in pure form in large quantities (Premier Tech, Quebec Canada), which provides a resource for researchers to obtain sequence-quality DNA. *G. intraradices* has a small genome of c. 16.0 Mb (Hijri and Sanders, 2003) and nearly 3000 EST sequences from *G. intraradices* have been deposited in GenBank, along with nearly 1500 from other *Glomus* species (Jun et al., 2002).

The ECM fungus, *L. bicolor* is commonly associated with many northern temperate forest trees including *Populus*. The fungal genetics for host interaction of *Laccaria* has been studied extensively and provides a useful system for studying the evolution of host and ecological specificity (Kropp and Mueller, 1999). The physiological ecology *of L. bicolor has* also been well studied among ECM fungi because its mycorrhizas are easily established in microcosms and in vitro experiments in dual culture with *Populus* or conifer seedlings (Kim et al., 1998; Tagu et al., 2001). This species is also often chosen for use in large-scale commercial inoculation programs in forest nurseries worldwide to enhance growth of tree seedlings (Selosse et al., 2000).

The haploid genome size of *L. bicolor* is estimated at c. 25 Mb and several cDNA libraries of vegetative mycelium (Peter et al., 2003) and from *Populus* ECM have been constructed (Martin et al., 2004). Currently, there is sequence from approx. 2000 ESTs and random genomic fragments (Podila et al., 2002; Peter et al., 2003).

Molecular analysis of Pi transport in AM symbiosis

A well-developed model system for molecular and genetic studies of the AM symbiosis is the legume, *Medicago truncatula* and the AM fungus, *G. versiforme*. To facilitate studies of Pi translocation, the two-compartment *in vitro* mycorrhizal culture system is used to study ERH which ramify into the other compartment separate from the roots (Declerck et al., 1996; St.-Arnaud et al.,

1996). These systems are being used extensively to investigate the molecular processes of Pi transport in AM fungi and in roots prior to and during symbiosis.

A cDNA clone, *GvPT*, has been isolated from a *M. truncatula*/*G. versiforme* cDNA library (Harrison and Van Buuren, 1995) that shares sequence similarity with the H^+/Pi symporter gene. Previous analysis of Pi uptake by hyphal germ tubes of the AM fungus *Gigaspora margarita* (Thomson et al., 1990) suggests that AM fungi possess both high-affinity (K_m 1.8–3.1 μM) and low-affinity (10.2—11.3 mM) Pi uptake activities. The K_m and localization of *GvPT* are consistent with those of a high-affinity system and suggest a role for *GvPT* in the acquisition of Pi from soil (Harrison, 1999).

AM fungus-mediated translocation of Pi from soil to plant presumably requires specific transporters that function in ERH and at the arbuscule membrane interface. ERH are expected to mediate uptake from the soil, and arbuscules are predicted to have an efflux transporter or channel, *GvPT* transcripts are detected in ERH, but are weakly detected in IRH and arbuscules (Harrison, 1999). *GiPT* is a *G. intraradices* homolog of *GvPT* that shares 90% identity at the nucleotide level and 95% identity at the amino acid level with the coding region of *GvPT* (Maldonado-Mendoza et al., 2001). The expression of *GiPT* is regulated in response to the Pi concentration surrounding the ERH. Transcript levels are elevated in response to μM levels of Pi, but not to mM levels or to the absence of Pi. This expression pattern is consistent with a role in the high-affinity uptake of Pi from the soil solution (Versaw et al., 2002).

Two genes, *MtPT1* and *MtPT2*, have been identified in *M. truncatula* as Pi transporters active in the AM symbiosis (Liu et al., 1998b). *MtPT1* and *MtPT2* are expressed in roots, but not in leaves and the expression of both is greater under conditions of Pi deprivation (Liu et al., 1998b). The transcript levels of both *MtPT1* and *MtPT2* decrease during AM development for either *G. versiforme* or *G. intraradices* and the decline is detected early in the colonization process. Therefore, it is unlikely that these transporters are involved in the uptake of Pi across the arbuscule cortical cell interface. The down-regulation of *MtPT1* following colonization of the roots with an AM fungus may result from signals from the fungus, or may be an indirect consequence of increased Pi levels in the plant (Chiou et al., 2000; Liu et al., 1998b).

Examination of the spatial expression patterns of *MtPT1* reveals that transcripts and the encoded protein are localized in epidermal cells and root hairs and that the protein is present in the plasma membrane of roots grown under low Pi conditions (Chiou et al., 2000). The expression pattern and location of *MtPT1* suggest that this transporter plays a role in uptake of Pi from the soil solution and not in Pi uptake into the vacuole. The tomato *LePT1* transcripts and encoded protein show similar expression and subcellular localization patterns as *MtPT1*, but *LePT1* transcripts are also detected in root cap, stele, and leaf parenchyma cells, as well as cells containing arbuscules (Liu et al., 1998a; Rosewarne et al., 1999). These differences suggest overlapping but distinct physiological roles for these transporters (Versaw et al., 2002).

Models for host fungus compatibility in AM symbiosis

The most explored approach to the study of host fungus compatibility is the coordinated genetic analysis of AM symbiosis and nitrogen-fixing *Rhizobium* through the identification of nod^-myc^- mutants in pea, *Medicago* and *Lotus japonicus* (Gianinazzi-Pearson et al., 1996; Hirsch and Kalpulnik, 1998; Peterson and Guinel, 2000). Mapping and possible function of mycorrhiza-specific genes have been attempted (Marsh and Schultze, 2001). So far, the findings are that signal transduction pathways for the two root symbioses have been linked (Van Rhijn et al., 1997; Stracke et al., 2002), but disease-producing interactions have not (Barker and Larkan, 2002; Salzer et al., 2000).

Interspecific fungal variation in AM symbiosis has been demonstrated with the tomato mutant *rmc* (Barker et al., 1998). In *rmc*, different AM fungal species either do or do not form mycorrhizas and may form either Arum or Paris type mycorrhizas (Gao et al., 2001). Intra-species variation in compatibility of *G. intraradices* isolates results in functional differences in Pi uptake in *rmc* (Poulsen, 2003). Mycorrhizal defective mutants do not enhance resistance to pests or

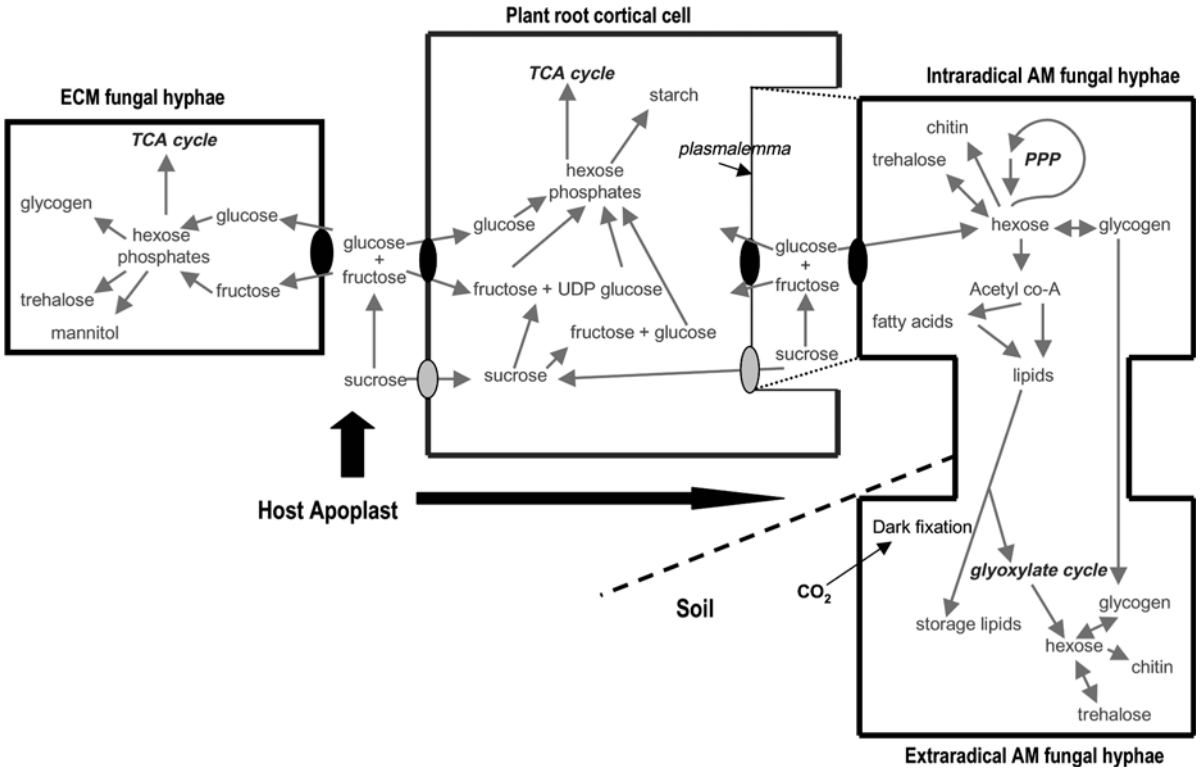

Figure 1. Simplified model of C metabolism in the ECM and AM symbiosis. The fluxes and pathways are from information reported in Bago et al. (2002), Nehls et al. (2001), and Pfeffer et.al. (1999; 2001). Although sucrose is the primary transport carbohydrate in plants, ECM and AM fungi have no system for sucrose import. Sucrose can only be used as a C source by ECM and AM fungi provided it is hydrolyzed by cell wall bound invertase. Roots also possess sucrose synthase capable of splitting the sucrose molecule. What differentiates AM fungal C metabolism from ECM metabolism is its preference for importing glucose over fructose and the accumulation of lipids as the primary storage reserve. Import of hexose by AM fungi only occurs in the intraradical phase.

pathogens (Barker et al., 1998; Graham, Ravnskov and Larsen, personal observation). Conversely, pest or pathogen resistance genes have not been demonstrated to confer mycorrhizal resistance.

In the initial stages of AM symbiosis, a non-targeted approach has been taken wherein gene expression early in the development of the symbiosis is investigated using differential mRNA screening procedures either differential screening of cDNA libraries or differential display (Delp et al., 1998, 2000; Murphy et al., 1997). These techniques identify genes that change in transcript abundance when comparing different stages of colonization or equivalently grown mycorrhizal and non-mycorrhizal plants. Subtractive hybridization molecular analysis of the changes in gene expression in *G. mosseae* during symbiosis has been used to isolate an AM fungal cDNA clone encoding an H^+-ATPase, *GmPMA1* (Requena et al., 2003). *GmPMA1* is highly expressed during pre-symbiotic development, but its expression does not change during symbiosis formation.

Molecular approaches to study of metabolism in AM symbiosis

The assumption is that carbon compounds move from plant to fungus by passive efflux across the plant plasma membrane and active uptake by the fungal plasmalemma (Ferrol et al., 2002a; Figure 1). The evidence that photoassimilate is directed toward cells with arbuscules is supported by studies showing up-regulation of genes involved in sucrose hydrolysis such as sucrose synthases and soluble acid invertase (Blee and Anderson, 1998; Ravnskov et al., 2003). NMR and radiorespirometry indicate that IRH can

take up and use hexoses, mainly in the form of glucose (Shachar-Hill et al., 1995; Soliaman and Saito, 1995). However, whether hexose transfer occurs across intercellular or intracellular membranes remains unresolved (Figure 1).

A cDNA encoding a hexose transporter whose expression increases in cells with arbuscules has been cloned from *M. truncatula* (Harrison, 1999). Functional characterization of this gene demonstrates its ability to take up glucose by a symport process, suggesting that this protein is not involved in efflux. A novel sugar transporter has been isolated from tomato leaves whose transcripts increase in a wild-type tomato, but not the colonization mutant, *rmc* (Ferrol et al., 2002b). These observations suggest that this transporter plays a role in loading sugars into the phloem at the leaf level in response to colonization with *G. mosseae*.

The presence of ATPases in the fungal membranes provides the best clue about the mechanism of transport. The membranes of the arbuscular branches lack ATPase staining while the intercellular interfaces show high levels of ATPase activity (Gianinazzi-Pearson et al., 1991, 2000). Five ATPases have been identified from *G. mosseae* and two from G. *intraradices* (Ferrol et al., 2000, 2002b). Expression of one of the sequences occurs in IRH of *G. intraradices* indicating active transport processes in these hyphae. The specific location of these transcripts remains to be determined.

In vivo NMR coupled with ^{13}C labeling has led to a wide array of important discoveries about carbon exchange with the fungus (Shachar-Hill et al., 1995; Pfeffer et al., 2001). ERH acquire hexose from the root and convert it into trehalose and then glycogen (Bago et al., 2000: Figure 1). Glycolysis, the TCA cycle and the pentose phosphate pathways are functioning in the IRH (Pfeffer et al., 1999). IRH synthesize and store large amounts of lipid as triacylglycerides (TAGS) (Bago et al., 2002; Pfeffer et al., 1999).

ERH are unable to take up exogenous hexose (Pfeffer et al., 1999; Figure 1). No storage lipid production occurs in ERH, thus the CHO requirement is via tranlocation of TAG from IRH and catabolism, and gluconeogenesis via the glyoxylate cycle (Bago et al., 2002), There is also some export of glycogen from the IRH. Translocation of carbon compounds from IRH to ERH appears to occur in tubular vacuoles along the entire length of the hyphae to provide the carbon to ERH (Ashford and Allaway, 2002).

ECM transport processes at the molecular level

Degenerate PCR primers from ESTs have been used to isolate a number of candidate genes for ECM transport processes (Chalot et al., 2002). The function and localization of transport proteins in mycorrhizal tissues are poorly known. Genomic and cDNA libraries of about 3000 ESTs have been sequenced and among the ESTs, 38 tags correspond to genes encoding carriers or channels that play a role in nutrient uptake from the soil solution, from the host plant apoplast, or transport toward the host plant (Chalot et al., 2002).

Elucidation of these transport mechanisms relies on heterologous complementation of deficient yeast mutants within transporter genes. Expression in yeast mutants of *HcAMT1, HcAMT2* and *HcAMT3* NH^+_4 transporters from *H. cylindrosporum* demonstrate they are members of the NH^+_4-transport protein family (Javelle et al., 2001). The high-affinity NH^+_4-transporter gene *HcAMT1* of *H. cylindrosporum* is expressed only under N-deficient conditions, while the low-affinity NH^+_4-transporter gene *AMT3* is highly expressed but not highly regulated by N (Javelle et al., 2003). Two amino-acid transporters, *AmAap1* from *A. muscaria* (Nehls et al., 1999) and *HcBap1* from *H. cylindrosporum* (Chalot et al., 2002), have been identified by functional complementation of a yeast strain deficient in amino-acid transporters. Their expression in yeast reveals that these transporters have two functions: uptake of amino acids from the soil solution and retention of amino acids under N deprivation conditions. Given the high number and diversity of amino-acid transporters in other fungal systems, characterization of more transporters is expected.

In ECM, CHO is delivered into the aplop-last at the plant-fungus interface (Hartig net), probably hydrolyzed by plant invertases, and then taken up into the fungus and plant symp-last (Figure 1). So far, only one hexose transporter system *(AmMst1)* has been identified from an ECM fungus, *A. muscaria* (Nehls et al., 2000, 2001; Wiese et al., 2000). Two

putative hexose transporters have also been identified from *Betula pendula* (Wright et al., 2000). Fungal uptake of hexoses is supposed to take place because the ECM represents a major CHO sink, but the fungus does not compete for the hexoses at the plant fungus interface (Chalot et al., 2002), Thus far, the results suggest active transport across fungal membranes, but the form of hexose transferred remains unresolved.

Techniques for transformation are available for selected ECM fungi, including *H. cylindrosporum* (Marmeisse et al., 1992; Pardo et al., 2003). The combination of identification of fungal transport genes with development of transgenics will provide a basis for discovering how carbon and nutrient release and transport events at the apoplastic interface are coordinated and controlled by the fungus and host.

Limitations to scaling of the mycorrhizal symbiosis to higher organizational levels

AM fungal biomass

Integrating mechanisms for metabolism of mycorrhizas with higher-level community and ecosystem processes requires techniques for identification and quantification of IRH and ERH biomass. Quantification of AM fungi in roots has been based around microscopic procedures and usually involves staining. Unfortunately, these methods are wholly inadequate for identification and do not readily scale-up to biomass. As previously discussed, spores are only partially reflective of the diversity of AM fungi, because many fungi reside within the root cortex and may sporulate rarely (Clapp et al., 1995; Merryweather and Fitter, 1998). Identifying AM fungi in roots is made more difficult by a lack of stable morphology and because of the small amount of AM fungal DMA in a rather high background content of DNA of other fungi in roots (Renker et al., 2003).

Most studies of mycorrhizal effects on host physiology measure the extent of root colonization by using stains that usually do not distinguish living from senescent fungal structures (e.g., trypan blue or acid fuchsin) and quantify colonized root segments by using the line intersection method (e.g., McGonigle et al., 1990). An important methodological improvement is the application of vital stains (e.g., succinate dehydrogenase and alkaline phosphatase in association with tetrazolium salts) to quantify living or active fungal structures, including external hyphae (e.g., Saito et al., 1993; Schaffer and Peterson, 1993; Tisserant et al., 1993). Although such methodologies are indeed an improvement, they are still only an indirect measure of the association. However, a recently developed approach for application of real-time PCR to quantify a single isolate of AM fungus in a root segment offers the opportunity for direct and specific quantification of selected taxa (Alkan et al., 2004). However, scaling-up the sampling for root and soil communities of AM fungi will require improved techniques for isolation of DNA and the design of multiple and nested primers procedures.

A decade ago, quantifying ERH was considered the domain of experiments performed with sandy soils. Since then, isolation and measurement procedures for quantifying ERH have advanced considerably (e.g., Jakobsen et al., 1992a,b; Miller et al., 1995). A major step in quantifying ERH has been the application of experimental designs using microcosms composed of membrane mesh dividers to create a hyphal compartment that separates ERH growth from root growth (e.g., Jakobsen, 1998; Schüepp et al., 1987). Today, 25-μm-diameter mesh bags are used routinely in microcosms and field studies for separating roots from ERH (Johnson et al., 2001; Schweiger and Jakobsen, 1999). These bags further provide a matrix that yields relatively pure ERH for quantification by molecular means.

In recent years, lipid analyses have been used to address a range of questions, including taxonomy, interactions with other fungal functional groups, assessment of biomass within both the root and soil and characterization of the amount of storage reserve (Graham, 2000; Larsen et al., 2000; Olsson, 1999). The use of signature phospholipid fatty acids (PLFA) and their neutral lipid fatty acid (NLFA) counterparts offers much promise for quantifying the biovolume and energy status of AM fungi in roots and soil. The PLFAs are constituents of biological membranes that can be used to estimate the biomass of AM fungi, because biovolume and cell-surface area are well correlated

(Tunlid and White, 1992). The NLFAs are the basic storage lipid of many fungi and serve as the primary energy reserve in fungi (Larsen and Bødker, 2001; Olsson, 1999). Recently, NLFAs have been used to investigate the energy status of fungi in roots (Larsen et al., 2000). Fungi differ in fatty acid composition, and some fatty acids appear to be specific for certain groups of fungi (Larsen et al., 1998). AM fungi contain the fatty acids 16:lω5, 20:4 and 20:5 that are absent in plants (Graham et al., 1995; Larsen et al., 1998). The fatty acid 16:lω5 can be used to study the biomass and nutritional status of AM fungi (Olsson et al., 1999). In contrast, 18:2ω6,9 dominates in most dikaryotic fungi (e.g., ECM fungi), but not in AM fungi (Muller et al., 1994). The application of signature acids for identification and quantification depends not only on their specificity but the presence of substantial quantities of the storage form in roots.

Advances in the quantification of AM fungi enable the addressing of new questions about AM fungal interactions with other root colonists (Graham, 2001). In particular, use of NLFAs in conjunction with PLFAs demonstrates that neutral lipids are a good indicator of competition between mycorrhizal fungi and root pathogens (Larsen et al., 2000). Although there is no difference in PLFA-estimated biovolume when mycorrhizal fungi and root pathogens occupy the same root segment, dramatic changes in NLFAs indicate that the presence of a pathogen reduces the energy status of the AM fungus and vice-versa. Such an approach can provide a powerful tool for assessing the contributions of AM fungi to the function of its host, where AM fungal biovolume is quantified by using the PLFA 16:lω5, and the corresponding NLFA is used for quantifying energy reserves. Specifically, using a ratio of signature NLFAs to PLFAs might enable identification of stress effects that are not revealed by quantifying biomass alone.

Some workers have reported that ergosterol can be used for quantifying biomass of AM fungi (e.g., Frey et al., 1992; Hart and Reader, 2002). However, these findings have been refuted based on several published reports of low levels of ergosterol in genera of the Glomales (Olsson et al., 2003). Ergosterol measured in colonized roots may be due to simultaneous infection by saprophytic fungi, which may contain high levels of ergosterol.

ECM fungal biomass

Much of what we know about ECMs is based on sporocarpic surveys; however, very little is known about vegetative structures such as rhizomorphs and hyphal mats. While sporocarpic surveys conducted over several growing seasons indicate what fungal species are present at a site (Taylor et al., 2003), the relevant contribution of ECM to nutrient uptake is usually estimated by proportion of root tips colonized by ECM fungi. Because the morphology of a root changes in response to the formation of a fungal mantle around the root tip, it is quite easy to distinguish colonized root tips from the non-colonized ones. It is generally accepted that ERH represent the functional exploratory and exploitation structure responsible for nutrient uptake and transport (Smith and Read, 1997); however, identification and quantification of this structure *in situ* is difficult.

Application of PCR-based molecular methods for identifying ECM fungi has demonstrated the deficiencies of classical methods where only a fraction of the ECM fungal taxa can be identified using sporocarps (e.g., Gardes and Bruns, 1996a,b; Jonsson et al., 1999). Any single mycorrhiza can potentially be identified to species either by PCR-RFLP of the rDNA-ITS or by DNA sequencing (Dahlberg, 2001; Martin et al., 2001). Often, a few of the ECM fungi account for the majority of abundance with the majority of fungi being rarely encountered (Dahlberg, 2001).

The stable isotopes ^{15}N and ^{13}C have been an important tool for verifying the identity of sporocarps (Taylor et al., 2003). For ECM, the discrimination should mimic that of the autotrophic associate (Henn and Chapela, 2001; Hobbie et al., 1999; Högberg et al., 1999). Although ^{15}N has been used to delineate saprophytie and ECM sporocarps, the results are more ambiguous than for ^{13}C, because many more interacting factors, including physiology, control discrimination (Taylor et al., 2003). This observation indicates that ^{15}N might be more important for delineating function than identity (Hobbie and Colpaert, 2003).

The method most likely to be used to quantify ECM biomass is measurement of total hyphal length or fungus-specific biomarkers. Ergosterol, a fungus-specific component of membranes, is believed to be a measure of metabolically active ECM fungal biomass (Ekblad et al., 1998; Nylund and Wallander, 1992). Also, because ergosterol is broken down rapidly, it is an effective measure of viable ECM biomass (Ekblad et al., 1998). Although there can be considerable variability in the amount of ergosterol associated with fungal biomass, ergosterol concentration appears to be fungal species specific and seasonally variable (Ekblad et al., 1998). Ergosterol is also positively correlated with chitin content, a cell-wall component of most fungi, along with dikaryotic fungal signature PLFA 18:2ω6,9 (Ekblad et al., 1998; Nilsson and Wallander, 2003; Wallander et al., 2001). Because saprophytic fungi in soil also produce ergosterol, the sterol is best used for quantifying ECM fungi in roots. However, mesh bags filled with organic-free sand can be used as traps for extramatrical hyphae of ECM (Wallander et al., 2001). Use of the ^{13}C signature of the hyphae in the bag showed that they were ECM, because hyphal and host signatures were similar (Henn and Chapela, 2001).

Molecular techniques enable the use of genes as biomarkers to identify hyphae directly from soil (Landeweert et al., 2003). Coupled with extraction of total soil DNA, molecular techniques such as DGGE (denaturing gradient gel electrophoresis), RFLP (restriction fragment length polymorphism), PCR and cloning sequencing have the potential to identify ECM hyphae in soil (Dickie et al., 2002; Guidot et al., 2002). These procedures reveal only the identity of an ECM fungal species. Use of real-time PCR also appears to have the potential for determining both ECM identity and biomass in soil (Landeweert et al., 2003), although quantitative extraction of DNA from soil is problematic.

Model systems to integrate molecular measurements at larger organizational scales

During the last half century, a reductionist approach to research has ruled the plant sciences, including mycorrhiza research. It is still unclear exactly how knowledge about lower-scale processes of the mycorrhizal symbiosis can be employed to predict larger-scale responses (Miller and Kling, 2000). More than a decade ago, Read (1991) argued that primary determinants of the kinds of mycorrhizas at the global scale are climate and (indirectly) litter decomposition. Since then, little progress has been made in identifying the lower-scale processes that are responsible for these higher-order observations. Read's basic premise is that climate-induced vegetation gradients and the associated soil nutrient-quality gradients have developed so that N and P are sequestered in forms not readily available to plants. A key component of this view is that natural selection has apparently favored associations between plants and fungal symbionts that can gain access to growth-limiting nutrients (Read, 1991; Read and Perez-Moreno, 2003). Unfortunately, recognition of the effects of climate and soil processes has not translated into scaling-up of mechanisms for nutrient transport to predict larger-scale ecosystem responses. Nevertheless, identification of lower-level processes that influence plant and mycorrhizal fungal growth across scales should now progress with the application of targeted molecular tools for those processes (e.g., Pi uptake, CHO metabolism).

Integrating studies of ECM and AM fungi requires a good understanding of the mechanisms controlling plant and fungus ecophysiology and their contributions to biogeochemical cycling. For example, how mycorrhizal fungi respond to and influence host photosynthate allocation, especially responses to alterations in soil nutrient quality and quantity can be monitored with specific reporter genes for C metabolism in the fungus and plant (see Figure 1). Although widely accepted that ECM and AM fungi contribute to ecosystem-level processes, such as carbon sequestration, the linkages and feedbacks between vegetation and nutrient cycles are not understood (e.g., Allen et al., 2003; O'Neill et al., 1991; Staddon et al., 2002). While feedbacks and linkages mediated by mycorrhizas are centered on lower-level processes, the outcomes of these processes can influence larger-scale performance expressed at the community and ecosystem levels. One of the major challenges for this coming decade will be the application of the tools of molecular biology to measure outcomes in a way that allows scaling-up to the ecosystem.

Because of the complexity of the symbiosis, a reductionist approach has not necessarily explained the contributions of mycorrhizas to higher-scale processes. Lower-level knowledge that does not scale-up readily is N assimilation by ECM (Read, 2002). Detailed understanding of ion uptake at the cell membrane may not scale-up readily because ion uptake is not the rate-limiting step in ECM ecosystems; rather, for most ECM plants the key step in acquiring N is mineralization of organic N complexes (Colpaert and van Laere, 1996; Read and Perez-Moreno, 2003). ECM fungi have little hydrolytic capacity for degrading polyphenolic compounds; hence, N uptake relies primarily on the activity of associated soil microbes (Bending and Read, 1997; Cairney et al., 2003). A reductionist approach would rely on knowledge of transporters and ion-uptake kinetics for scaling-up. In isolation, such an approach would be of little value in resolving the contributions of ECM to nutrient cycling (Read, 2002). This example demonstrates that the point from which one scales-up to higher organizational levels must be carefully chosen.

In AM-dominated ecosystems, primarily grasslands, mineral nutrient needs are generally accepted to differ from those of ECM systems, in that N is replaced by P as the primary limiting nutrient for plant growth (Read, 1991). Because of warmer climate conditions, as well as a concomitant increase in evapotranspiration, soil N mineralization and nitrification rates are enhanced. Also, normally Pi is sequestered in response to warmer climatic conditions. Pi availability is reduced in response to evapotranspiration-mediated elevation of soil solution salts and increased soil pH (Read, 1991; Read and Perez-Moreno, 2003). These same conditions result in a greater proportion of N released to soil solution in the form of the mobile NO^{-}_{3} ion. Because of the high mobility of NO^{-}_{3}, no depletion zone develops, and P is typically the primary limiting nutrient in AM plants.

Colonization appears to be a good indicator for carbon demand by the fungus (Graham, 2000; Miller et al., 2002; Wright et al., 1998b). However, through the use of various host and fungal combinations. Smith et al. (2003, 2004) have demonstrated that colonization may not be a good predictor for P uptake or the host growth response. The importance of the observation that dependence on ERH as the primary means of P uptake is coincident with down-regulation of genes for P transporters in plants, cannot be underestimated (Smith et al., 2003, 2004; Versaw et al., 2002). These observations are fully supported by the discoveries of 'mycorrhiza-specific' P transporters residing in both plants (Harrison et al., 2002; Paszkowski et al., 2002; Rausch et al., 2001) and AM fungi (Harrison and Van Buuren, 1995; Maldonado-Mendoza et al., 2001).

Unlike with ECM, the research reported by Smith et al. (2003, 2004) indicates that lower-level organizational mechanisms may readily scale-up for nutrient uptake in AM plants, provided the spatial and temporal variations in the field are well defined. Furthermore, variation in Pi uptake kinetics for the few plant and fungal combinations so far tested lend support for a mechanism that gives rise to diversity at the community level (Smith et al., 2003, 2004). When the potential number of plant and fungal partners is considered (Bever et al., 1996), the variations may explain differences in gene expression, nutrient-uptake kinetics and growth responsible for the 'functional diversity' in plant and fungal communities (Burleigh et. al., 2002).

Integrating lower-level processes into landscapes

Knowledge of the contributions of mycorrhizas to below-ground processes is pivotal to understanding carbon and nutrient cycling, as well as vegetation dynamics. Developing a predictive capability for ecosystem response to environmental change will require experiments at a minimum of two scales, to both develop and test ecological models (Huston, 1999). Experiments can be at the molecular scale (to observe plant and fungus gene expression) or at the interfacial scale (to quantify ion uptake); they can also be at the scale of the individual plant or fungus, their populations and communities (to quantify the responses of mechanisms). Studies can include ECM or AM influences on host growth response, biomass allocation, resource use and partitioning, or population dynamics. Experimental designs should (1) integrate multiple mechanisms to the landscape and include such measures as mycorrhizal influences on net primary production,

evapotranspiration and nutrient cycling, or (2) integrate from measures of biodiversity to ecosystem function. Extension to lower-level processes that determine ecosystem dynamics must be accomplished with smaller-scale experiments. Lower-scale experiments need to be designed to test each component under a wide range of experimental conditions that might be experienced in the field. For mycorrhizal studies, the design could use microcosms, mesh dividers, mesh bags and isotope tracers. These kinds of studies are usually conducted under controlled conditions, but more recently have been successfully extended to field studies (Johnson et al., 2001, 2002a,b; Schweiger and Jakobsen, 1999; Wallander et al., 2001).

Mycorrhizas and ecosystem genomics

Despite the fact that AM and ECM are important contributors in the cycling of carbon, N and P, their detailed functioning in dynamics of nutrient exchanges is still in its infancy. The future integration of the contributions of mycorrhizas to community and ecosystem processes will require the tools of the developing research area referred to as 'ecosystem genomics'. This new research field will allow for the integration of reductionist molecular biology with systems ecology, especially at the process level. Integration will require methods for robust, high-throughput nucleic-acid extraction for roots and mycorrhizal fungi. Especially important will be the development of quantitative, species-specific molecular assays for roots and fungi, including microarray-based methods for quantifying the expression of key mycorrhizal genes residing in host and fungus. Such advances will enable fungal genotyping and functional gene analysis. These advances will be driven by exponentially expanding sequence databases, aided by rapidly developing computational and bioinformatics resources. In addition, the developing field of metabolic profiling using high throughput techniques, such as high resolution GC-MS and NMR coupled with bioinformatics, represents an exciting approach to analyze the complexities in biochemical pathways as found in mycorrhizal associations (Pfeffer et al., 2001; Roessner et al., 2001). The application of these tools will foster an unprecedented increase in our precision in quantifying the mycorrhizal symbiosis, a necessary step for integrating molecular scale processes of the mycorrhizal symbiosis into whole systems.

Acknowledgements

JHG was supported by a Faculty Professional Development Grant from the University of Florida, Institute of Food and Agricultural Sciences. RMM was supported by the United States Department of Energy, Office of Science, Office of Biological and Environmental Research, Climate Change Program, under contract W-31-109-Eng-38. We thank three anonymous reviewers for their constructive comments.

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Plant and Soil (2005) 274:101–125
DOI 10.007/s11104-004-2725-7

Cluster roots: A curiosity in context

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Received 16 February 2004. Accepted in revised form 31 August 2004

Key words: alternative oxidase, carboxylates, citrate, mineral nutrition, phospho*enol*pyruvate carboxylase, phosphorus, proteoid roots, rhizosphere, root exudation

Abstract

Cluster roots are an adaptation for nutrient acquisition from nutrient-poor soils. They develop on root systems of a range of species belonging to a number of different families (e.g., Proteaceae, Casuarinaceae, Fabaceae and Myricaceae) and are also found on root systems of some crop species (e.g., *Lupinus albus*, *Macadamia integrifolia* and *Cucurbita pepo*). Their morphology is variable but typically, large numbers of determinate branch roots develop over very short distances of main root axes. Root clusters are ephemeral, and continually replaced by extension of the main root axes. Carboxylates are released from cluster roots at very fast rates for only a few days during a brief developmental window termed an 'exudative burst'. Most of the studies of cluster-root metabolism have been carried out using the crop plant *L. albus*, but results on native plants have provided important additional information on carbon metabolism and exudate composition. Cluster-root forming species are generally non-mycorrhizal, and rely upon their specialised roots for the acquisition of phosphorus and other scarcely available nutrients. Phosphorus is a key plant nutrient for altering cluster-root formation, but their formation is also influenced by N and Fe. The initiation and growth of cluster roots is enhanced when plants are grown at a very low phosphate supply (viz. ≤ 1 μM P), and cluster-root suppression occurs at relatively higher P supplies. An important feature of some Proteaceae is storage of phosphorus in stem tissues which is associated with the seasonality of cluster-root development and P uptake (winter) and shoot growth (summer), and also maintains low leaf [P]. Some species of Proteaceae develop symptoms of P toxicity at relatively low external P supply. Our findings with *Hakea prostrata* (Proteaceae) indicate that P-toxicity symptoms result after the capacity of tissues to store P is exceeded. P accumulation in *H. prostrata* is due to its strongly decreased capacity to down-regulate P uptake when the external P supply is supra-optimal. The present review investigates cluster-root functioning in (1) *L. albus* (white lupin), the model crop plant for cluster-root studies, and (2) native Proteaceae that have evolved in phosphate-impoverished environments.

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Introduction

The root systems of species that develop cluster roots have a unique capacity for altered branch-root development. Vast numbers of branch roots ('rootlets') are initiated which are compacted into specific regions along the axes of growing roots (Jeffery, 1967; Purnell, 1960). The individual structural and functional components of cluster roots are certainly not unique in cluster-rooted species, but common, to some extent, in root systems of most vascular plants. However, in the case of cluster-root forming species, adapted to nutrient-impoverished soils, cluster-root development represents the epitome of many common root traits that are effective in enhancing the acquisition of immobile nutrients, especially mineral-bound inorganic phosphorus (Marschner, 1995; Vance et al., 2003). The elaborate surface area associated with cluster roots is hardly designed for P uptake *per se*, but functions as an interface, providing a large surface for the release of large amounts of nutrient-solubilising compounds, and also ensures efficient recovery of solubilised P (Gardner et al., 1983).

Cluster-root development is almost ubiquitous amongst species belonging to the Proteaceae, and is an important nutrient-acquisition strategy on severely nutrient-impoverished soils in the kwongan in the southwest of Australia and in the fynbos of South Africa (Dinkelaker et al., 1995; Lamont, 1982; Pate et al., 2001). The soils in southwest Australia and South Africa are amongst the most nutrient-impoverished in the world (McArthur, 1991; Pate and Dell, 1984; Specht and Specht, 1999) which suggests that species with cluster roots are particularly well adapted to such soils (Pate, 1994). Cluster roots were first noted on *Banksia* spp. growing in the botanical gardens in Leipzig (Germany) by Engler (1894) as roots that were 'very much branched'. A detailed description of cluster roots in Proteaceae waited until Purnell (1960), who coined the term 'proteoid' roots, described dense clusters of rootlets of limited growth in genera of Australian Proteaceae she examined. Since then, the term 'proteoid-like' roots, 'cluster' roots and 'root clusters' have been applied to similar structures that have been found on root systems in several other families (Dinkelaker et al., 1995; Lamont, 1981, 1982, 2003; Skene, 1998; Table 1).

Whether cluster roots develop or not, is under genetic control, but their initiation, growth and exudation of nutrient-solubilising compounds is highly plastic, and influenced by a range of physical, chemical and perhaps even biological factors (Lambers et al., 2003; Lamont, 2003). The influence of phosphorus is probably the most important factor determining cluster-root formation and inducing physiological and biochemical processes that allow solubilisation and uptake of phosphorus.

The cluster-root adaptation for enhancing the acquisition of soil nutrients is particularly prominent in native species of the Proteaceae, which include approximately 1600 species in 73 genera (Lamont, 2003). Outside the Proteaceae, there are some 30 species of cluster-root forming genera (reviewed by Dinkelaker et al., 1995; Lamont, 2003; Skene, 1998; Table 1). Cluster-rooted plants are generally non-mycorrhizal (Brundrett and Abbott, 1991; Neumann and Martinoia, 2002). However, as more species are being examined, more exceptions are being found, e.g., *Placospermum coriaceum* (P. Reddell, pers. comm.), *H. verrucosa* (Boulet and Lambers, in press) and *Banksia ericifolia* (Pattinson and McGee, 2004). Several cluster-root forming species that form weak mycorrhizal associations sometimes also have N_2-fixing symbioses (i.e. *Casuarina*, *Acacia*, *Lupinus*, *Kennedia*, *Viminaria* and *Myrica*) (with *Rhizobium* or *Frankia*) (Brundrett and Abbott, 1991; Skene, 1998). It remains to be established, however, if these weakly mycorrhizal associations (see Graham and Miller, this volume) are important for phosphorus acquisition, growth and functioning of cluster-root forming species.

Cluster roots may once have been thought of as a curiosity in slow-growing woody species; however, their discovery in more and more species outside the Proteaceae (Table 1) and the growing knowledge of their functioning in P acquisition from sparingly available sources has triggered renewed interest. Evidence is also emerging that Proteaceae (e.g., *H. actities*) can access N from complex nitrogenous compounds, including proteins (S. Schmidt et al., 2003). The aim of this review is to draw together the key elements of cluster-root form and functioning as related to the acquisition of phosphorus, primarily in those species adapted to extremely nutrient-impoverished

Table 1. Families and representative species that produce cluster roots

Family	Representative species	Reference(s)
Betulaceae	*Alnus incana*	Hurd and Schwintzer (1996)
Casuarinaceae	*Casuarina cunninghamiana*	Diem et al. (1981); Reddell et al. (1997)
	C. glauca	Arahou and Diem (1997); Zaïd et al. (2003)
	Gymnostoma papuanum	Racette et al. (1990)
Cucurbitaceae	*Cucurbita pepo*	Waters and Blevins (2000)
Eleagnaceae	*Hippophae rhamnoides*	Skene (1998)
Fabaceae	*Lupinus albus*	Gardner et al. (1982)
	L. cosentinii	Trinick (1977)
	L. luteus	Brennan and Bolland (2003)
	Viminaria juncea	Lamont (1972a)
Moraceae	*Ficus benjamina*	Rosenfield et al. (1991)
Myricaceae	*Comptonia peregrina*	Hurd and Schwintzer (1997)
	Myrica cerifera	Louis et al. (1990)
	M. gale	Crocker and Schwintzer (1993)
Proteaceae	*Banksia grandis*	Lambers et al. (2002a)
	Banksia integrifolia	Grierson and Attiwell (1989)
	Dryandra sessilis	Roelofs et al. (2001)
	Hakea obliqua	Lamont (1972b)
	Hakea prostrata	Shane et al. (2003a)
	Leucadendron laureolum	Lamont et al. (1984)
	Telopea speciosissima	Grose (1989)

Note: the list of species given here is by no means exhaustive and the reader is referred to Lamont (1982, 2003); Dinkelaker et al. (1995) and Skene (1998).

environments, but also in other species, including the crop species *Lupinus albus* (white lupin), for which the physiology, biochemistry and molecular biology of organic anion (carboxylate) exudation has been investigated more thoroughly. The reader is also referred to comprehensive reviews by Dinkelaker et al. (1995), Lamont (1982, 2003), Neumann and Römheld (2000), Neumann and Martinoia (2002) and Skene (1998).

Structure and functioning of cluster roots

Cluster-root morphology and anatomy have been reviewed thoroughly by Dinkelaker et al. (1995), Lamont (1981, 1982, 2003), Skene (1998) and Watt and Evans (1999b), therefore, we cover only key subject matter. There are two main types of cluster roots; 'simple' (i.e. bottlebrush-like), as in *Hakea* (Lamont, 1972b; Purnell, 1960; Figure 1A) and *Lupinus* (Clements et al., 1993; Trinick, 1977) and *Telopea* (Wenzel et al., 1994), and 'compound' (i.e. mat-forming) as in *Banksia* and *Dryandra* species (Jeffery, 1967; Lamont, 1981; Purnell, 1960; Figure 2A). Compound cluster roots are an assemblage of many simple cluster roots, and have a 'Christmas-tree' like morphology (Pate and Watt, 2002; Figure 2A).

Individual cluster roots are typically 1–3 cm in length (e.g., *L. albus*; Gardner et al., 1981; Watt and Evans, 1999a, b) but can also be much longer (e.g., up to 200 mm in *H. prostrata*, Lamont, 2003 and *H. ceratophylla*; Figure 3A and B). Cluster-root initiation is periodic and is particularly obvious in *Hakea* spp. where parental root axes, each with many cluster roots, take on a 'beaded' appearance (e.g., *Hakea prostrata*, Figure 1A) , the youngest root clusters formed near the root tip. Each cluster root comprises large numbers of rootlets ranging in length from approximately 0.5 to 35 mm (Dinkelaker et al., 1995; Lamont, 1982, 2003). In species of the Proteaceae the rootlet numbers can be so large that the cluster root may take on a 'bottlebrush-like' appearance (Dinkelaker et al., 1995; Lamont, 1972b, 1982; Purnell, 1960; Figure 1A and B). In 'simple' cluster roots of *H. prostrata*

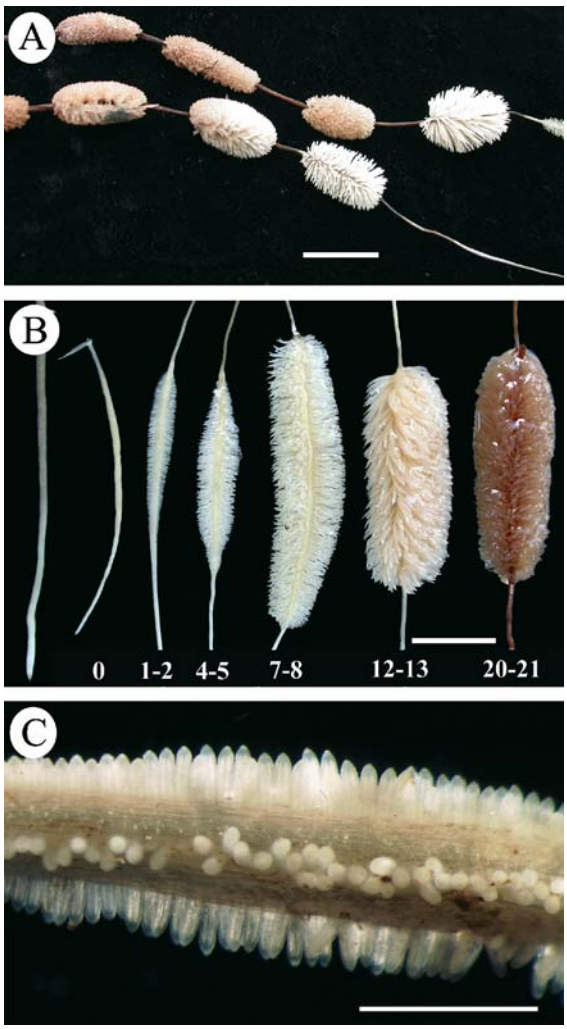

Figure 1. Cluster roots of *Hakea* spp. grown in nutrient solution at extremely low P supply (≤1 μM). (A) *Hakea petiolaris*, periodic cluster-root initiation behind the tip of the growing root axes produce 'beaded' roots, each comprising many individual 'bottlebrush-like' cluster roots separated by unbranched regions. The youngest cluster roots are at the far right of photograph. White bar is 20 mm. (B) Six stages of cluster-root development in *H. prostrata*, labelled from left to right, are arranged according to their age, i.e. number of days following rootlet emergence from the swollen axis (day 0) until cluster-root senescence (day 20 to 21). The root at the far left is a non-cluster root. White bar is 10 mm. (C) Young cluster root of *H. prostrata* approximately 2–3 days after rootlet emergence. Thousands of densely spaced rootlets emerged from the parental root axis in longitudinal rows (three longitudinal rows shown); the number of longitudinal rows corresponds to the number of protoxylem poles in vascular tissue. White bar is 5 mm.

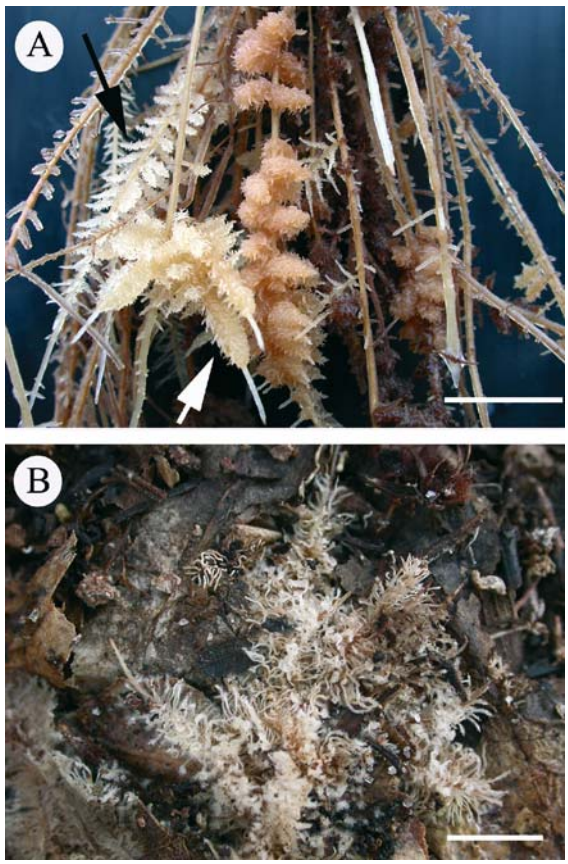

Figure 2. Compound cluster roots of *Banksia grandis*. (A) Root system of hydroponically grown plant at extremely low P supply (≤1 μM) showing young developing (black arrow) and older mature (white arrow) cluster roots. White bar is 20 mm. (B) Cluster roots found colonising decomposing litter layer in the field. White bar is 20 mm.

the packing of rootlets per unit root length is enormous (Figure 1B), and because rootlets are directly adjacent there may be hundreds of these short, determinate rootlets per cm root axis (Skene et al., 1998). Determinate roots (see Figures 14 to 24 in Varney and McCully, 1991) as defined by McCully (1999) stop growing; their meristem 'grows out' and tissues become differentiated to the tip. Skene (1998, 2003) has assigned four characteristics that define whether or not a dense grouping of branch roots is indeed a 'cluster root' as follows: (1) there is a dense clustering of rootlets, (2) there is an exudative burst with associated physiology, (3) there is determinate development of rootlets, and (4) the shape of mature (groupings) roots are ellipsoid and uptake of nutrients in enhanced (e.g., *H. prostrata*, Figure 1A and B).

It is becoming increasingly clear as we gain knowledge about species that develop cluster

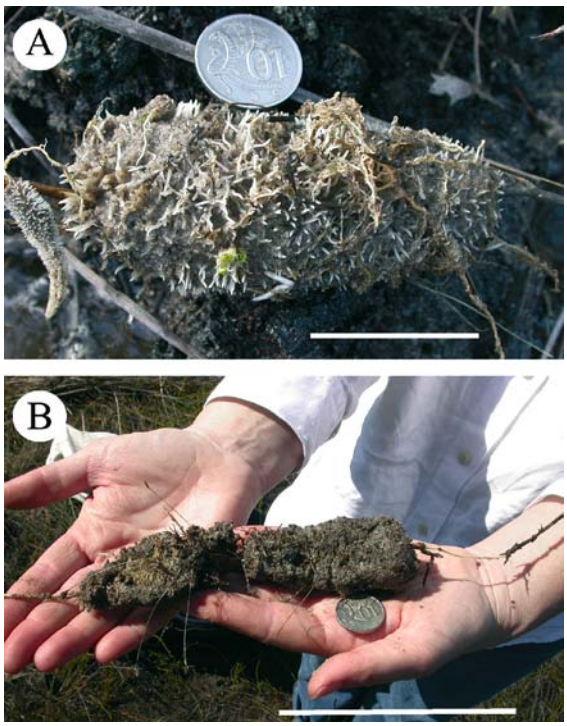

Figure 3. Cluster-root development in *Hakea ceratophylla* (Sm.) R.Br. (staghorn hakea) (Proteaceae) growing in the field during the wet, winter and spring months, in the Mediterranean climate of the southwest of Western Australia. (A) A large cluster root (centre) with thousands of growing rootlets was found near the soil surface. Another, younger cluster root can also be observed on the far left. White bar is 30 mm. (B) When mature, cluster roots tightly bind rhizosphere soil that can not be removed without damaging the rootlets. White bar is 120 mm.

roots that a continuum of cluster-root morphologies exists regarding the frequency of rootlets (reviewed by Lamont, 2003). The frequency of rootlets can range from a 100 rootlets (per mm root length) in very dense cluster roots of Proteaceae (e.g., *Banksia* and *Hakea* spp., Jeschke and Pate, 1995; Lamont, 1982) to somewhat less dense rootlet packing (e.g., 10 rootlets per mm) in cluster roots of species of Fabaceae (e.g., *Kennedia* spp., Adams et al., 2002; *Viminarea juncea*, Lamont, 1972a). The lower limit to define cluster roots of *L. albus* can be one rootlet per mm root axis (Gilbert et al., 1999), but depending upon the level of P stress *L. albus* generally produces 1–5 rootlets per mm (Keerthisinghe et al., 1998). Cluster roots are likely more widely formed than currently described (Hocking and Jeffery, 2004), especially as researchers become increasingly aware of what to look for (e.g., cluster roots on squash plants (*Cucurbita pepo*) during Fe deficiency, Waters and Blevins, 2000). Irrespective of the species that produce cluster roots, or the shape and frequency of rootlets along a continuum of morphology, these specialised root structures are all thought to provide a similar function (i.e. to enhance the acquisition of immobile nutrients such as P and Fe).

Only a small number of investigations have examined the anatomy of cluster roots. The meristems of rootlets originate in near synchrony from pericycle cells located over protoxylem poles which is similar to the development of 'normal' branch roots (Esau, 1960; Lamont, 1972b; Skene et al., 1996; Watt and Evans, 1999a). Purnell (1960) and Lamont (1972b) have shown that rootlets of *H. prostrata* each have two protoxylem poles and that additional xylem elements develop during rootlet maturation (Figure 4A and B). Watt and Evans (1999a) found in *L. albus* that vascular tissues are differentiated to the tip within 4 days of rootlet initiation. In *H. prostrata* this process may require approximately 7–10 days (Shane et al., 2004b). Each rootlet develops numerous root hairs (e.g., approximately 800 root hairs mm^2 root surface, Lamont 2003) that envelop the mature rootlet tip (Jeffery, 1967; Lamont, 1972b, 1982; Purnell, 1960; Skene et al., 1996; Skene, 1998; Figure 5A and B). In soil-grown *H. prostrata* (Figure 4C), *B. collina* (Purnell, 1960), *Grevillea robusta* (Skene et al., 1996) and *Telopea speciosissima* (Wenzel et al., 1994) root hairs may be branched, adhering tightly to soil particles and organic matter, even after careful washing (Jeffery, 1967; Lamont, 1982). Considering the ratio (approximately 30×) of cortical area to stellar area in *H. prostrata* rootlets they seem particularly well designed for something other than axile transport (Figure 4A and B). The vascular connections must be complex between rootlets and axes and offer exciting areas for future investigation. Moreover, the developmental anatomy of rootlet senescence remains unknown which raises the question of how the numerous vascular connections between senescing rootlets and parental axes are sealed.

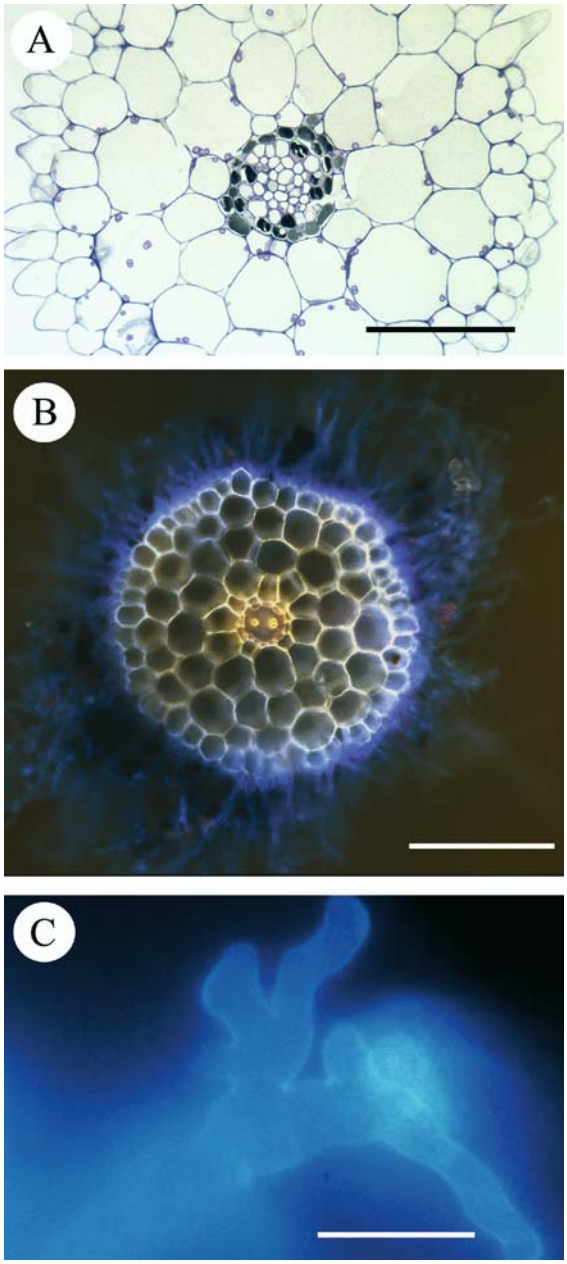

Figure 4. (A) Transverse section of resin-embedded, approximately 5-day-old rootlet from *Hakea prostrata* stained with Toluidine blue pH 4.4. Endodermal cells contain darkly stained tannins (black) and purple starch grains are found in most cortical cells. The area of the cortex is approximately 30× larger than that of the stele. A root hair is developing on some epidermal cells. Black bar is 200 μm. (B) Autofluorescence of a transverse section of a fresh, approximately 10-day-old rootlet from soil-grown *H. prostrata*. Two xylem poles can be observed in the tiny stele and root hairs cover the surface of the rootlet. White bar is 300 μm. (C) Higher magnification of the rootlet in B showing branched root hairs. White bar is 50 μm.

Factors influencing initiation and growth of cluster roots

Cluster roots may account for as much as 40–65% of the total root biomass (Dinkelaker et al., 1995; Lamont, 1981), and may contribute up to 80% of the new season's root growth when plants grow in nutrient-poor soils (Lamont, 1972b, 1973; 1982). For Proteaceae growing in a Mediterranean environment, the length of time during which cluster roots are initiated and grow may last for only 2–4 months, compared with growth in wetter regions where functional dense root mats may persist much longer (Dinkelaker et al., 1995; Lamont, 1982; Purnell, 1960). However, individual cluster roots may be physiologically active for little more than a week in *L. albus* (Hagström et al., 2001; Keerthisinghe et al., 1998; Watt and Evans, 1999a) and perhaps 2–3 weeks in *Hakea* species (e.g., *H. undulata* in soil, Dinkelaker et al., 1995; *H. prostrata* in soil, Lamont, 1976; *H. prostrata* in nutrient solution, Shane et al., 2004b), after which they senesce. The surface colour of rootlets generally changes from white to grey-brown as they mature, and that colour shift has been used to distinguish living from senesced rootlets (Louis et al., 1990). However, brown roots are not always dead; in fact, the underlying parental axis in cluster roots remains intact after rootlets senescence.

When soil moisture does not restrict root growth, cluster-root development is usually most concentrated in upper soil layers, generally just below and within the litter layer (Dinkelaker et al., 1995; Jeffery, 1967; Lamont, 1982; Purnell, 1960). Spatial patterning of cluster-root growth in the field is illustrated by the development of a superficial cluster-root 'mat' in species of *Banksia* (Jeschke and Pate, 1995; Figure 2B) and *Dryandra* to exploit the nutrients in decomposing litter layers (Dinkelaker et al., 1995; Jeffery, 1967; Lamont, 1982; Specht, 1981). The general notion is that cluster-root growth is enhanced where nutrients are likely enriched (Lamont, 1973; Lamont et al., 1984; Purnell, 1960); this is supported by the observation of cluster roots of *B. prionotes* growing 5 m below the surface in soil rich in $FePO_4$ (Pate et al., 2001).

Phosphorus is a key element that strongly influences the initiation and growth of cluster roots, e.g., in *Macadamia integrifolia* (Proteaceae,

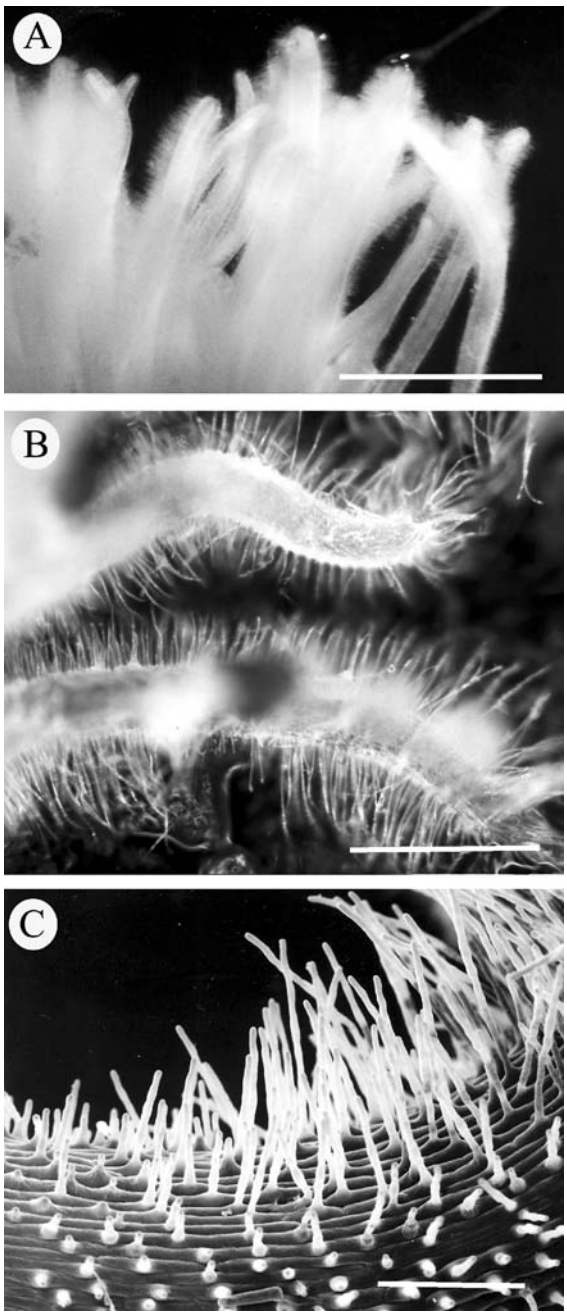

Figure 5. Root-hair development on rootlets. A) Hairy rootlets *Banksia prionotes* that were found colonising a leaf litter layer. Root hairs envelop the tips of mature rootlets. White bar is 2 mm. (B) Abundant root-hair development occurs on mature rootlets of *Hakea prostrata* grown at extremely low P supply (≤1 μM) in hydroponics. White bar is 1 cm. (C) Cryo-scanning electron micrograph showing the surface of a fully hydrated, frozen, rootlet of *H. prostrata*. Almost every epidermal cell develops a root hair. White bar is 125 μm.

Aitken et al., 1992), *L. albus* (Fabaceae, Gardner et al., 1983; Keerthisinghe et al., 1998), *Myrica cerifera* (Myricaceae, Louis et al., 1990) and *Gymnostoma papuanum* (Casuarinaceae, Racette et al., 1990). However, low N supply (*H. prostrata*, Lamont, 1972b), a limiting supply of K (*L. albus*, Liang and Li, 2003) or Fe (*Casuarina glauca*, Arahou and Diem, 1997; Zaïd et al., 2003; *Cucurbita pepo*, Waters and Blevins, 2000; *Ficus benjamina*, Rosenfield et al., 1991; *L. albus*, Hagström et al., 2001; Liang and Li, 2003; White and Robson, 1989) also enhance cluster-root development. We now know that cluster-root proportions also varies with changes in whole plant growth rate in *H. prostrata*, even though no corresponding change in internal [P] was measured (Shane et al., 2003a). Studies that alter, e.g., aeration (Racette et al., 1990) and pH (Peiter et al., 2001) influence cluster root growth. Parameters such as aeration and pH that in turn influence plant nutrition and hence growth rates need to be interpreted with some caution (Lambers et al., 2003). When the response of cluster-root proportions (of total root mass) have been assessed against a wide range of P supplies an optimum of cluster-root development generally appears as enhanced cluster-root mass at a low supply of P, above that of control plants that have no added P (e.g., *B. ericifolia*, Handreck, 1991; *H. prostrata*, Lamont, 1972b; Shane et al., 2004a, c; *Telopea speciosissima*, Grose, 1989). In response to a low availability of P many species, including cluster-rooted species (e.g., *H. prostrata*, Shane et al., 2003a, 2004c) acclimate by increasing their root mass ratio (Brouwer, 1963). In fact, it would be surprising if low P availability did not also lead to increased root-hair length (Bates and Lynch, 1996; Jungk, 2002) and root hair-density (Ma et al., 2001) in cluster-rooted plants, but this has not been measured and would be of interest to determine in future studies.

Investigations with cluster-rooted species have raised interesting questions as to whether internal or external P, and if local root or systemic shoot signals (see Dodd, this volume) modify cluster-root initiation and growth. In *L. albus*, foliar feeding of P to leaflets suppressed cluster-root development to a similar degree as when P was supplied in the rooting medium (Gilbert et al., 1997; Marschner et al., 1986, 1987). This indicates that cluster-root development in *L. albus* is

controlled by internal P, and not soil P. Furthermore, split-root studies with *L. albus* (Shane et al., 2003b) and *H. prostrata* (Shane et al., 2003a) indicate that shoot P concentration is more important than root P for controlling cluster-root initiation and growth. In reciprocal grafting studies with *L. albus* and *L. angustifolius* (Lambers et al., 2003; Marschner, 1995) the results indicated that for cluster roots to be developed, the root stock must be derived from a cluster-root forming species. Therefore, cluster-root initiation in *L. albus*, and likely in all cluster-root forming species, is under genetic control and modified systemically (Lambers et al., 2003). Table 2 illustrates a wide range of responses amongst four species grown in a split-root design with localised P supplies. In *Hakea* spp. cluster roots developed mostly in the low-P compartment, while in *L. albus* nearly equal numbers of cluster roots were produced in both high- and low-P compartments (Table 2).

How internal and/or external factors lead to cluster-root development at regular intervals along the parent axis, even when plants were grown in well-stirred nutrient solutions lacking P, is uncertain (Figure 1A). Watt and Evans (1999a) suggested that a central signalling cascade, perhaps involving auxins (Gaspar et al., 2002; discussed below), account for the synchronous development of cluster roots. If this signal were delivered in pulses (Watt and Evans, 1999b) and the response to the signal was localised (Skene, 2000) it might account for the spacing of cluster roots at regular intervals. In *Arabidopsis*, Zhang et al. (1999) have shown that regulation of genes involved in root branching are influenced both locally and systemically by [NO_3^-]

Table 2. Cluster-root growth in response to localised P supply in a split-root design

Species	Cluster root fresh mass on high-P side (mg)	Cluster root fresh mass on low-P side (mg)
Hakea prostrata	0	307
Hakea trifurcata	50	175
Lupinus albus	183	162
Lupinus pilosus	1310	702

Note: Data for *H. prostrata* from Shane et al. (2003a) and for other species kindly provided by Tim Scanlon (unpublished).

and this may be relevant here, since Shane et al. (2003b) suggested that phloem sap [P] may provide long-distance signals (see Dodd, this volume) that influence cluster-root development. The fact that many free-living rhizosphere bacteria (e.g., *Pseudomonas*) release auxins that may induce or promote branch-root growth and development (Glick et al., 1994) suggests that free-living soil micro-organisms might initiate or enhance cluster-root growth. Except for the species already noted to develop mycorrhizas and nodules, there are no reports of root-colonising bacteria and very few reports of fungi in physiologically active cluster roots, but it should be noted that the number of studies that have examined this is limited (reviewed by Lamont, 2003). Furthermore, internal signals could operate locally in a manner proposed by Skene (2000) whereby a 'primed pericycle' responds locally to internal signals (e.g., increased auxin concentration, Skene, 2001) which is similar to the localised inhibition of auxin transport that promotes nodule development (Mathesius et al., 1998).

Exogenous application of synthetic auxins to the shoots of P-sufficient *L. albus* plants overrides the suppression of cluster-root growth by high P supply to roots (Gilbert et al., 2000; Skene and James, 2000). Inhibitors of auxin transport suppress cluster-root growth in P-deficient *L. albus*. This suggests that auxins are involved in cluster-root initiation; they probably play a role in the final stage(s) in the signal-transduction pathway (Gilbert et al., 2000). Root-produced cytokinins probably also plays a role in the transduction pathway, as they do between sensing P supply and increased leaf growth (Kuiper et al., 1988, 1989). Additional information concerning the antagonistic influence of cytokinins on cluster-root growth (Neumann et al., 2000) and interactions of auxins and ethylene on cluster-root growth (Gilbert et al., 2000; Waters and Blevins, 2000) and the expression of plant-hormone associated expressed sequence tags (Uhde-Stone et al., 2003) are supplying a wealth of insight into the response of cluster roots to phytohormones.

Mechanisms of phosphorus mobilisation by cluster roots

Phosphorus acquisition by roots can be enhanced by increasing the surface area of the

root system or the rate at which phosphorus reaches the root surface (Gardner et al., 1983). A combination of strategies allows species that form cluster roots to acquire more P from soils that are low in labile P, compared with species that do not from cluster roots (Braum and Helmke, 1995; Hocking et al., 1998). Based on mineral concentrations in xylem sap measured from roots of *B. prionotes*, Jeschke and Pate (1995) discovered that cluster roots acquired 10 times more P than non-cluster roots. The cluster roots' *modus operandi* is of increasing interest as we approach the time when the P stores for fertiliser production are dwindling (Vance et al., 2003). Below, we consider three major components of the cluster-root 'design', i.e. morphological, exudative and metabolic which, in combination, are ideal for the acquisition of highly immobile nutrients like P.

Cluster-root morphology

Both the 'simple' and 'compound' cluster roots that are typical of *Hakea* and *Banksia* species, respectively (e.g., *H. prostrata*, *B. grandis*, Figures 1A and 2B) are very good examples of fine roots that are designed to enhance the acquisition of phosphate from relatively small volumes of soil (Gardner et al., 1983; Lamont, 1972b; Skene, 1998). Whereas the usefulness of localised prolific root branching in soil, in response to the presence of highly mobile nutrients like NO_3^- has been questioned (Robinson, 1994; Van Vuuren et al., 1996), it may well be important for capturing relatively immobile nutrients like P. On a mass basis, cluster roots explore potentially 25 to 33 times more soil volume (Dell et al., 1980) compared with non-specialised roots, and if the root-hair area is included this estimate increases dramatically to 140 times (see Table 1 in Lamont, 2003). Because depletion zones for nutrients overlap between adjacent hairy rootlets, the efficiency of cluster roots (e.g., of *L. albus*) to acquire labile P per unit root length is less than that of, e.g., soybean roots (Watt and Evans, 2003). Nevertheless, using ^{32}P added to soils, Braum and Helmke (1995) showed that *L. albus* mobilises a significant amount of its P from the non-labile fraction that is unavailable to soybean. The acquisition of non-labile P is not conditional on a species' ability to develop a larger surface area of roots, as is the strategy used by most species grown under low P conditions (Marschner, 1995), but the large surface area of cluster-roots in a small volume of soil provides a means to concentrate nutrient-solubilising compounds (e.g., carboxylates, Gardner et al., 1983). Indeed, exudates facilitate the mobilisation of P from non-labile soil P fractions (e.g., insoluble Al-, Fe-, and Ca-phosphates) that are unavailable to roots of most other species (Braum and Helmke, 1995; Jones, 1998; Lamont, 2003).

Cluster-root exudation

All plant roots are, to some extent, capable of 'chemical mining' of the soil, and can exude a number of chemical compounds to liberate soil minerals such as phosphate (Hinsinger, 2001; Hinsinger et al., 2003; Jones et al., 2003; Vance et al., 2003). Root exudates comprise enzymes, water, carboxylates, sugars, phenolic compounds, hormones and mucilage (Dinkelaker et al., 1995; Grierson, 1992; Jones, 1998; Marschner, 1995; Neumann and Römheld, 2002; Randall et al., 2001) which function in a range of plant processes (e.g., allelopathic interactions, signalling in plant-microbe interactions (see Miller and Cramer, this volume) and plant defence, Lambers et al., 1998). Here we concentrate on exudates that modify rhizosphere chemistry for enhancing nutrient acquisition.

Carboxylates exuded from roots are important for promoting P mobilisation (Gardner et al., 1982) because they complex metal cations that bind phosphates (e.g., Al, Fe and Ca), or displace phosphate from the soil matrix by ligand exchange (Gerke et al., 2000a, b; Jones, 1998). The release of inorganic P through ligand exchange or complexation of metal ions holding P (e.g., Ca^{2+} in rock phosphate or Fe^{3+} in $Fe(OH)_3$) is more effective by tri-carboxylates (e.g., citrate) than by mono- and di-carboxylates (Dinkelaker et al., 1989; Jones 1998; Jones et al., 2003). This is because citrate has a greater affinity for trivalent and divalent metals Fe^{3+} and Ca^{2+} (i.e. formation constants of 12.5 and 3.1, respectively; see Table 3 in Randall et al., 2001) compared with that of, e.g., oxalate and malate, and can better displace P from charged surfaces (Randall et al., 2001).

Under P-deficient conditions, rates of carboxylate exudation from non-cluster roots (e.g., *Brassica napus*, Hoffland et al., 1989; *Cicer arietinum*, Veneklaas et al., 2003) and from cluster roots are several-fold faster (e.g., *L. albus*, Gardner et al., 1983; Neumann et al., 1999, 2000; Shen et al., 2003; *Hakea* and *Dryandra* spp., Dinkelaker et al., 1995; Roelofs et al., 2001; Shane et al., 2003a, 2004b). However, rates of carboxylate exudation per unit mass or area need to be faster from non-specialised roots in order to reach local concentrations that are as high as those in the rhizosphere of cluster roots, and even then the recovery of solubilised P will not be as efficient, because of a longer diffusion pathway of both carboxylates and P from/to the root surface. For example, *L. angustifolius*, which does not form cluster roots, is far less efficient at acquiring P from low-P soils than *L. albus* is (Bolland, 1997) even though rates of citrate exudation from its roots are faster (Römer et al., 2000). *L. angustifolius* is, however, better at obtaining P from low-P soils compared with wheat, which exhibits very slow carboxylate-exudation rates (Veneklaas et al., 2003).

P-deficiency stimulates carboxylate synthesis and release, which is qualitatively similar to its influence on cluster-root initiation and growth, i.e. citrate exudation is inversely related to plant P status (Keerthisinghe et al., 1998). Cluster-root carboxylate exudation is, however, far more sensitive to P status than cluster-root initiation and growth (Keerthisinghe et al., 1998; Shane et al., 2003a, b). The exudation rates of carboxylates (e.g., citrate) from cluster roots are typically 2 orders of magnitude faster (Lambers et al., 2003; Roelofs et al., 2001; Watt and Evans, 1999a) than those from roots of non-cluster-root forming species deficient in P and/or other nutrients (Jones, 1998; Jones et al., 2003). Split-root studies with *H. prostrata* and *L. albus* indicate that suppression of carboxylate (e.g., citrate) exudation is probably a systemic response to a low shoot P status (Shane et al. , 2003a, b).

A wealth of important information for understanding cluster-root functioning and exudation of carboxylates has come from developmental studies of growing cluster roots. Dinkelaker et al. (1997) linked a greater than 10-fold increase in carboxylate concentration (e.g., malate increased from approximately 20–900 μM) in the rhizosphere of cluster roots of native Proteaceae (e.g., *H. undulata*) to the time when rootlets were 6–8 days-old (sustained for 2–3 days); following the 'exudative burst' only trace amounts of carboxylate were detected. Localised exudate sampling (see Hoffland et al., 1989; Neumann and Römheld, 2000) has clearly demonstrated spatial variation of carboxylate-exudation rates, as dependent upon the age and stage of root development (Keerthisinghe et al., 1998; Neumann et al., 1999). The rates of citrate exudation increase approximately four times from 107 to 472 pmol g^{-1} fresh mass s^{-1} (Keerthisinghe et al., 1998) from so-called 'mature' cluster roots (i.e. 10–60 mm behind the root tip, Keerthisinghe et al., 1998; Neumann et al., 1999). Precise time-line investigations of citrate efflux rates from *L. albus* (Hagström et al., 2001; Watt and Evans, 1999a) show that the citrate is released from the mature rootlets in an exudative burst that lasts approximately 2 days and coincides with the cessation of rootlet growth when rootlets are approximately three to four days old. Furthermore, exudation rates of citrate from cluster roots of *Lupinus* spp. follow a diurnal pattern, with rates during the light period being twice that in the dark (e.g., *L. albus*, Watt and Evans, 1999a; *L. luteus*, Hocking and Jeffery, 2004). Cluster roots of *H. prostrata* also release citrate and malate during an exudative burst after rootlets have finished elongating (7–13 days old) at similar or faster rates (e.g., approximately 350–2000 pmol citrate g^{-1} root fresh mass s^{-1}, Shane et al., 2003a, 2004b), but the possibility of diurnal patterns has yet to be evaluated. In P-deficient *L. albus*, carboxylate exudation can be further stimulated by a low Fe supply (Hagström et al., 2001; McCluskey et al., 2004). The rate of citrate exudation from these low-P, low-Fe cluster roots is up to 1000 times faster (depending upon the study and growth conditions, see Table 3; McCluskey et al., 2004). The uniqueness of the exudative burst of carboxylates from cluster roots has been reviewed by Skene (2003), but comparisons with growing, non-specialised roots in other species is difficult considering we have only limited quantitative data. For example, carboxylate exudation from roots of *Brassica napus* (Hoffland et al., 1989) and *Cicer arietinum* (Wouterlood et al., 2004) are increased by 2- to 4-fold from younger as compared to older regions of roots. Root exudation

is associated almost exclusively with relatively young white roots that may or may not have root hairs. Although rootlets of some species are reportedly covered in root hairs by the time the exudative burst of citrate occurs, root hairs are not essential for exudation as illustrated by malic acid exudation from root tips of wheat (Ryan et al., 1997); however, very little is known about the role of root hairs in exudation and P uptake by cluster roots, and this warrants further investigation.

The acquisition of P by cluster roots is further enhanced by release of extracellular acid phosphatases (APases), e.g., in *Casuarina cunninghamiana* (Reddell et al., 1997), *Dryandra sessilis* (Grierson and Comerford, 2000), *H. undulata* (Dinkelaker et al., 1997) and *L. albus* (Adams and Pate, 1992; Neumann et al., 1999, 2000; Ozawa et al., 1995; Wasaki et al., 2003). In *L. albus* a novel acid phosphatase is specifically induced under P deficiency (Gilbert et al., 1999; Miller et al., 2001). Considering the remarkable proliferation of 'compound' cluster roots of *Banksia* and *Dryandra* species in decomposing litter, which is enriched in organic forms of P (Lamont, 1982; Pate and Watt, 2002), acid phosphatases exuded by cluster roots may contribute significantly to P acquisition (Dinkelaker et al., 1997). Exudation of phosphatases is likely an important adjunct to carboxylate exudation from cluster roots, because inorganic P, liberated by acid phosphatases, is more likely to be taken up in the presence of citrate, which can suppress re-adsorption and precipitation of inorganic P (Braum and Helmke, 1995; Gerke et al., 1994). Additional support comes from work showing that citrate accumulation, in concentration ranges detected in the rhizosphere of cluster roots, is particularly effective in the solubilisation of organic P forms, which thereafter can serve as a substrate for APase (Hens et al., 2003). Under these conditions the efficiency of organic P mobilisation is greatly enhanced, since the low solubility of organic P in soils and of the APase enzyme protein are major factors limiting the acquisition of organic P by roots (Adams and Pate, 1992; Neumann and Römheld, 2000).

Under conditions of P deficiency, cluster roots of both *L. albus* (Neumann et al., 2000) and Proteaceae (*H. undulata*; Dinkelaker et al., 1995) exude phenolic compounds (isoflavonoids) that can mobilise P by reduction of mineral-bound phosphates (e.g., Fe-bound phosphates, Neumann and Römheld, 2000). Cluster roots of P-stressed *Ficus benjamina* (Rosenfield et al., 1991), *L. albus* (White and Robson, 1989) and squash (*Cucurbita pepo*) also show an enhanced capacity for Fe^{3+} reduction (Waters and Blevins, 2000). Considering the potential of phenolic compounds in exudates to reduce and liberate micronutrients (Dinkelaker et al., 1995), inhibit microbial degradation of exuded carboxylates, and influence the rate of carboxylate exudation (Neumann et al., 2000) we still know very little about them.

Mobilisation of P by carboxylates in soil (depending on the initial pH), may also be enhanced by acidification of the rhizosphere in P-deficient plants (Tang et al., 2001). Depending upon soil type and nutrition, the cluster-root rhizosphere may become acidified (Dinkelaker et al., 1995; Yan et al., 2002), but whether or not rhizosphere acidification coincides with carboxylate exudation is uncertain; alternatively, other cations might be released with the carboxylates. If an exudative burst of carboxylates and H^+ do coincide (e.g., *L. albus*, Dinkelaker et al., 1989), then concomitant extrusion of H^+ (e.g., together with citrate) could assist in accessing insoluble forms of P (e.g., Ca-phosphates) and micronutrients if the soil pH is initially relatively high (Marschner, 1995). Since most cluster-rooted plant species identified so far, are naturally adapted mainly to low-pH soils, it remains to be established whether the reported H^+ exudation (Dinkelaker et al., 1989, 1997) can really be regarded as an adaptation for improved P acquisition or whether it is simply a component of the mechanism for carboxylate export, which may be at least partly substituted by the release of other cations such as K^+ (Dinkelaker et al., 1995, Roelofs et al., 2001; Sas et al., 2001).

There are clearly rhizosphere micro-organisms associated with cluster roots, and in addition to the potential for stimulation of cluster-root growth by rhizosphere micro-organisms noted earlier, their presence may also be linked to the acquisition of P and other nutrients. Wenzel et al. (1994) located phosphate-solubilising bacteria associated with the cluster roots and non-cluster roots of *Telopea speciosissima*, and Pate et al.

(2001) identified citrate-consuming bacteria associated with soil extracts of *B. prionotes*. It has been shown that different bacterial populations interact with different root zones (sheathed and bare regions) in field-grown maize (Gochnauer et al., 1989). In *L. albus*, Marschner et al. (2002) have also found that bacterial communities differ between cluster and non-cluster roots, and also vary with cluster-root age and plant age. Interestingly, bacterial community structure was correlated with the exudation of cis-aconitate, malate and citrate. Furthermore, Weisskopf et al. (2004) have shown that bacterial abundance decreases during the stage of cluster-root development associated with the exudative burst of citrate, but that bacterial diversity and community structure were mostly related to root proximity, and to a lesser extent with cluster-root age. Questions concerning the functioning of the bacterial communities associated with cluster roots and how bacterial populations might influence nutrient acquisition would make for fascinating experiments.

It is well known that cluster roots can bind a substantial amount of rhizosphere soil (Grierson and Attiwell, 1989; Jeffery, 1967; Figure 3A and B), which is impossible to remove without damaging the rootlets. Considering the ubiquitous nature of mucilage exudation from roots which is thought to play a key role in plant nutrition (McCully, 1999) virtually nothing is known of the exudation of mucilage from cluster roots. There are only reports by Dell et al. (1980) and Skene et al. (1996) that mucilage is exuded from root-cap cells of two species of Proteaceae, and much anecdotal evidence for copious production of mucilage by developing cluster roots.

Roots are, to some extent, capable of chemically 'designing' a rhizosphere in response to soil type (Ström et al., 1994). For example, in *B. grandis* the carboxylate composition in cluster-root exudates depends on the form of P in the soil (Lambers et al., 2002a). When P was supplied as aluminium phosphate, the major carboxylates were the larger di- and tricarboxylates. When P was supplied as iron phosphate, a mixture of smaller mono- and less of the larger di- and tricarboxylates was exuded (Lambers et al., 2002a). Furthermore, in *C. arietinum* and *L. albus* the malate/citrate ratio exuded from roots is correlated with the soil pH (Veneklaas et al., 2003). Citrate dominates when malate is rather ineffective at mobilising P. Studies examining the response of cluster roots to different soil types are lacking, but considering the interesting findings thus far, cluster-root carboxylate exudation appears to be highly plastic, and presents cluster roots as active soil miners, rather than simple accumulators of soil nutrients.

Cluster-root metabolism

Carboxylates exuded from roots are often tricarboxylic acid (TCA) cycle intermediates (e.g., citrate, cis-aconitate and malate) or derived from them (e.g., trans-aconitate) (Figure 6). In addition, oxalate (e.g., root tips of buckwheat, *Fagopyrum esculentum*, Zheng et al., 1998) and malonate (e.g., chickpea, *Cicer arietinum* L., Ohwaki and Sugahara, 1997) may be exuded. As already noted, cluster-root exudation of carboxylates is increased in response to P deficiency, whereas the rates of several metabolic reactions that require P as a substrate decrease (e.g., carbon flux through glycolysis; Neumann et al., 1999; Rychter and Mikulska, 1990). In P-deficient *L. albus*, the fast rates of citrate exudation from mature cluster roots are associated with enhanced *in vitro* activities of enzymes involved in carbon supply (e.g., 3-fold increase in phospho*enol*pyruvate carboxylase (PEPC), Neumann et al., 2000; Figure 6) and carboxylate synthesis (Johnson et al., 1994, 1996a, b; Neumann and Martinoia, 2002; Watt and Evans, 1999a). This suggests that some of the exuded carboxylates are produced locally, presumably from imported sucrose, rather than transported via the phloem.

Formation of auxin-induced cluster roots in P-sufficient plants is very interesting because these induced clusters do not show enhanced citrate exudation which suggests that the two responses are regulated independently (Watt and Evans, 1999b). A similar ability to uncouple structure from functioning has also been observed for auxin-induced rhizodermal transfer-cell development in *Lycopersicon esculentum*; these induced transfer cells do not exhibit enhanced expression of membrane-H^+-ATPases (W. Schmidt et al., 2003). Taken together this evidence suggests that it is necessary for metabolism to be specialised in roots for enhancing exudation of carboxylates to

mobilise additional P. On the other hand, cluster-root morphologies may vary as dependent on external or internal environment, without concomitant metabolic changes.

The evidence for metabolic specialisation in cluster roots of *L. albus* (Johnston et al., 1994, 1996a, b; Keerthisinghe et al., 1998; Neumann et al., 1999; Watt and Evans, 1999a) supports at least three views: (1) enzymes (activities and transcript levels) involved in carbon metabolism (e.g., PEPC (Johnston et al., 1994, 1996a, b; Uhde-Stone et al., 2003; Vance et al., 2003) and PEP-phosphatase (Kihara et al., 2003)) are up-regulated during citrate exudation from cluster roots; (2) enzymes involved in carbon metabolism are down-regulated just before the peak in citrate exudation from cluster roots (e.g., aconitase (Kihara et al., 2003; McCluskey et al., 2004; Neumann and Römheld, 1999; Neumann et al., 1999)), isocitrate dehydrogenase and pyruvate kinase (Kihara et al., 2003)); (3) transport, rather than net synthesis, is the rate-determining step for exudation of carboxylates (e.g., citrate (Watt and Evans, 1999a, b)).

Carbon may enter mitochondria as either pyruvate or malate (Figure 6); the latter is produced by the concerted action of PEPC and MDH in the cytosol. However, only malate can enter the mitochondrial TCA cycle, whereas pyruvate is first converted to acetyl-CoA, malate or oxaloacetate before it enters the TCA cycle (Lambers, 1997; Figure 6). Cytosolic PEPC has been implicated as a key enzyme facilitating citrate exudation (Johnson et al., 1996a, b; Ryan et al., 2001; Watt and Evans, 1999a). The role of PEPC is to replenish TCA intermediates that have been siphoned off (e.g., for exudation), by catalysis of the carboxylation of PEP to form oxaloacetate, which is then reduced to malate (Johnson et al., 1994) before entering mitochondria. Johnson et al. (1996a) assessed the quantitative importance of PEP-carboxylase-mediated non-photosynthetic CO_2 fixation for citrate exudation by cluster roots of P-deficient *L. albus* *in vivo*. They found that 25 and 34% of the fixed carbon was exuded as citrate and malate, respectively (as determined for the whole root system). Furthermore, Johnson et al. (1996b) showed that enhanced citrate exudation by whole root systems of *L. albus* grown without P coincided with elevated PEPC activity *in vitro*, and with increased expression of mRNA encoding PEPC and with PEPC protein abundance. However, Keerthisinghe et al. (1998) and Watt and Evans (1999a) found that *in vitro* PEPC activities on a protein basis in cluster roots of *L. albus* were not correlated with the measured rates of citrate exudation at all stages of cluster-root development. In cluster roots of P-deficient *H. prostrata* Shane et al. (2004b) found that protein abundance of PEPC varied only marginally during cluster-root development. Why would there be little change in PEPC abundance? Calculations show that PEPC activity in cluster roots of *H. prostrata* (Shane et al., 2004b) would be expected to reach a maximum activity when respiration peaked, and then to decline gradually during the exudative burst of citrate and malate (i.e. 4- to 5-fold faster rates compared with those of non-cluster roots). These values may have been overestimated slightly, but they convincingly demonstrate that there may not be a necessity for a major change in PEPC activity during cluster-root development and carboxylate exudation in *H. prostrata*. It appeared that PEPC activity in cluster-root tissue of *H. prostrata* resulted in a gradual loading of (iso)citrate during development, followed by a burst of citrate and malate exudation when roots matured; the role of PEPC gradually shifted from one involved in respiration to one involved in carboxylate exudation. The stored carboxylates provided some of the released citrate and malate during the fast exudation rates (Shane et al., 2004b). The progressive accumulation of citrate and isocitrate in cluster-root tissue is in line with the hypothesis that carbon flux via oxidative decarboxylation and respiration (Figure 6) is reduced in favour of an increased carbon flow via PEPC for cluster-root carboxylate production. PEPC use for carboxylate synthesis increases, finally leading to accumulation of citrate and malate in mature cluster roots of *H. prostrata* (Shane et al., 2004b) and *L. albus* (Johnston et al., 1994; Kihara et al., 2003; Neumann and Römheld, 1999).

In contrast with enhanced citrate biosynthesis as a means to increase net citrate production, reduced activity of citrate catabolism by aconitase (that converts citrate to isocitrate in the TCA cycle) is associated with enhanced exudation of citrate from cluster roots of P-deficient (Neumann and Römheld, 1999) and Fe and

P-deficient *L. albus* (McCluskey et al., 2004). Neumann and Martinoia (2002) proposed that reduced activity of citrate turnover by inhibiting the cytosolic enzyme ATP-citrate lyase (Figure 6), may also provide a means for citrate to accumulate in cluster root tissues prior to the exudative burst. A reduction in activity of ATP-citrate lyase may also underlie the dramatic shift from malate production to citrate accumulation in cluster roots of *L. albus* (Neumann et al., 2000). In cluster roots of *H. undulata* (Dinkelaker et al., 1997) and *H. prostrata* (Shane et al., 2004b) which exude large quantities of malate along with citrate during the exudative burst, malate exudation would be enhanced by the action of ATP-citrate lyase on newly synthesised or stored citrate by converting it into malate (*via* oxaloacetate and malate dehydrogenase) in the cytosol (Langlade et al., 2002; Neumann and Martinoia, 2002; Figure 6). The other by-products of the ATP-citrate lyase reaction (i.e. acetyl-CoA; Figure 6) might be directed towards synthesis of lipids, and terpenoid and phenolic compounds for exudation, providing an anaplerotic supply of acetyl-CoA under conditions of limited acetyl-CoA production via pyruvate kinase (Kania et al., 2003; Kihara et al., 2003).

Can we really extrapolate from the *in vitro* measurements of enzyme activity in P-deficient plants to activities *in vivo*? According to Ryan et al. (2001) we must interpret the results of *in vitro* assays with caution, because *in vivo* enzyme activity is influenced by other cellular conditions, especially in P-deficient plants (Moraes and Plaxton, 2000; Ryan et al., 2001). For example, PEPC is regulated *in vivo* by, e.g., post-translational phosphorylation, oligomerisation, and protein turnover (Chollet et al., 1996). In addition, PEPC can be activated *in vitro* by G-6-P, and deactivated by L-malate and DL-isocitrate (Moraes and Plaxton, 2000).

In *L. albus*, the amount of citrate exuded into the rhizosphere may account for up to 23% of whole-plant net photosynthesis (Dinkelaker et al., 1989). Considering that most of this citrate is released from only the cluster-root fraction of the total root system (which may contribute up to 40% of the total root mass in P-deficient *L. albus*), a considerable carbon sink is generated in the mature citrate-exuding cluster roots (Massonneau et al., 2001). In fact, there is a positive correlation between sucrose synthase activity and citrate-exudation rates in mature (citrate-exuding) cluster roots of *L. albus* (Massonneau et al.,

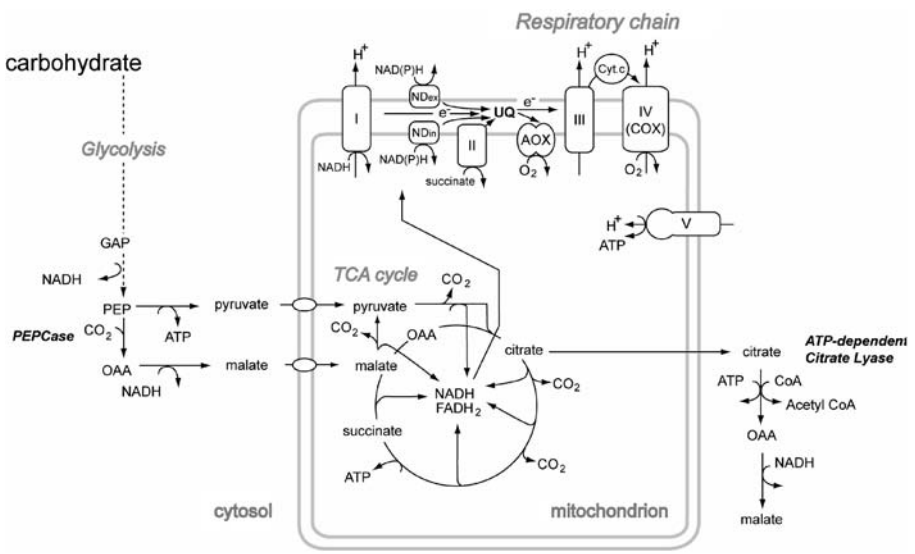

Figure 6. The entry of carbon into mitochondria, and reactions of the TCA cycle and the organisation of the four, electron-transporting complexes (I–IV) of the respiratory chain in higher plants. All complexes are located in the inner mitochondrial membrane. Dark CO_2 fixation in cells of root tissues requires PEPC (anaplerotic pathway). If citrate or similarly oxidised carboxylates are exuded, exudate production requires the oxidation of NADH produced during carboxylate synthesis, which may require alternative oxidase (AOX) activity.

2001). Moreover, the sucrose synthase pathway consumes half of the ATP used by the invertase pathway which is energetically important under conditions of cluster-root growth (i.e. P deficiency). Plants use PEPC, malate dehydrogenase (MDH), NAD-dependent malic enzyme (ME), and pyruvate dehydrogenase and citrate synthase (CS) to provide citrate anaplerotically (Johnson et al., 1994, 1996a, b; Neumann and Römheld, 2000; Ryan et al., 2001; Watt and Evans, 1999a). Whether or not enhanced *in vitro* activities of enzymes would translate into enhanced citrate biosynthesis and exudation *in vivo* in *L. albus* is not certain. In studies with transgenic tobacco plants (*Nicotiana tabacum*) that over-expressed CS, citrate accumulated in root tissues and citrate exudation from roots was enhanced (Lopez-Bucio et al., 2000). However, in other studies with transgenic tobacco that also showed enhanced expression of CS, Delhaize et al. (2000, 2003) found that citrate did *not* accumulate in tissues, and citrate exudation was *not* enhanced, even though mitochondrial CS protein levels were five times greater than those in control plants.

Under conditions of P deficiency, low levels of cellular P*i* may restrict the cytochrome (*cyt*) pathway (Theodorou and Plaxton, 1993). Moreover, *in vitro* activities of *cyt* oxidase may be severely decreased under P*i* deficiency (e.g., in *Phaseolus vulgaris*; Rychter and Mikulska, 1990). The continued flux through part of the TCA cycle when intermediates like citrate are being siphoned off during the exudative burst, with a concomitantly enhanced production of NADH, requires an active mitochondrial electron transport chain (Figure 6). In plants, the cyanide-resistant, alternative oxidase (AOX) pathway branches from the main electron transport pathway, and is not linked to ATP production beyond the branching point (Millenaar and Lambers, 2003; Figure 6). In *L. albus* (Kania et al., 2003) and *H. prostrata* (Shane et al., 2004b) the amount of AOX protein in cluster roots increases prior to the exudative burst of citrate. In cluster roots that exude a substantial amount of carbon as citrate, the increased expression of AOX presumably reflects an increased *in vivo* activity. This is probably needed to ensure continued electron flow in the electron transport chain, and oxidise the NADH generated during the synthesis of citrate in the anaplerotic route from the carboxylation of PEP to the production of citrate in the TCA cycle (Lambers et al., 2002b; Figure 6). Further studies are required that use the O_2-fractionation technique to determine the AOX activity of intact cluster-root tissues (Day et al., 1996; Guy et al., 1992; Ribas-Carbo et al., 2005).

What controls the brief and specific efflux of citrate (and malate) from cluster roots during the exudative burst? It is controlled either (1) by the net rate at which citrate is synthesised or imported into cluster root cells (i.e. the balance between its synthesis/import and breakdown/export), or (2) by the rate of citrate release from the cluster roots. In terms of (1), Keerthisinghe et al. (1998) and Neumann et al. (1999) showed that the internal concentration of citrate in developing cluster roots of *L. albus* does not always correlate with the rates at which citrate is exuded. In cluster roots of *H. prostrata* which exuded malate and citrate, the concentration of (iso)citrate increased internally, and then decreased at the time of the exudative burst of citrate and malate (Shane et al., 2004b). In terms of (2), citrate efflux from cluster roots probably occurs via anion channels (Neumann et al., 1999; Randall et al., 2001; Ryan et al., 2001; Watt and Evans, 1999a, b) such as those activated in wheat (*Triticum sativum*) root tips exposed to Al^{3+} (Ryan et al., 1997). The findings of Neumann et al. (1999) that anion-channel blockers reduced carboxylate exudation by half when applied to cluster roots of *L. albus* supports that contention. Recently, Kania et al. (2003) have demonstrated that a decline of the cytosolic pH by external application of low-molecular-weight organic acids (e.g., propionate), according to the ion-trap principle could induce intense citrate and malate exudation in young cluster roots and also in non-cluster roots during the time when exudation of these carboxylates is usually very low.

It is unlikely that all the different root tissues in the cluster rootlets are similarly involved in the production and release of citrate, but every cluster-root study uses information derived from whole-cluster-root-tissue homogenates. In terms of the exudative interface at the root surface, it is possible that citrate is produced and exported only by epidermal cells and their root hairs, as shown for the release of mucilage from root cap cells (e.g., *Zea mays*) (McCully, 1999) and for

fast rates of H^+ exudation and Fe-reductase activity in rhizodermal transfer cells of sunflower (*Helianthus annuus*) when grown at very low Fe supply (Kramer et al., 1980). If citrate is assumed to be produced and exuded from epidermal cells (and/or root hairs) only, then an analysis of whole tissue extracts masks any changes in epidermal cell metabolism. On the other hand, if citrate were synthesised and exported from deeper tissues, i.e. several inner layers of cortical cells, then a transient increase in tissue citrate concentration (or other carboxylates) might be measured during the exudative burst (i.e. considering citrate would have to traverse several cell layers before leaving the root surface). Citrate efflux rate would then depend on transporters in the outer periclinal membranes of epidermal cells.

Carboxylates, such as citrate and malate exist as anions at the near-neutral pH typical of the cytosol of plant cells (see Figure 3 in Neumann and Römheld, 2000). Evidence indicates that carboxylates are released down an electrochemical gradient via separate anion-permeable channels (Neumann et al., 1999; Ryan et al., 2001; Vance et al., 2003; Zhang et al., 2004). Anion-channels involved in aluminium-induced carboxylate exudation have been identified in apices of wheat roots (Kollmeier et al., 2001; Zhang et al., 2001). The work of Zhang et al. (2004) has provided the first patch-clamp characterisation of a citrate channel in cluster roots which is inhibited by the same ion channel blockers as employed in the study of Neumann et al. (1999) with intact roots. The recent identification of a putative Al-activated gene *ALMT1* encoding a malate channel (Sasaki et al., 2004) certainly brings nearer the possibility of applying biotechnology to crop plants for combating Al toxicity and increasing carboxylate exudation and P acquisition-efficiency (Ryan et al., 2003).

The fact that whole-cluster-root-tissue homogenates reveal little of the cellular heterogeneity that exists in complex tissues such as roots has propelled efforts to understand the roles played by some of the rootlet cell types. Uhde-Stone et al. (2003) have shown, that enhanced *in situ* expression of PEPC and MDH in P-deficient *L. albus* was localised in the cortical cells. More studies are required that carefully follow the time course of cluster-root development and citrate exudation (e.g., Watt and Evans, 1999a), and link this information with altered metabolism at the level of specific tissues using single-cell methodologies. The techniques of single-cell sampling and analysis (Tomos and Leigh, 1999), cryo-SEM with X-ray microanalysis (McCully et al., 2000), and *in situ* hybridisation (Uhde-Stone et al., 2003) may all assist in exploring the heterogeneity of cell metabolism in complex tissues of cluster roots. Another important aspect concerns the fact that almost all our knowledge on cluster-root function and physiology comes from nutrient-solution experiments under P-limiting growth conditions, and studies done in soil are rare (Dinkelaker et al., 1997; Kamh et al., 1999). Apart from limited P availability, under natural conditions cluster-rooted plant species are probably exposed to a wide range of factors with potential impact on carboxylate metabolism and exudation, such as aluminium toxicity, high levels of manganese and availability of different N forms. Nothing is known on how cluster-root formation and function is influenced by the simultaneous action of these factors under natural growth conditions.

Ecophysiology of cluster roots and P nutrition in Proteaceae

Cluster-root development and phosphorus toxicity

There is no doubt that Proteaceae are able to function well at very low leaf [P] (in *H. prostrata* 0.33 mg P g^{-1} DM, Shane et al., 2004a) compared with other species (approx. 1 mg P g^{-1} DM in crop plants) (Lambers et al., 1998; Marschner, 1995), but there are no physiological explanations for this. Furthermore, Proteaceae typically develop foliar symptoms of phosphorus toxicity, even at very low rhizosphere [P] (e.g., in *B. ericifolia*, Ozanne and Specht, 1981; Parks et al., 2000; *B. grandis*, Lambers et al., 2002a; *H. prostrata*, Shane et al., 2004a, c). Previous studies have characterised the development of P-toxicity symptoms, but the physiological basis for its development in Proteaceae remained unclear. The symptoms of P toxicity described for Proteaceae include early leaf senescence, poor growth and necrotic and chlorotic regions on leaves (e.g., *B. ericifolia*, Handreck, 1991; Parks et al., 2000;

B. grandis, Lambers et al., 2002a; *B. serrata*, Groves and Keraitis, 1976; *Grevillea* cv. 'poorinda firebird', Nichols and Beardsell, 1981; *H. prostrata*, Shane et al., 2004a). These symptoms are rather similar to those described for other species, including crop plants (e.g., wheat, Bhatti and Loneragan, 1970; Loneragan et al., 1966). A comparison of the leaf [P] at which P toxicity occurred in *H. prostrata* (approx. 10 mg g^{-1} DM) with that of other species showed that this concentration agrees closely with development of P-toxicity symptoms in many other Proteaceae (e.g., *Banksia* species, e.g., *B. aemula*, *B. oblongifolia* and *B. robur*, Grundon, 1972; *B. ericifolia*; Handreck, *B. serrata*, Groves and Keraitis, 1976; 1991; *H. gibbosa*, Grundon, 1972) and crops (see Table 2, Shane et al., 2004a). In crop species, the probability of developing symptoms of P toxicity increases at leaf P concentrations >10 mg P g^{-1} DM (Marschner, 1995). This shows that enhanced leaf sensitivity to [P] in Proteaceae does not explain their greater susceptibility to P-toxicity symptoms at low external P supply.

The physiology of P toxicity is not well understood, and symptoms are thought to result, either directly, from interference with leaf water relations at high cellular P concentration (Bhatti and Loneragan, 1970), or from non-specific interactions of high cellular P with Zn or Fe (Marschner, 1995). In *H. prostrata*, the distribution of [P] amongst different cell types in leaves was heterogeneous. At high P-supply rates, P accumulated in vacuoles of palisade cells which was associated with decreased photosynthetic rates (Shane et al., 2004a). The interaction of P with Zn and/or Fe also produces symptoms in leaves that resemble micronutrient deficiencies (Lambers et al., 2002a; Marschner, 1995; Robson and Pitman, 1983).

To avoid temporarily the toxic effects of excessive P accumulation in the cytosol, P can be stored in vacuoles (e.g., as P*i* in higher plants; Bieleski, 1973; Raghothama, 1999), and it can preferentially accumulate in different organs (e.g., stems; *B. prionotes*, Jeschke and Pate, 1995; *B. ericifolia*, Parks et al., 2000; *H. prostrata*, Shane et al., 2004a). In addition to tolerating high levels of P by transporting it between compartments, roots may also down-regulate the uptake of P (Harrison and Helliwell, 1979; Raghothama, 1999; see Raghothama this volume), by decreasing the expression of genes encoding P transporters (Smith et al., 2003). For many species belonging to the Proteaceae with a Mediterranean distribution the development of cluster roots, and hence nutrient uptake, is restricted to wet winter months, whereas shoot growth mainly occurs during dry summer months (e.g., *B. prionotes*; Jeschke and Pate, 1995). In the Western Australian kwongan vegetation, cluster roots form only in the wet winter season (Lamont, 1976; Purnell, 1960). Since nutrient mobilisation and nutrient uptake by cluster roots are separated by several months from shoot growth in Proteaceae growing in the south-west of Australia, nutrients acquired in winter must be stored, to be re-mobilised during summer shoot growth. The capacity of stem tissues to store P is limited. When P uptake exceeds the tissue's storage capacity, P-toxicity symptoms develop in leaves (Parks et al., 2000; Shane et al., 2004a). In most natural habitats the capacity of stem and root tissues for P storage would exceed the P uptake. By contrast, in hydroponics or fertilised soil cultures the availability of P can be drastically enhanced, and exceed the capacity of stem and root tissues to store it. Then P accumulates in leaves, leading to symptoms of P toxicity. What distinguishes the development of P toxicity in Proteaceae from that in other species is not the symptoms, but its occurrence at relatively low external P concentrations compared with that which induces P toxicity in other species.

Shane et al. (2004c) showed that *H. prostrata*, a P-sensitive species belonging to the Proteaceae, has a very low capacity to down-regulate its phosphorus uptake. A low capacity to reduce P uptake at enhanced P supply would account, in part, for the P-toxicity symptoms in many Proteaceae (Grose, 1989; Lambers et al., 2002a; Parks et al., 2000; Shane et al., 2004a). Since other 'woody' species (e.g., *Betula verrucosa*, Harrison and Helliwell, 1979) do show a capacity to reduce P-uptake rates, observations on the woody *H. prostrata* are not typical for woody species in general. Phosphorus-sensitive species (e.g., *H. prostrata*) can sense internal P status (as evidenced, e.g., by changes in root mass ratio and cluster-root development; Shane et al., 2004c). Future investigations may identify changes in the signal-transduction pathway between P sensing and expression of P transporters to provide molecular evidence for enhanced P sensitivity.

Phosphate uptake

A low P-absorption capacity might be expected in slow-growing plants adapted to infertile soils, because (1) diffusion to the root surface is the major rate-limiting step in low-P soils, and this cannot be overcome by increased absorption capacity (Nye and Tinker, 1977), and (2) P demand to support growth is the major determinant of P-absorption rate (Clarkson and Hanson, 1980). In *H. prostrata* net P-uptake rates decline only where cluster-root formation had been suppressed by the pre-experiment P regime; in *L. albus*, net P-uptake rates declined well before cluster roots were suppressed (Shane et al., 2004c), in contrast with the findings by Esteban et al. (2003) who showed that phosphate/arsenate uptake by *L. albus* was relatively insensitive to plant P status.

In the root environment, phosphorus is absorbed by a combination of a constitutively expressed low-affinity P-uptake system, and an inducible high-affinity P-uptake system (Epstein et al., 1963; Glass, 2002; Smith et al., 2003). Plants that receive a sub-optimal P supply generally up-regulate the amount and activity of the high-affinity P transporters, and thus increase the rate of P uptake per unit root mass (Raghothama, 1999). Conversely, at higher P supply the P-uptake rates can be decreased by down-regulating the activity or numbers of transporters (Raghothama, 1999; Smith et al., 2003). The system responsible for P*i* uptake by cluster roots appears to be similar to that used in non-cluster roots (Smith et al., 2003), and involves $H_2PO_4^-/H^+$ symporters, belonging to the *Pht1* family. In *Arabidopsis thaliana* and many other species, most of the genes of the *Pht1* family that are expressed in roots are up-regulated in P-deficient plants (see Raghothama, this volume; Smith et al., 2003). The findings of Liu et al. (2001), that *LaPT1* and *LaPT2* genes encode P*i* transporters in cluster and non-cluster roots of *L. albus* supports the contention that the P-uptake mechanism is similar in non-cluster and cluster roots.

Depending upon root age and whether or not rates are expressed on an area or mass basis, ^{32}P-uptake and V_{max} for cluster roots of P-deficient *L. albus* (Keerthisinghe et al., 1998; Neumann et al., 1999, 2000) and species of Proteaceae, e.g., *B. grandis* (Malajczuk and Bowen, 1974); *Protea compacta* and *Leucadendron uliginosum* (Vorster and Jooste, 1986), and *B. marginata* (Green, 1976, as cited in Table 3 of Lamont, 1982) are faster, slower or the same as those of non-cluster roots. In *H. prostrata*, net P-uptake rates were measured at five stages of cluster-root development, and compared with the rates of non-cluster roots (defined as the 1–3 cm region of root axes proximal from the tip; see Figure 5 in Shane et al., 2004b). Interestingly, at the stage of root development coincident with the burst of carboxylate exudation (12–13-day-old cluster roots), net P-uptake rates by cluster roots and non-cluster roots were the same.

Both Keerthisinghe et al. (1998) and Roelofs et al. (2001) suggested that the dense packing of rootlets may prevent an accurate determination of P-uptake rates by cluster roots, because of diffusion limitations and inaccessible regions of the root surface. Conversely, differences of P uptake rate between cluster and non-cluster roots may depend upon the comparison, and raises the important issue of what root is used as a 'non-cluster root', and whether that root is really comparable with a cluster root. Perhaps in the early stages of cluster-root development both types of roots are comparable, but mature cluster roots have an enormous number of root hairs, and likely have a more mature vascular system for P transport. Both of these characteristics, which are not present in so-called 'non-cluster roots', favour faster rates of net P uptake by cluster roots. The enormous redundancy of rootlets within each cluster root likely provides very efficient recovery of P with no further requirement for physiologically enhanced P-uptake rates in cluster rootlets beyond that of the non-cluster roots.

Many studies that measure P-uptake rates by cluster and non-cluster roots have assessed the P-uptake rates using high [P], and probably test the low-affinity P-uptake system. Considering the low [P] that predominates in soil solutions, future studies might assess the influence of external [P] for growth on net P-uptake rates by the high-affinity mechanism (Epstein et al., 1963), because measurements in the low concentration range are of greater ecological significance. A low K_m (e.g., 1.2 μM in *H. prostrata*, Shane et al., 2004c) and *L. albus* (Neumann et al., 2000) is appropriate

for P uptake from low-P soils. However, when the soil solution concentration is very low (as in soils of south-western Australia) and absorbed P is not rapidly replaced by P moving towards the root, then the diffusion towards the root becomes the limiting step, and kinetic parameters have little effect on P uptake (Clarkson, 1985).

Concluding remarks

Cluster roots are much more than just a curiosity. Studies on cluster roots in *L. albus* and in members of the Proteaceae assist in unravelling the complexities of how roots function in nutrient acquisition, especially of phosphate. It is increasingly evident that cluster-root functioning needs to be considered in broader terms; that is, as an integration of specialised morphology, anatomy, biochemistry and physiology. Although the properties of cluster roots are not unique to plants that develop cluster roots, they are elegantly combined to provide cluster roots with remarkable abilities to access poorly available forms of soil P. Further examination is now required to identify the specific cell types that are directly involved in carboxylate synthesis for exudation, and whether or not these cells have a specialised metabolism. In broader terms, we may uncover valuable information about how roots in general acclimate to nutrient stress, and increase their activity in mining the soil for nutrients.

In Proteaceae, which constitute the bulk of cluster-root forming species, further investigations will help identify key processes that have been the target of natural selection for plants that have evolved in phosphate-impoverished environments. Proteaceae are amongst the first Angiosperms to appear in the fossil record in Australia, and thus offer a unique opportunity to study plant adaptations to nutrient-impoverished soils. Indeed, the southwest of Australia is unique, because it is one of the world's 'hotspots' for diversity of higher plants. Furthermore, a relatively large proportion of the species from these nutrient-poor soils, including virtually all Proteaceae, do not produce a symbiotic association with a mycorrhizal fungus. However, this generalised view is changing slightly, as more species come under investigation. For example Boulet and Lambers (in press) have suggested that *H. verrucosa*, which naturally occurs on ultramafic soil, rich in nickel, has 'reverted' to the mycorrhizal habit of its ancestors, which are believed to have been mycorrhizal. They speculate that this might reduce the uptake, thus preventing nickel toxicity. This highlights another area for further investigation. The knowledge gleaned from these studies will assist in conservation of species that are adapted to severely nutrient-impoverished soils.

Acknowledgements

We thank Professor Margaret McCully (CSIRO, Plant industry, Canberra, Australia) for Figures 1C, 4A, B and C and 5C and three anonymous reviewers for their very helpful comments. M.W. Shane was the recipient of an International Postgraduate Research Scholarship and a University of Western Australia Postgraduate Award. This work was supported by the Australian Research Council.

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Plant and Soil (2005) 274:127–140
DOI 10.007/s11104-004-2754-2

The roots of carnivorous plants

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Received 30 April 2004. Accepted in revised form 31 August 2004

Key words: carnivorous plants, insectivorous plants, morphology, nutrition, root

Abstract

Carnivorous plants may benefit from animal-derived nutrients to supplement minerals from the soil. Therefore, the role and importance of their roots is a matter of debate. Aquatic carnivorous species lack roots completely, and many hygrophytic and epiphytic carnivorous species only have a weakly developed root system. In xerophytes, however, large, extended and/or deep-reaching roots and sub-soil shoots develop. Roots develop also in carnivorous plants in other habitats that are hostile, due to flooding, salinity or heavy metal occurance. Information about the structure and functioning of roots of carnivorous plants is limited, but this knowledge is essential for a sound understanding of the plants' physiology and ecology. Here we compile and summarise available information on:
(1) The morphology of the roots.
(2) The root functions that are taken over by stems and leaves in species without roots or with poorly developed root systems; anchoring and storage occur by specialized chlorophyll-less stems; water and nutrients are taken up by the trap leaves.
(3) The contribution of the roots to the nutrient supply of the plants; this varies considerably amongst the few investigated species. We compare nutrient uptake by the roots with the acquisition of nutrients via the traps.
(4) The ability of the roots of some carnivorous species to tolerate stressful conditions in their habitats; e.g., lack of oxygen, saline conditions, heavy metals in the soil, heat during bushfires, drought, and flooding.

Introduction to carnivorous plants

Plants benefit in many ways from animals; e.g., animals play a role as pollinators and as dispersers of fruits. However, animals may also contribute to a plant's nutrition by being caught and digested. This phenomenon of carnivory has fascinated the scientific community ever since Darwin drew attention to it (Darwin, 1875). Although carnivorous plants can obtain water and at least some minerals from the soil, they also extract nutrients from captured animals. Carnivorous plants attract their victims by means of scent, colouration and nectar (Lloyd, 1942). They are able to trap and retain their victims, kill them, and digest their soft tissues, and take up at least part of their contents (Juniper et al., 1989; Lloyd, 1942). This whole process is achieved by highly specialized leaves, which have been transformed into various types of traps. Therefore, the leaves may take over functions that are usually restricted to the roots of non-carnivorous plants. The general features of carnivorous plants have been reviewed in detail by Juniper et al. (1989). Five types of traps can be distinguished:

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- *Adhesive traps* produce sticky mucilage that is able to glue little animals to the leaves. Such '*fly-paper*' traps may actively roll over their victims (*Drosera*, *Pinguicula*) or they remain motionless (*Byblis*, *Drosophyllum*).
- In *steel traps*, the leaf forms two lobes, which snap around animals that touch sensitive trigger hairs on the surface of the lobes (*Dionaea*, *Aldrovanda*).
- In *pitcher traps*, leaves have been transformed to a pit that contains a pool of digestive enzymes (*Darlingtonia*, *Nepenthes*, *Sarracenia*, *Cephalotus*, *Heliamphora*). Animals are attracted towards the rim, and glide into the pit along the slippery inner surface, which prohibits escape.
- Even more sophisticated are the *eel traps* that attract prey through a system of claw-like cells and inward-pointing hairs into a bulb where it is digested and absorbed (*Genlisea*).
- The *suction traps* have the form of bladders that arise from leaves. Little animals that touch sensitive hairs near the entrance are sucked into the bladder through a trap door, due to the lower hydrostatic pressure inside, and are then digested (*Utricularia*).

Carnivory has been detected in at least 470 plant species from 9 families and 6 orders of vascular plants (Schlauer, 1986). Carnivory has evolved independently 6 (following the system of Takhtajan, 1969) or seven (Albert et al., 1992) times. Despite their taxonomic diversity, many carnivorous plants have several features in common:

- They are generally tolerant of nutrient-poor soils (Juniper et al., 1989).
- They tend to be poor competitors (Gibson, 1983a; Nash, 1973).
- They tend to prefer sunny habitats (Juniper et al., 1989; Pissarek, 1965; Schnell, 1980)
- Many are calcifuges and tolerate low soil pH (Carow and Fürst, 1990; Juniper et al., 1989; Rychnovska-Soudkova, 1953, 1954).
- Many are tolerant to waterlogging, growing in swamp-like habitats and peat bogs (Schnell, 1976), but others are able to survive severe water stress (Dixon and Pate, 1978; Harshberger, 1925, Lloyd, 1942) and even fires (Carlquist, 1976a; DeBuhr, 1976; Dixon and Pate, 1978; Roberts and Oosting, 1958).
- Since the observations of Nitschke (1860) and Burbidge (1897), it has been a general belief that carnivorous plants only have weakly developed roots or no roots at all. However, a wide variety of root systems can be found. These roots have not lost their ability to absorb minerals from the soil; rather, nutrients obtained from prey can be regarded as an additional source.

In contrast with the great interest in various aspects of their reproduction, nutrition and the morphology of their leaves and traps, information about the roots of carnivorous plants is relatively scarce. Here, we review the few studies that deal with the under-ground organs of carnivorous plants, especially with their roots *sensu stricto*, and describe their morphology and physiology.

The roots of carnivorous plants

In carnivorous species, a wide range of root types occur; schematic drawings of the various roots and under-ground organs of a great variety of carnivorous plants are included in Pietropaolo and Pietropaolo (1986), but quantitative data about size and morphology are found only very rarely in the literature; roots are mainly described as frail, weak or strong. We therefore take over these descriptions in this review. For our own observations, we use the term 'weak' for inconspicuous roots that are much smaller than the above-ground parts of the plant, and 'strong' for roots that are well developed and more than half the size of the shoots.

The roots may function permanently or only during part of the year (Slack, 2000). Some species have deep roots that probably allow continuous water uptake (França, 1925; Menninger, 1965), and others have only poorly developed roots (Nitschke, 1860) or no roots at all (Slack, 2000; Taylor, 1994). The absence of roots does not necessarily mean, however, that the functions of roots are not needed; in some plants, the stem and leaves have replaced their functions, as discussed below.

The radicle

Whereas the root systems of adult carnivorous plants are very diverse, the radicle is quite similar, and only weakly developed in the few species that have been investigated.

In *Drosera*, the radicle is replaced by stem-borne roots soon after germination. Interestingly, it develops extremely long root hairs, which are long lived (Smith, 1931). *Byblis* and *Drosophyllum* have an extensive root system as a mature plant; the radicle, however, is hardly able to anchor the seedling to the substrate, so that even with a total length of 30 mm the plant can be displaced easily (Juniper et al., 1989). Similarly thin is the radicle of *Nepenthes gracilis*. It forms a bunch of long root hairs within the testa, but even so, together with the hairs, the root is too feeble to anchor the seedling or to supply it with sufficient water and nutrients (Green, 1967).

Aquatic carnivorous plants do not have roots

Aquatic carnivorous plants never develop roots. Best known examples are *Aldrovanda* which bears snap traps (Adamec, 2003), *Polypompholyx* and many *Utricularia* species with suction traps, and the closely related *Genlisea* that catches its prey with an eel trap. They grow submerged in water, except for the flowers, which are above the water. Their stem or rhizome floats freely and carries green leaves and traps. They take in the few available minerals through their stems and leaves (Slack, 2000). In *Utricularia*, there are also many terrestrial and epiphytic species that grow in moist soil and in decomposing organic matter from other plants (Taylor, 1994). *Genlisea*, growing in loose sandy soil, is submerged only during part of the year (Slack, 2000). Again in both *Utricularia* and *Genlisea*, no roots are developed. Specialised underground shoots or leaves have replaced their roots.

Roots of hygrophilic carnivorous plants

The moisture of swamps and peat bogs creates a hostile substrate for most plants, because the roots may be poorly aerated (Armstrong, 1979). In addition, organic matter may be mineralised only incompletely, so that the nutrient availability in the substrate is poor. During the decomposition processes, and also due to the action of *Sphagnum* mosses, humic acids are generated which decreases the soil pH down to 3 (Naucke, 1990). Only few plants tolerate these conditions in peat bogs and swamps, and those that survive, for instance through their strategy of carnivory, require specific root adaptations.

Roots of hygrophilic carnivorous plants are usually only short-lived, and they are reduced, frail and thin. Vestigial root systems occur in species of *Drosera* and *Pinguicula* (drawings of these reduced root systems are given by Kutschera et al. (1992), *Dionaea*, all Sarraceniaceae, *Cephalotus* and the carnivorous Bromeliaceae (Figure 1). However, the total of the underground organs (rhizomes with outgrowing roots and tubers) can be quite extensive in some species (Figure 2). In addition, they produce tannic acids and other impregnating substances, like in *Dionaea* (Guttenberg, 1968). However, this feature is not restricted to wetland plants, but occurs also in the dryland plant *Byblis gigiantea* (Lloyd, 1942). There are even examples for well developed deep roots, e.g., in *Triphyophyllum peltatum* (Dioncophyllaceae), which grows in the humid rain forests of West Africa in very poor soils. This species has relatively recently been added to the list of carnivorous plants (Green et al., 1979), and further details about the anatomy and physiology of its roots have not yet been published. In *Drosera rotundifolia*, roots are also used for vegetative propagation. Some of the few roots run parallel to the surface of the soil and form root suckers (Kutschera et al., 1992).

The ecological adaptations to oxygen-deficient soils in some carnivorous plants are reflected in the anatomy of their roots. The cortex may be thin and inconspicuous, with practically no exodermis, as in *Dionaea* (Fraustadt, 1877; Smith, 1931). In contrast, the inner cortex may develop many gas-filled intercellular spaces, which often

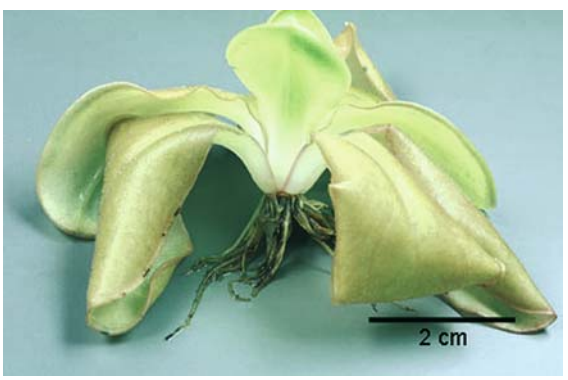

Figure 1. Piguicula moranensis, a tropical plant with large fleshy leaves, has frail and thin roots during its lifetime.

Figure 2. *Sarracenia* hybrid, although hygrophytic, has extended sub-soil rhizomes from which small roots arise.

collapse to lacunae due to the lysis of cells, and hence allow for the aeration of the tissue (Freidenfelt, 1904; Guttenberg, 1968; Oels, 1879). This is the case in many species of *Drosera, Pinguicula* and *Sarracenia*. *Darlingtonia* has numerous small intercellular spaces around virtually every cortical cell (Figure 3).

The stele of the carnivorous plant roots investigated so far usually is oligarch. In *Dionaea*, the young root has a di- or tetrarch central cylinder; later a multilayered pericambium develops that forms groups of tracheids and phloem elements (Smith, 1931). In *Drosera*, the central cylinder is very similar to that of *Dionaea* in the young roots; in older roots with secondary thickening, however, amphivasal bundles are formed (Guttenberg, 1968).

Root hairs are very prominent in the few species that have been examined. In *Drosera* species, they are extremely long (viz. 15 mm); in *Dionaea* they are up to 1.9 mm long, persistent and so heavily impregnated with cutin that root hairs can be dissolved only in chromic acid (Smith, 1931).

Concerning symbiosis with mycorrhizal fungi, it is a common belief that there is no association with roots of neither hygrophilic nor xeromorophic carnivorous plants (MacDougal, 1899; Peyronel, 1932, reviewed by Juniper et al., 1989). The only exception would be *Roridula*, which forms

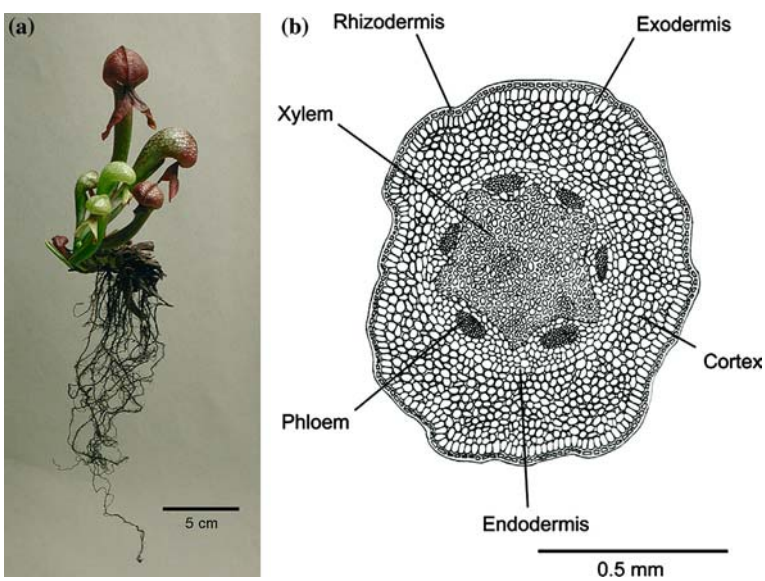

Figure 3. *Darlingtonia californica* has a relatively large root system that servives fires and heavy metal contaminated soils (a). Transections show the cortex containing numerous small intercellular spaces that encloses the compact hexarch central cylinder with xylem and phloem. Rhizodermis and exodermis are thick walled and heavily impregnated (b).

vesicular arbuscular mycorrhizas (Midgley and Stock, 1998), but this genus nowadays is no longer regarded as carnivorous (Ellis and Midgley, 1996). Recent research showed, however, that such vesicular arbuscular mycorrhiza occurs in *Drosera intermdia* (Fuchs and Haselwandter, 2004).

Roots of dryland carnivorous plants

Carnivorous plants of both moist and arid habitats may develop an extended system of underground organs. This is, however, exceptional, but does occur in *Drosophyllum lusitanicum*, which grows in poor soils derived from sandstone in southern Spain and Portugal, where the summers are hot and dry. During this time, *Drosophyllum* sometimes senesces (Flísek and Pásek, 2000), but more frequently it is found in a turgescent shape, including fresh and sticky mucilage on the traps (Juniper et al., 1989; own unpublished observations). Due to its well developed root system (França, 1925), *Drosophyllum* can survive 5 months without any rainfall, even on southfacing hillsides (Juniper et al., 1989). Water uptake by the roots presumably continues, because the plants have no water storage organs (Juniper et al., 1989). Studies of the root anatomy showed some peculiarities of the endodermis; the primary state of the endodermis is only very short and soon transition into a secondary endodermis is initiated by suberization of the cell walls; no further lignification of the cell walls occurs, but the endodermis remains in this intermediate state throughout the life of the root (Guttenberg, 1968). The rhizodermis, on the other hand, lignifies very soon in young roots (Figure 4).

The Australian *Byblis* species and *Ibicella lutea* from the southern part of North America are found in dry habitats similar to those of *Drosophyllum*; they also have well developed roots (Juniper et al., 1989). Even more pronounced are the roots of *Nepenthes pervillei* that grows on rocky cliffs; they are described as long and reaching into deep cracks, where they are believed to find moisture and nutrients (Juniper et al., 1989) (Figure 5). Australian dryland *Drosera* species also form extensive root systems. The roots of the recently discovered *Drosera caduca* are contractile, as in many Alliaceae (R. Barrett and M. Barrett, unpublished observation), so that the tubers become drawn into the soil.

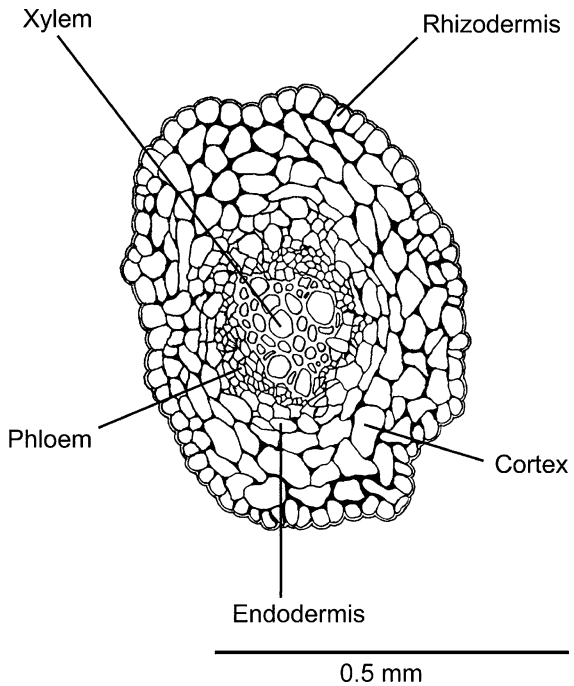

Figure 4. *Drosophyllum lusitanicum* develops lateral roots with lignified rhizodermis, collenchymal cells in the cortex and a secondary endodermis with heavily suberized cell walls that encloses the diarch central cylinder with xylem and phloem.

Figure 5. *Nepenthes* hybrid tends to climb but still has long and deep-reaching roots.

Specialized roots occur in tuber-forming *Drosera* species of Australia that are adapted to hot and dry summers; plants die back to a well developed underground stem tuber from which they emerge again when the rainy season begins (Pate and Dixon, 1982). The tuber is covered by leaf scales. From their bases, root-like structures grow

downwards; these were termed 'leaf-rhizoids' ('Blattrhizoide') by Diels (1906). These structures might be reduced roots, because they have several features in common with roots; they grow out of the axis, their apical cells are enforced, and they carry starch grains in the second cell layer behind the tip; the meristem follows only behind. In addition, they produce many outgrowths which were described as 'hairs' (Diels, 1906). On the other hand, they have no calyptra which is why Goebel (1923) described them as "dubious roots" ("zweifelhafte Wurzeln"). In addition to the rhizoids, small horizontal roots emerge from the bases of the leaf scales, which enable the stems to be used for the propagation of the plants; they can form a new shoot and produce a tuber (Slack, 2000).

Like in roots of hygrophilic carnivorous plants, also in some dryland species special anatomical features can be found: In *Drosera macrantha* and *D. trinerva* (Oels, 1879), the cortex is developed and even divided into an outer and an inner cortex, which differ considerably; the cells of the outer cortex have thick cell walls, usually without secondary thickening; the parenchymatic cells of the cortex have spiral fibres in their walls similar to the cells of the *velamen radicum* of orchids. These cells can reinforce the exodermis, especially in those cases where the rhizodermis has only fragile cell walls (Freidenfelt, 1904).

Temporary roots

Temperate species of *Drosera* and *Pinguicula* develop roots only during part of the year. In some *Drosera* species, the roots die in autumn, and the winter buds are anchored to the substrate only by the dead remainders (Slack, 2000) (Figure 6). In spring, new leaves and new roots are formed at the same time. In most temperate *Pinguicula* species of the northern hemisphere, the roots die in winter, but the dead parts are completely lost, so that the dormant bud (the hibernaculum) is freely mobile. This can be seen either as a strategy for efficient dispersal or it is a mechanism to avoid damage when the water in the soil freezes and expands (Slack, 2000). As an exception, *P. alpina* has perennial roots (L. Adamec, personal communication).

In the Mexican montane butterworts occuring on limestone, e.g., *Pinguicula gypsicola*, the roots die in winter which is the dry season. The death of the roots is accompanied by a complete change of the shape of the plants; in summer, new roots and broad and sticky carnivorous leaves are formed which both die during the dry season. The leaves are replaced by non-carnivorous and succulent leaves. With the rain in spring, new roots and new carnivorous leaves are formed (Slack, 2000).

Figure 6. In *Drosera rotundifolia*, only dead roots remain during winter and keep the plants anchored to the substrate.

Physiological traits of the roots of carnivorous plants

Virtually all carnivorous plants are poor competitors (Juniper et al., 1989); therefore they occur in extreme habitats, where the majority of plants cannot grow. The ecological conditions of the habitat require specialized physiological adaptations. Here, only the properties of the roots will be discussed.

The problems of waterlogged habitats were mentioned above. Roots of hygrophytes have adapted to hostile soil conditions of low pH and low oxygen by the development of aerenchyma

and/or by the reduction or even lack of roots. In contrast, in the habitat of the Australian *Byblis*, heat, drought and fires occur regularly. The fires destroy all parts of the plant above the ground. The underground organs are protected by the soil, and have been described to endure low-intensity fires (DeBuhr, 1975). Similarly, North American *Dionaea muscipula* (Roberts and Oosting, 1958), *Darlingtonia californica* (R.M. Austin, unpublished data, cited in Juniper et al. (1989); Barbour and Major, (1977)), *Sarracenia* (Plummer, 1963; Plummer and Kethley, 1964), and some *Drosera* species (Pate and Dixon, 1982) survive fires by means of underground organs. Some species, like *Dionaea muscipula* (Schulze et al., 2001), appear to need regular fires, which serve to remove competitors. *Sarracenia flava*, for instance, is outcompeted by wiregrass, *Sporobolus teretifolius*, if the habitat is not burnt regularly (Eleuterius and Jones, 1969; Plummer and Kethley, 1964). The tolerance to low-temperature fires is so widespread amongst carnivorous plants that it is even seen as part of the carnivorous syndrome by Juniper et al. (1989). In peaty soils, the heating during bushfires is restricted to the surface (Heyward, 1938, cited in Roberts and Oosting, 1958), so that underground parts of the plants can survive.

The roots of *Darlingtonia californica* (Figure 3) encounter another kind of stress in addition to fires. They grow only in cool running fresh water; the roots therefore are exposed to temperatures around 10 °C, even during the hottest time of the year, when the air temperature exceeds 25 °C (Juniper et al., 1989). Cultivation experiments show that the roots have an absolute requirement for this low temperature; they die if exposed to temperatures above 10 °C for a prolonged time, and seedlings are even more sensitive (Slack, 2000). The shoot, on the other hand, is not very sensitive to high temperatures (Slack, 2000). The reason for this extraordinary sensitivity of the roots to high temperatures is probably a very low and limited optimal temperature of their ion pumps in root cells (Ziemer, 1973). Similar extreme specializations to a certain temperature are known for many other hygrophytes (Sapper, 1935), but it is unusual for only one organ of the plant.

Heavy metals are another stress factor that is resisted by the root systems of some carnivorous plants. For example, *Darlingtonia californica* (Juniper et al., 1989), *Nepenthes rajah* (Gibson, 1983b), *N. xalisaputrana* (Clarke, 2001), *N. villosa* (Kaul, 1982) and *Stylidium* spp. (Darnowski, 2002; 2003) grow in serpentine habitats. Serpentine soils are rich in Ni, Cr and Mg, which are all toxic for plants when a threshold concentration is exceeded; these soils are also very low in available macronutrients (Kruckerberg, 1954). *Sarracenia purpurea* ssp. *purpurea*, *Drosera rotundifolia* and *Pinguicula* sp. were found growing on ultra basic, heavy metal rich deep mantle rocks (D'Alessi, 2004). In general, plants have developed various strategies to avoid or to tolerate the toxic influence of heavy metals, and in every case the roots play a key role (Greger, 1999; Meharg, in press), but it is not known which strategy is used by carnivorous plants.

Saline soils are tolerated by some carnivorous plants. This is the case for *Dionaea muscipula*, which sometimes grows in brackish, acid swamps (Juniper et al., 1989) and probably also for *Drosophyllum lusitanicum* (Harshberger, 1925). In addition, four species of *Nepenthes*, i.e., *N. albomarginata*, *N. reinwardtiana*, *N. treubiana* and *N. mirabilis* (Juniper et al., 1989), grow at the sea side in spray zones of south-east Asian coasts, where there are no competitors. The distribution pattern of recent *Nepenthes* species indicates that the common ancestor of the genus was a halophyte with a coastal distribution in the Cretaceous period. Only later, descendents also colonized the interior of the land (Speirs, 1981).

It is generally believed that carnivorous plants are calcifuges, and that, for most, Ca is toxic. Exceptions include several Australian *Drosera* species that grow on calcareous sands in alkaline conditions (*D. erythrorhiza* (Dixon and Pate, 1978; Pate and Dixon, 1978), *D. falconeri* Tsang, 1980). Also some *Pinguicula* species (Studnicka, 1981) grow on rocks of calcium sulfate in Mexico (*P. gypsicola,* Taylor and Cheek, 1983), in central Europe (*P. vulgaris* ssp. *bohemica* Hadac, 1977) and *P. alpina* (Adler et al., 1994) and in southern Spain (*P. vallisneriifolia* Diaz-Gonzales et al., 1982). *Nepenthes clipeata* from Borneo (Slack, 1986) is another example of a calcicole. The toxicity of Ca was correlated to the pH value of the soil for *Drosera rotundifolia* by Rychnovska-Soudkova (1953), because it appears

that a low pH allows plants to tolerate higher concentrations of Ca. This question deserves, however, additional investigations.

How can carnivorous plants survive without roots?

In a mesomorphic plant, the root fulfills four main functions: anchorage, water uptake, nutrient uptake, and storage of nutrients and photoassimilations. In rootless plants and in plants with only a small root system, these root functions are taken over by other organs, i.e., by the stems and/or by leaves.

The stem takes over the function of roots

In *Utricularia*, roots in the anatomical sense occur neither in aquatic nor in amphibian, terrestrial and epiphytic plants, but the stems usually develop special organs that may accomplish the function of the roots (Taylor, 1994). The stems have adapted in that they anchor the plants to the substrate, probably absorb available minerals and water, and store water and nutrients for times of drought. Four different modifications of the stem have been described (Taylor, 1994), (Figure 7):

- Horizontal stems, so-called stolons, serve for the propagation of the plant.
- Colourless shoots that grow downwards into the soil ('mud shoots', i.e. 'Schlammsprosse' (Adler et al., 1994; Schubert and Went, 1986)). They anchor the plants to the substrate and probably also contribute to their nutrition by taking up minerals. These organs are easily recognised as shoots, because they bear numerous bladders, the traps, similar to the green shoots *sensu stricto*.
- Root-like formations of the stem are so-called 'rhizoids'. They are specialized shoots with limited growth that emerge from the base of the flowering axis, but carry no leaves. They fix the plant within the masses of other floating weeds (Hegi, 1906).
- Stem tubers that form from stolons serve as storage organs to survive drought (in *Utricularia alpina*, *U. menziesii*, *U. inflata*, *U. reniformis*, *U. mannii*, *U. reflexa* and many other species). The tubers are of various morphological origin; in *U. alpina* they consist of huge parenchymatic cells containing an enormous vacuole (our own observations). In terrestrial species, they also serve to anchor the plants. Moreover, they contribute to the propagation of the plant which leads to the formation of large clones (Taylor, 1994).

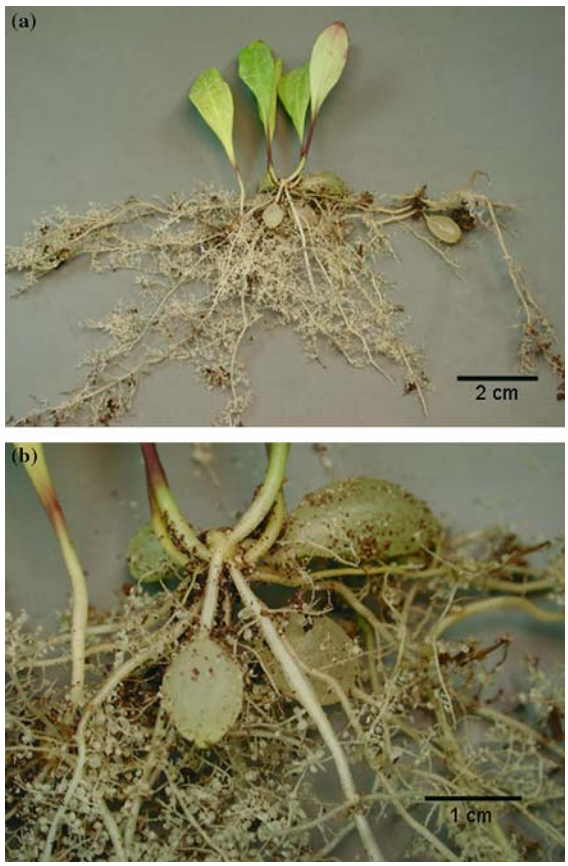

Figure 7. *Utricularia alpina*: Morphology of the green and colourless shoots (a). Sub-soil shoots are transformed to tubers and to horizontal and downward-pointing rhizomes that carry traps (b).

Stem tubers are found in some Australian species of *Drosera*, such as *D. erythrorhiza*, *D. zonaria* or *D. hamiltonii* (Slack, 2000). In *D. erythrorhiza* the first tuber is formed under ground at the end of a positive geotropic shoot, the 'dropper', which has its origin in the radicle just above the hypocotyl (Dixon and Pate, 1978; Pate and Dixon, 1978). It is a storage organ and serves as the perennating structure during dry periods and bushfires. With the onset of tuber sprouting, rhizomes emerge from the vertical stem, each terminating in a 'daughter tuber'. They contribute to the clonal propagation of the plant. The tubers

Table 1. Carnivorous plant species, their traps and selected characteristics of their roots[a]

Family	Genus	Number of species	Type of trap	Root system[b]
Sarraceniaceae	*Heliamphora*	5	Pitcher	Weak
	Sarracenia	8	Pitcher	Weak to medium
	Darlingtonia	1	Pitcher	Medium to large
Nepenthaceae	*Nepenthes*	68	Pitcher	Weak to medium
Droseraceae	*Drosophyllum*	1	Fly-paper	Medium to large
	Drosera	110	Fly-paper	Weak and large
	Dionaea	1	Snap trap	Weak, but fleshy
	Aldrovanda	1	Snap trap	None
Roridulaceae	? *Roridula*	2	Fly-paper	Large
Dioncophyllaceae	*Triphyophyllum*	1	Fly-paper	Large
Byblidaceae	? *Byblis*	2	Fly-paper	Large
Cephalotaceae	*Cephalotus*	1	Pitcher	Weak to medium
Lentibulariaceae	*Pinguicula*	52	Fly-paper	Weak
	Utricularia	180	Suction trap	None
	Biovularia	2	Suction trap	None
	Polypompholyx	2	Suction trap	None
	Genlisea	15	Eel trap	None
Martyniaceae	*Ibicella*	1	Fly-paper	Large
Stylidiaceae	? *Stylidium*	136	Fly-paper traps in some species?	Medium
Bromeliaceae	*Brocchinia*	1	Pitcher	Weak
	Catopsis	1	Pitcher	Weak

[a]Based on Schlauer (1986), Juniper et al. (1989), Carow and Fürst (1990), Hartmeyer (1997), Mabberley (2000), Darnowski (2002), and own observations.
[b]Quantitative data are not available. The statements are to be seen in relation to a typical mesophytic plant of a temperate climate. The question marks indicate that for these genera the carnivorous habit is not proven.

are replaced by new ones, which grow inside the old tubers, during each vegetative period, using their stored assimilates for their own growth.

Leaves take over the function of roots

The uptake of water through the leaves was suggested for *Drosophyllum lusitanicum*. The leaves are covered by mucilage-secreting glands from the trap. They are supposed to contribute to the water supply of the plant by absorbing water from fog and humid air (Mazrimas, 1972). A similar suggestion was made for *Pinguicula,* which has specialized glands on the lower surface of the leaves (Lloyd, 1942), but this has never been proven.

The uptake of nutrients from captured prey is principally carried out by the leaves of all carnivorous plants, as it is the leaves that have transformed into traps. They catch and digest their prey, and usually they also absorb the digested substances, although this has not yet been proven for every species, and for some it remains a matter of debate (Juniper et al., 1989; Slack, 2000).

Organic substances of origin other than from animals are also utilized by leaves, as was shown for *Drosera* and *Pinguicula*. *Drosera rotundifolia* as well as some species of *Pinguicula* catch pollen grains with their traps. The pollen is digested (in some species only after the pollen grains have germinated on the trap), and the absorbed nutrients support the growth of the plants considerably (R.M. Austin, 1875, published in Juniper et al., 1989; Harder and Zemlin, 1968). Similarly, the nutrient-rich foliar leachate from the canopy above may feed the leaves, as was suggested by Juniper et al. (1989) for three woodland species of *Drosera*, i.e., *D. schizandra*, *D. prolifera* and *D. adelae*. To possibly more efficiently benefit from the leaching, these plants have developed remarkably broad leaves (Juniper et al., 1989; Lavarack,

1979). In *Heliamphora nutans* large amounts of decaying leaves were found in the pitchers (Studnicka, 2003).

The function of anchorage is achieved by leaf tendrils in many species of *Nepenthes* and in *Triphyophyllum peltatum*. These species are climbers and sometimes even become true epiphytes in later stages of their lives, losing all contact with the soil. Their leaf tendrils fasten them to their supports.

The function of storage of assimilates and water is probably accomplished in *Pinguicula gypsicola* by special leaves that are non-carnivorous but thick and succulent, and occur only during the dry winter before the new carnivorous leaves develop (Slack, 2000).

Traps versus roots: which one takes up what?

The ecological significance of carnivorous nutrition has been discussed ever since carnivory was proven (Darwin, 1875). After 130 years of research, we are aware of a wide range of data concerning the physiological dependence of the plants on their prey, which reaches from almost total dependence to almost total independence. In some groups nutrients can be taken up from the substrate by the roots as well as from animal prey through the traps (e.g., *Pinguicula vulgaris* Aldenius et al., 1983); other species obviously need their prey for sufficient growth (e.g., *Aldrovanda vesiculosa* Adamec, 2000), and some are even suppressed by a nutrient-rich medium (e.g., *Dionea muscipula* Roberts and Oosting, 1958). In general it appears that many, if not all carnivorous plants can survive without animals as prey; many species suffer, however, by reduction of their reproductive organs; they grow more slowly and they lose their vigour (Adamec, 2000; Pringsheim and Pringsheim, 1967; Roberts and Oosting, 1958). In greenhouses, where these experiments have been conducted, this response may be of no significance. In their natural habitat, the carnivorous plants may not be able to tolerate the hostile soil conditions without prey, and hence be unable to compete with the non-carnivorous plants. Only few data about ion uptake in carnivorous plants are available. A comprehensive review of the mineral nutrition of carnivorous plants is given by Adamec (1997).

In some plants, the killed animals may contribute little to their nutrition, as for instance in the South African *Roridula* (Roridulaceae); the killed animals are not digested, the nutrients come either from the soil (Carlquist, 1976b), or through the leaves, but in this case digestion is carried out by bacteria (Midgley and Stock, 1998) or symbiotic hemipterans (Ellis and Midgley, 1996). Because of it lacking digestion, the genus *Roridula* is no longer considered as a carnivorous plant *sensu stricto* (Lloyd, 1942). The West African *Triphyophyllum peltatum* obtains its mineral nutrients also from a well developed root system. In addition, it gets nutrients from animals from sticky trap leaves that are grown during part of the year; *Triphyophyllum* is therefore considered as a 'part-of-the-time' carnivorous plant (Green et al., 1979). *Pinguicula gypsicola* and other Mexican butterworts growing on limestone are also carnivorous during part of the year only (Slack, 2000). The same is true for *Drosera caduca* developing insect-trapping lamina in the juvenile growth stages of the leaves. After a few weeks the photosynthetic petiole elongates and broadens considerably (R. Barrett and M. Barrett, unpublished observations).

Darlingtonia californica appears to be quite independent of the nutrient status of the soil. It shows good growth in both nutrient-poor and rich soils, with or without insects, provided its very special temperature requirements (see above) are met (Juniper et al., 1989). *Pinguicula vulgaris* (Aldenius et al., 1983) and *Utricularia uliginosa* (Jobson et al., 2000) can also be cultured on either rich or poor substrates; both species can take up nutrients from the soil. In *Pinguicula vulgaris,* Aldenius et al. (1983) found that maximum size (68 mg average dry mass) is achieved on a nutrient-rich medium, with additional animal feeding. On a rich medium without insect supply, average dry mass was 46 mg. Insect-fed plants on a poor medium reached 33 mg, completely unsupplied control plants were 22 mg. Recent research on *Drosera capillaris*, *D. aliciae* and *D. spathulata* showed that their roots can take up inorganic nutrients like Ni, P, Ca and Mg, but their uptake has to be stimulated by nutrients transported from the leaves, i.e., by successful prey capture (Adamec, 2002).

Some carnivorous plants appear to have a limited capacity for nutrient absorption from the soil, and therefore depend on animals to a greater extent: *Utricularia gibba* (Pringsheim and Pringsheim, 1967) can survive on an inorganic medium, but grows very slowly. Better growth occurs, when beef extract, peptone, glucose and acetate are added to the medium. The same is the case with *Dionaea muscipula* on a nutrient-rich soil: without animals plants produce no new roots, only few flowers, no fertile seeds, and die (Roberts and Oosting, 1958). Therefore, in the natural habitat of *Dionea*, only 8–25% of the total N comes from the soil. The greatest amounts are found in dense vegetation, where the traps work less effectively (Schulze et al., 2001). The closely related *Aldrovanda vesiculosa* is able to survive without animal prey, but shows only poor growth (Adamec, 2000).

The amount of nutrients obtained from either prey or from the soil seems to vary substantially. *Sarracenia leucophylla* can get 60 times more ions from the prey than from the soil (Gibson, 1983b). *Nepenthes mirabilis* gets about 60% of its N from insect prey, whereas in *Cephalotus* it is only 30% (Schulze et al., 1997). In *Drosera rotundifolia* about 50% of the total N is of animal origin (Millett et al., 2003), and in *D. hilaris* 68% (Anderson and Midgley, 2003). The protocarnivorous *Roridula gorgonias*, which needs symbiotic hemipterans for digestion, even up to 70% of N comes from animals (Anderson and Midgley, 2003).

For another group of plants, applied mineral nutrients (i.e. fertilizers) can be fatal: *Sarracenia alata*, for instance, grows on soil containing sufficient concentrations of N, P and K; it is, however, very sensitive to fertilizer additions, and dies when growing in such nutrient-enriched areas (Eleuterius and Jones, 1969).

Nutrition can also influence the morphology of some carnivorous plants, and the size and number of their traps. In some species of *Sarracenia* (Ellison and Gotelli, 2002) and of *Nepenthes* (Smythies, 1963) more, and more efficient pitchers are produced on a nutrient-poor medium. On a richer medium the leaf bases become flattened and hence more suitable for photosynthesis, whereas the pitchers are reduced.

Another interesting observation is that plants may take up only some specific nutrients through the roots, whereas others come through the leaves from the prey. This is the case for some Australian *Drosera* species that grow in habitats subjected to fires. The soil in this habitat in general is very poor, but enriched in K after a fire. *Drosera* is thought to take up the K^+ by its roots, and the other nutrients from insects (Dixon and Pate, 1978; Pate and Dixon, 1978), but this effect has not been quantified. *Nepenthes pervillei* sends its roots into rock cliffs where the cyanobacterium *Lyngbia* (Oscillatoriaceae) grows. *Lyngbia* fixes atmospheric dinitrogen, which is suggested to be absorbed by the roots, whereas other nutrients may come from animals that are caught in the few functioning traps (Juniper et al., 1989).

Conclusions and future aspects

Roots and root-substituting stems play an important role in the functioning of many carnivorous plant species, yet so far little information is available on their morphology and their physiological traits as well as on possible adaptations to their ecological situation. Much of our knowledge is based on the work of amateur botanists, therefore part of the cited literature is derived from popular journals. Since roots are the 'hidden half' of every plant (Waisel et al., 2002), our understanding of the carnivorous habit can only be improved substantially if we gain further information on the morphology of the roots and their capacity to take up nutrients from the soil as well as to survive the extreme conditions of their habitats.

Acknowledgements

We are grateful to our greenhouse specialist, Walter Pois, for the provision of carnivorous plants and for his most patient help and advice. The photographs were made by Gregor Eder. Edith Mayer and Ingeborg Lang helped us with the formatting of the illustrations. We thank Dr Gerhard Draxler for valuable discussion. This work was supported by a grant from the Jubiläumsfonds der Stadt Wien for the Austrian Academy of Sciences to I.K. Lichtscheidl.

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Plant and Soil (2005) 274:141–161
DOI 10.007/s11104-004-8070-z

Roles of aquaporins in root responses to irrigation

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Received 9 August 2004. Accepted in revised form 21 December 2004

Key words: aquaporins, hydraulic conductivity, irrigation, mechanosensitive-gating, MIP, NOD26, pH regulation, rectification, roots, water channels, water stress

Abstract

Due to current environmental issues concerning the use of water for irrigation, the improvement of crop water-use efficiency and a reduction in water consumption has become a priority. New irrigation methods that reduce water use, while still maintaining production have been developed. To optimise these techniques knowledge of above- and below-ground plant physiological responses is necessary. During growth, plant roots are exposed to cycles of wetting and drying in normal rain-fed and irrigation situations. This review concentrates on the below-ground aspects, in particular the water permeability of roots. Significant research has been conducted on the root anatomy and hydraulic conductivity of desert plants subjected to wetting and drying. Major intrinsic proteins (MIPs), most of which show aquaporin (water-channel) activity are likely to be involved in balancing the water relations of the plants during water deficit. However, many MIPs seem to allow permeation of other small neutral solutes and some may allow permeation of ions under certain conditions. The ability of the plant to rapidly respond to rewetting may be important in maintaining productivity. It has been suggested that aquaporins may be involved in this rapid response. The down-regulation of the aquaporins during dry conditions can also limit water loss to the soil, and intrinsic sensitivity of aquaporins to water potential is shown here to be very strong in some cases (NOD26). However, the response of aquaporins in various plant species to water deficits has been quite varied. Another component of aquaporin regulation in response to various stresses (hypoxia/anoxia, salinity and chilling) may be related to redistribution of flow to more favourable regions of the soil. Some irrigation techniques may be triggering these responses. Diurnal fluctuations of root hydraulic conductance that is related to aquaporin expression seem to match the expected transpirational demands of the shoot, and it remains to be seen if shoot-to-root signalling may be important in regulation of root aquaporins. If so, canopy management typical of horticultural crops may impact on root hydraulic conductance. An understanding of the regulation of aquaporins may assist in the development of improved resistance to water stress and greater efficiency of water use by taking into account where and when roots best absorb water.

Abbreviations: MIP – major intrinsic protein; TIP – tonoplast intrinsic protein; PIP – plasma membrane intrinsic protein; L_p – hydraulic conductivity; L_o – hydraulic conductance; P_f – osmotic water permeability; PRD – partial root zone drying; RDI – regulated deficit irrigation

Introduction

Terrestrial plants transpire enormous quantities of water as a necessary part of gaining carbon

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dioxide for photosynthesis. It has been estimated that terrestrial plants recycle over half the amount of global precipitation per annum (Chahine, 1992). A large proportion of this water, probably over 50%, will traverse aquaporins, in root membranes, leaf membranes and in membranes associated with sugar and water storage. It is clear that stomata play the major role in regulating transpiration and there behaviour in time and in response to various environmental and internal signals will determine the efficiency of water use (carbon gained/water loss). However, the roots must be able to carry the supply of water to match demand from the shoots and in this respect roots can determine the operating conditions of the shoot in terms of the shoot water potential and xylem solute concentrations.

Although water transport through roots is passive, we cannot consider roots as invariable water supply systems. The root system hydraulic conductance is variable, the cause of the variability depending on the time scale. For longer time scales hydraulic conductance is determined by changes in the size of the root system, patterns of root growth and changes in root anatomy. We can scale root conductance to root surface area, root length, root dry weight or leaf surface area, to extract various forms of root hydraulic conductivity (see discussion in Tyree, (2003)), but this scaling does not eliminate the variability. Within the time scale of a day variability in root hydraulic conductivity is mainly due to the activity of aquaporins as the conduits of passive water flow across root membranes. Aquaporins, either directly or indirectly via cell control systems, may respond to water potential, nutrient status, soil temperature, root metabolic status and probably shoot water status via hormone signalling.

This review is written in the context of the likely role of changes in root water transport properties, and the possible role of aquaporins, also know as major intrinsic proteins (MIPs), in plant responses to irrigation. In recent years irrigation technology has improved to reduce water wastage and reduce plant vigour while maintaining yield. Regulated-deficit irrigation (RDI) involves maintaining plant water status at a deficit relative to maximum water potential at particular stages of plant development (Kriedemann and Goodwin, 2003). This method depends on the monitoring of plant and soil water status. Yield loss can occur, particularly if the plant becomes stressed during flowering (Mitchell and Goodwin, 1996). In this review we examine the links between root hydraulic properties in response to wetting and drying cycles, and the possible role played by root MIPs most of which display aquaporin activity.

Possible secondary impacts of irrigation on the root environment include salinisation, changes in nutrient concentrations, particularly with fertigation, changes in root temperature, and hypoxia. The effects of these factors on root MIPs have also been examined to varying degrees in the literature, both in terms of changes in root water permeability and in terms of expression patterns of MIP genes. The central role that MIPs play in root physiology is becoming clear from the transcriptome studies where inevitably MIPs are amongst those genes that are up- or down-regulated during stress responses (Klok et al., 2002; Maathuis et al., 2003; Seki et al., 2001). Some very interesting associations have been found with cation deficiency and salinity (Maathuis et al., 2003) and nitrogen sources (Fizames et al., 2004). These associations have generally been interpreted in terms of aquaporins functioning as water conducting pores, but aquaporins may also function to transport ammonia/ammonium, urea, and possibly potassium and anions (nitrate and chloride) (see below). Despite the large size of the MIP gene family in plants, it seems that the isoforms are not redundant as indicated by different patterns of transcript expression under stress (citations above) and from gene silencing studies (Javot et al., 2003).

Chemical signals associated with water stress

The method of applying different watering regimes to different sides of a plant's root system, known as partial root-zone drying (PRD), has been developed commercially for vineyards (Dry, 1997; Loveys et al., 1998). This method imposes a mild water stress that will reduce vigour without any significant reductions in yield. As one half of the root system is always well watered, the risk due to severe water stress is greatly reduced (Dry and Loveys, 1998). The partial closure of stomata increases plant transpiration

efficiency by reducing transpiration to a greater degree than the reduction in carbon dioxide assimilation (During, 1990). The reduction in shoot growth rate and gas exchange was not associated with a reduction in the water potential of the leaf (Dry and Loveys, 1999; Dry et al., 2000). However, in grapevines it was associated with increased levels of abscisic acid (ABA) and higher pH in the xylem sap compared to well watered controls (Stoll et al., 2000). Stoll et al., (2000) demonstrated, using deuterium-enriched water, that water movement occurred from the root system in the wet soil to the root system in the dry soil. This rehydration of the roots was proposed to be necessary to facilitate the movement of ABA from the drying roots to the shoots as a signal to partially close the stomata.

Zhang and Davies (1987) demonstrated that the ABA concentration of root tips from *Pisum sativum* and *Commelina communis* increased when they were air-dried. When the roots were placed in a solution of ABA there was a reduction in stomatal conductance, which increased with increasing concentrations of ABA in the solution. The roots of *Helianthus annuus* that protruded through the base of the pot were air dried to simulate partial drying. This resulted in an increase in ABA concentration in the protruding roots, xylem sap and leaves, compared with control plants (Neales et al., 1989). These results indicate that the ABA was synthesised in the roots in response to drying. Gowing et al., (1990) excised the roots from the dry side of a split-rooted plant, and observed that shoot growth recovered to a similar level to those plants for which the dry side was rewatered. This again indicated that the shoot growth was responding to a negative signal, such as ABA produced in the roots. If the roots had been grown in soil there is the possibility that the ABA can be taken up by the plants from the soil solution (Hartung et al., 2002). Some ABA in the root apoplast that may exude into the soil solution is a consequence of normal pH gradients and the equilibria of ABA as a weak acid. The exodermis of maize roots appears to be an effective barrier to apoplastic transport of ABA, while the endodermis is not (Schraut et al., 2004). The synthesis of ABA in the leaves only occurs when the leaf turgor approaches zero (Hartung et al., 2002). Therefore, for stomatal closure to occur before any changes in leaf water potential the ABA must be imported from the roots via the xylem.

Drying of the soil also increases the pH of the xylem sap from about pH 6.1 to pH 6.7 (Wilkinson and Davies, 1997), which is associated with closure of the stomata and reduced leaf growth (reviewed in Sauter et al., 2001). The alkaline pH prevents the ABA from being sequestered away from the apoplast and its site of action on the guard cells. A reduction in cytokinins (zeatin and zeatin riboside) in the shoot may also be associated with the closure of the stomates during PRD (Stoll et al., 2000). This was reinforced by the reversal of effects associated with drying roots by applying benzyladeneine to the shoots.

Results examining the association between ABA and root L_p are contrasting. Davies et al., (1982) observed an ABA stimulated water uptake by excised wheat roots under low water flux conditions, but no increase was observed when there were initially high flux rates. Also there was a decrease in the hydraulic conductance (L_o) when the whole root system was measured. ABA had no effect on the root L_p of aspen (*Populus tremuloides*) seedlings measured in a pressure chamber (Wan and Zwiazek, 2001). However, ABA ($5 \times 10^{-5} M$) added to the nutrient solution of whole seedlings and detached shoots did reduce stomatal conductance. In contrast, Hose et al., (2000) demonstrated that the L_p of root cortical cells of maize increased 7 to 27-fold in response to the addition of exogenous (+)-*cis*-*trans*-ABA at levels expected in a stressed plant (100–1000 n*M*). The radial root L_p measured with the root pressure probe increased by 3-fold (hydroponically grown roots) and 4-fold for (aeroponically grown roots). No other forms of ABA increased the L_p. ABA had a greater effect on the cell L_p indicating the cell-to-cell pathway was affected more than the apoplastic pathway. Hose et al. (2000) surmised that the ABA may affect the expression or activity of aquaporins. These results were reinforced by Sauter et al., (2002) using maize roots and measuring conductivity with the suction technique. The ABA applied at a concentration of 100 n*M* increased the radial movement of water. Quintero et al., (1999) demonstrated that the effect of ABA on the exudation rate of xylem sap depended on the Ca^{2+} concentration in the whole root. ABA increased root L_p to a greater extent at higher Ca^{2+}

concentrations. This is consistent with the ameliorating effect of external Ca^{2+} on stress-induced reductions in root L_o (Cabanero et al., 2004). However, mercuric chloride (1 μM) (an inhibitor of water-channel activity, discussed in the aquaporin section) inhibited the water flow in both the presence and absence of ABA, but from the graphical representation it appears to reduce the flow to a greater extent in the presence of ABA (Quintero et al., 1999).

Lovisolo et al. (2002) used split-rooted plants to examine the effect of ABA on the whole plant conductance of grapevines (*Vitis vinifera*). By withholding water to half of the roots there was no reduction in the conductance or the water potential of the leaf and stem, whereas the completely stressed plants suffered a substantial reduction. In contrast, the level of ABA in the leaves and the stomatal conductance of both treatments were similar. This appears to indicate that there is no interaction between ABA and whole plant conductance. However, the conductance of the roots in the water stressed half of the plants might be reduced, but compensated by increased conductance of the roots in the well watered half. In addition, when the shoot was inverted in the half-stressed plants to cause a reduction in the conductance, independent of soil water availability there was no impact on the level of ABA in the shoot or leaves. This research only examined the conductance of the whole plant, the sum of all components, rather than the root and cell-to-cell components examined by Hose et al. (2000). The ABA may be involved in the finer regulation at the cell-to-cell level, indicating a possible role in aquaporin regulation. The influence of ABA on the expression of aquaporin genes will be discussed in a later section.

Root hydraulics

Pathways of water transport

The hydraulic conductivity of roots is highly variable (Passioura, 1984; Passioura and Munns, 1984; Passioura and Tanner, 1984, Tsuda and Tyree, 2000). Before it was known that gating of aquaporins could be responsible for this variability, pressure responsive valves through which water must pass were proposed to occur in roots (Passioura and Munns, 1984). Variability, in part, may be due to the relative contributions of the individual components of water transport (Passioura, 1984). Tyree (2003) provides a more rigorous discussion on the different models or root water transport. A model that can account for the different properties of radial pathways across the root is the composite transport model (Steudle, 1994). The model comprises apoplastic, symplastic and transcellular pathways operating in parallel. The apoplastic pathway is outside of the cells' plasma membrane; the symplastic pathway is through the cytoplasm of cells connected by plasmodesmata; the transcellular pathway is across cell membranes (Steudle, 2000a, b). Water channels (aquaporins) would be involved in regulating the movement of water in the transcellular pathway (Steudle, 2000a). The combination of symplastic and transcellular is known as the cell-to-cell pathway. The composite transport model may explain why hydrostatic gradients result in much higher root L_p than for osmotic gradients (Steudle, 2000a, b; Steudle and Frensch, 1996; Steudle and Meshcheryakov, 1996; Steudle and Peterson, 1998; Steudle 1993, 1994), but Tyree (2003) has questioned whether the osmotic hydraulic conductivites are really as low as reported based on rates of solute diffusion relative to the time constant of water equilibration.

The movement of water through the apoplast is driven by hydrostatic gradients, while across a membrane-delimited (transcellular) pathway both hydrostatic and osmotic gradients are involved. Osmotic gradients are not directly involved in apoplastic transport, as the wall structures do not select against most solutes, unlike cell membranes. When plants are transpiring the hydrostatic gradient dominates due to the tensions developed in the xylem from capillary forces in leaf cell walls as water evaporates from these walls into the leaf air spaces. The water movement driven by hydrostatic gradients can flow via both the apoplastic and the cell-to-cell pathway, the proportion depending on the relative hydraulic conductances of the two pathways. When the transpiration rate is slow, during the night or under water-limiting conditions, the osmotic flow may dominate due to the pumping of ions to the stele not being diluted by large hydrostatic-driven water flows.

The path taken by water is influenced by the anatomy of the roots. The apoplastic pathway can be inhibited by the presence of a Casparian band, which is a deposit of suberin or lignin in the cell wall (Zeier and Schreiber, 1997). The Casparian band occurs in the endodermis and exodermis in the radial and transverse walls of the cell (Steudle and Peterson, 1998). Suberin lamellae may also occur on the tangential walls to further inhibit apoplastic flow. The apoplastic *barriers* should be considered as a variable apoplastic conductance that can be altered in the medium to long-term by the plant in response to the root environment (Hose et al., 2001). For example, it appeared that the exodermis in rice (*Oryza sativa*) roots with a physical barrier against radial oxygen loss (Colmer, 2003) that includes Casparian bands, did not reduce radial water flow attributed to the apoplast (Ranathunge et al., 2004). The formation of these *barriers* to the apoplastic movement of water is often associated with the imposition of stress such as water deficits and the aging of the plant (Steudle and Meshcheryakov, 1996). The suberised layers may assist in reducing water loss to the soil during water deficits. Roots of woody species in general have an L_p that is an order of magnitude smaller than herbaceous plants due to greater suberisation. In addition the proportion of flow along the apoplastic pathway is greater, as demonstrated by a much larger difference between the hydraulic and osmotic water flows (Steudle, 2000a).

Leading from this discussion there is still the over-arching question, which is not often clearly answered in the literature: *What is the proportion of radial flow under normal transpiration accounted for by aquaporins?* The answer probably depends on the developmental stage of the roots, the conditions the roots were exposed to, and the species (perhaps even different genotypes of a species). There is sometimes confusion in the literature because hydraulic conductivities used to account for aquaporin contribution are compared between hydrostatic- and osmotic-induced water flows. However, as a range, the cell-to-cell pathway with aquaporins playing a dominant role, could account for between 20% and 85% of root L_o under a hydrostatic gradient and under non-water stressed conditions (discussed in Martre et al., 2001; Maurel et al. 2002, see also references under Chilling stress). Martre et al. (2002) showed that *Arabidopsis* plants that were double-antisense with reduced expression of PIP1 and PIP2 had up to 68% reduction in root L_o.

In addition to the radial transport there is axial transport facilitated by tracheids and vessel members of the xylem. Both components lack a protoplast and generally lack the end walls between adjacent cells to reduce the resistance to water flow. The diameter of the channel affects the L_p according to Poiseuille's law. In the roots of maize (*Zea mays*) the xylem had the least resistance to water movement (Frensch and Steudle, 1989). Nobel and North (1993) and Steudle and Meshcheryakov (1996) also suggest that under wet conditions the radial L_p is the limiting component of water movements. Steudle and Meshcheryakov (1996) demonstrated this by cutting the roots of oak (*Quercus robur and Q. petraea*) when attached to the root pressure probe. The half times of water exchange were decreased by a factor of approximately five following the cutting of the root. However, under water stress the water flow can be interrupted by the presence of embolisms within the xylem (North and Nobel, 1991).

Roots as variable conductors – the response of roots to soil drying and rewetting

During periods of water stress, plants must conserve water. Roots could respond by preventing water loss to the soil when the water potential of the soil becomes more negative than the water potential of the root. The process of decreasing L_o to limit water loss, followed by a rapid increase in conductance once soil moisture is restored has been called *rectification* (Nobel and Sanderson, 1984). This meaning is distinct from the more common meaning to indicate that there is an almost instantaneous polarity in flow, that is, that the hydraulic conductivity may be lower for water efflux from roots (or cells) than for water uptake in the short term. We will refer to the former as *variable conductance* (as per variable resistance according to Lo Gullo et al., 1998) and the latter as *intrinsic rectification*. Intrinsic rectification has been examined in cells using the pressure probe (Steudle and Tyerman, 1983; Tyerman and Steudle, 1982) and in isolated membranes containing aquaporins (Figure 2a),

and provided that osmotic gradients are similar for flow in both directions there does not seem to be any intrinsic rectification. Much of the work on variable conductance that results in the time-dependent rectification described by Nobel and Sanderson (1984) has examined desert succulents that do not have specialised roots to cope with the extreme conditions. During drying conditions the L_p of the roots of *Agave deserti* declined, partly because of the collapse of cortical cells, increased suberisation, and embolisms in the xylem vessels (North and Nobel, 1991). The roots also shrink as a result of the collapse of the cortical cells that reduces the contact between the soil and roots. Upon rewetting the young nodal roots of *A. deserti* showed a 50% recovery after 2 days, and an almost complete recovery of conductivity after 7 days compared with the lateral roots, which only recovered to 21% of the initial conductivity (North and Nobel, 1991). The recovery to 50% of the initial conductivity occurred prior to any new root growth. Tyree et al., (1995) suggest that plants with good time-dependent rectifying properties would have a low radial solute permeability and a high reflection coefficient to cause an increase in solute concentration in the xylem vessels which would reduce the force driving water out of the roots. Where hydraulic redistribution occurs (e.g. hydraulic lift) there will be flow reversal in some roots that are exposed to soil with a lower water potential. These roots are supplied by roots that take up water in regions of the soil with higher water potential. Flow reversal in this situation probably has advantages to the plant and hence restriction of water efflux by either time-dependent or intrinsic rectification would not seem to be of an advantage. Perhaps the critical factor to consider here is the time scale over which time-dependent rectification occurs.

North and Nobel (1996) examined the conductivities of different tissues in the roots of *Opuntia ficus-indica* growing in pots in a glasshouse. The conductivity, radial and axial, of distal roots was not significantly affected by soil drying compared with that of mid-roots. This was explained by the presence of a soil sheath in the distal region. The cortex of the distal section had an increased conductivity due to the death of the cortical cells. In the mid-root section the conductivity of the cortex was not affected, as the cortical cells were dead prior to drying. In both sections the conductivity of the endodermis and periderm decreased. This was associated with an increase of suberin lamellae in the endodermal cells and an increased number of layers in the periderm. The conductivity of the vascular tissues also declined. After a period of approximately seven days of drying the lack of soil and root contact became the limiting component in water movement (Nobel and North, 1993). Eventually the water potential of the soil decreases to below the minimum attainable by the plant so that the water is no longer available to the plant. The cactus *Opuntia acanthocarpa* did display a reduction in L_p in both the distal and mid-root regions, with the distal region having an initially lower L_p under wet conditions (Martre et al., 2001). Upon rewetting for 8 days the L_p returned to 60% of the initial value. The effect of the gap between root and soil decreases as the root cells again become turgid, and the root L_p becomes the limiting component again. The drying process will produce anatomical changes that are irreversible, particularly in older roots (North and Nobel, 1991). The apoplastic component of water transport increased during re-wetting (North and Nobel, 1996) due to the death of the cortical cells. To fully restore the radial L_p following rewetting there must be new apical growth and new lateral roots (North and Nobel, 1995).

Given the importance of new root growth in recovery from water stress it is worth examining the potential role of aquaporins in root growth. This has been examined by Hukin et al., (2002). Using a segmental analysis of maize root growth they found a correlation between the reduction in cell L_p of proximal expanding cells and reduction in growth caused by 20 μM $HgCl_2$. The distal root tip cells did not respond to $HgCl_2$ and were in symplastic connection with the phloem, while proximal, older cells had a higher L_p and appeared to rely on the apoplast for their water supply. These cells had greater sensitivity to $HgCl_2$ and also showed higher expression of ZmPIP1;2 and ZmPIP2;4.

Martre et al. (2001) examined the impact of mercury-sensitive water channels on the L_p of *Opuntia acanthocarpa* roots. The addition of 50 μM $HgCl_2$ to the bathing solution of the roots did not impact on the L_p of the mid-root

region under any water regimes. However, with $HgCl_2$ the distal region's L_p decreased by 32%, but no decrease was observed under dry conditions. After rewetting for 1 day the L_p in the presence of $HgCl_2$ was again reduced by 21% compared with that of the control, with a similar reduction still present after eight days of rewetting. The reductions were reversible with 2-mercaptoethanol, indicating that the mercury did not have toxic effects on the plant cells (Martre et al., 2001). However, there still remains the possibility that mercury inhibition occurs by other mechanisms in addition to direct blockade of the water pore. For example, elevation of cytosolic calcium concentration or acidification of the cytosol in response to mercury could also reduce water channel activity (Gerbeau et al., 2002). Gaspar et al., (2001) also found that cell turgor in maize roots was much reduced in epidermal cells by low concentrations of mercury (11 μM).

The potential involvement of water channels may be underestimated as not all are sensitive to mercury (e.g., NtAQP1, Biela et al., 1999) and the mercury penetration may be restricted by apoplastic barriers (Barrowclough et al., 2000; Gaspar et al., 2001). This could be the reason that the mid-root region, with more suberised layers of periderm, was not affected by mercury. Similar results were observed using *Agave deserti* (North et al., 2004). During drying the overall root L_p decreased by 30–60% and was not decreased further by the addition of $HgCl_2$, whereas following rewatering the L_p was restored and was sensitive to $HgCl_2$ in the distal and basal regions of the root. Overall, aquaporins appear to be involved in contributing to the decrease in conductivity during drying and the subsequent increase during rewetting.

There are also species where the axial conductivity may be also restricting overall water movement. *Sorghum bicolour* seedlings grown in vermiculite suffered a 4-fold reduction in root L_p when water was withheld (Cruz et al., 1992). This appeared to be caused by significant suberisation and lignification of the exodermis and endodermis. In stressed roots the axial L_p did not increase as root segments were removed, indicating that the axial flow was blocked. In stressed roots the cross walls of the late metaxylem persisted even in mature sections of the root. This axial resistance contributed to the reduction in total root L_p under stressed conditions (Cruz et al., 1992). However, in *Agave deserti* there was actually an increase in the axial L_p of water-stressed plants, suggested to be due to the maturation of xylem vessels compensating for any loss of conductance due to embolisms (North and Nobel, 1995). During seven days of water stress, the radial, rather than the axial conductivity of young nodal roots was limiting total conductivity, even when embolisms were present in the xylem (North and Nobel, 1991).

Lo Gullo et al. (1998) described the decrease in conductance in response to water stress as a variable-resistor effect, rather than rectification. They observed that *Olea oleaster* (wild olive), a typical Mediterranean plant, suffered an 84% reduction in root L_p (normalised by leaf surface area). Following 48 h after irrigation the conductance had returned to 66% of the wellwatered control seedlings. Recovery was not completed within 96 h after irrigation. A severe water stress that caused an increase in suberised layers of exodermis and endodermis resulted in the recovery being delayed further. The recovery was linked to the emergence of new lateral roots and the growth of pre-existing root tips. Severely water stressed *Populus tremuloides* seedlings had a root L_p a third of that of control plants, which did not recover within 24 h of rewatering (Siemens and Zwiazek, 2003). The water-stressed plants had a higher proportion of flow using the apoplastic route, indicating that the cell-to-cell pathway was most affected by stress.

Potential secondary impacts of irrigation

Salt stress or nutrient stress can induce rapid changes in root L_p and cell-membrane L_p in some species (Azaizeh and Steudle, 1991; Azaizeh et al., 1992; Carvajal et al., 1996, 1999; Clarkson et al., 2000; Munns and Passioura, 1984; Radin and Mathews, 1989). The inhibiting effect of salinity on root L_o (50–80%) seems to be ameliorated by calcium, since it can determine the restoration of L_o (Carvajal et al., 2000), and there is a link between the effect on water flux and calcium uptake (Cabanero et al., 2004).

When macronutrients that are in the form of anions are deficient in the nutrient solution, it is generally observed that root L_o is reduced

(Radin and Eidenbock, 1984; Radin and Mathews 1989; reviewed in Clarkson et al., 2000). Carvajal et al., (1996) found evidence in wheat plants, deprived of nitrogen or phosphorus, for a reduction in the osmotic L_p of roots that was associated with a reduction in the activity or number of aquaporins that were sensitive to $HgCl_2$. In the control plants, grown with a complete nutrient solution, the presence of $HgCl_2$ (50 μM) reduced the osmotic L_p and the sap flow to levels similar to that of the nutrient deprived plants. Hoarau et al. (1996) found that maize seedlings treated with potassium nitrate had higher root L_o (for hydrostatic gradients) than plants treated with potassium chloride. Furthermore, a rectification was observed (water influx > efflux) with osmotic experiments with nitrate-fed plants compared with chloride-treated plants. The lack of certain nutrients may result in a reduced synthesis of proteins, including aquaporins, or changes in membrane properties. However, the changes appear to be too rapid for this to be the case. For example, after increasing phosphorus supply to *Cucurbita pepo* L. plants, root L_o increased immediately by 22% (Reinbott and Blevins, 1999). It is also not necessarily the lack of nitrogen that will cause a reduction in root L_o, but rather the form of the nitrogen presented. Stimulation of root L_o by nitrate has been observed in French bean (*Phaseolus vulgaris* L.) (Guo et al., 2002), and ammonium supply decreased the root L_o of *Cucumis melo* L. by 50% (Adler et al., 1996). Root exudation from detopped *Zea mays* L. was rapidly enhanced when either nitrate, ammonium or glutamine were supplied in the solution bathing the roots (Barthes et al., 1996). Using inhibitors of nitrogen assimilation enzymes it was concluded by Barthes et al (1996) that the effect was through enhancement of L_p (osmotic) by a link to nitrogen assimilation activity, and glutamine was suggested to be directly involved. It has been proposed by Clarkson et al. (2000) that the incoming (apoplast) concentration of anionic nutrients is somehow sensed and translated into changes in aquaporin activity. Since anion influx across the plasma membrane requires active transport, it is suggested that there is a link between energised anion uptake and aquaporin activity (Clarkson et al., 2000). The association between nutrient anion uptake and aquaporin activity is very interesting and warrants further detailed study.

Chilling of roots can cause water stress in the shoots because of a reduction in the root L_o (Markhart, 1984; Melkonian et al., 2004; Vernieri et al., 2001). The reduced root L_o is partly a result of an increase in viscosity of water at low temperature (e.g., Cochard et al., 2000), but there is also a component of the reduction that is under biological control (Bloom et al., 2004, Fennell and Markhart, 1998; Lee et al., 2004a; Melkonian et al., 2004). For chilling tolerant and sensitive *Lycopersicon esculentum* cultivars the temperature dependence of root water flow gave an activation energy of about 9 kcal mol^{-1}, which is much higher than would be expected for water flow through aquaporins in the roots or solely via the apoplastic pathway (Bloom et al., 2004). On the other hand, if it is accepted that aquaporins function in water transport across root membranes the high activation energy may indicate that gating of aquaporins is temperature sensitive, ie they turn off at lower temperatures. Lee et al. (2004a) observed a larger reduction in root L_p in response to an osmotic gradient (77%) than for a hydrostatic gradient (34%) in cucumber, and this was suggested to be due to closure of aquaporins. A correlation was observed between the reduction in root cell L_p under chilling and increased production of hydrogen peroxide in the roots (Lee et al., 2004b), but reduced respiration may also affect the activity of aquaporins (Kamaluddin and Zwiazek, 2001; Zhang and Tyerman, 1999). Hydrogen peroxide at concentrations commonly used in assessment of its role in signalling, also reduced the cell L_p (Lee et al., 2004b). Given the likely signalling role that hydrogen peroxide and reactive oxygen species (ROS) have in plant roots (Foreman et al., 2003; Mori and Schroeder, 2004), it would be worthwhile investigating further its role in regulation of aquaporins. There is also the possibility that aquaporins play a role in facilitating the passage of hydrogen peroxide (and ROS?) across the plasma membrane (Henzler and Steudle, 2000).

Subsequent to the initial chilling-induced reduction in root L_o recovery occurs to various degrees depending on species (Fennel and Markhart, 1998; Markhart et al., 1979; Melkonian et al., 2004; Vernieri et al., 2001). Broccoli

(*Brassica oleracea*) grown at lower temperatures showed a reduction in activation energy of water flow through roots which was interpreted as a modification to root membranes (Markhart et al., 1979), but could also be interpreted now as a greater contribution from aquaporins. Using stem-girdled *Phaseolus vulgaris* plants Vernieri et al., (2001) excluded a shoot-generated chemical signal, including ABA, in the recovery of root L_o. They concluded that recovery of root L_o was a major component of recovery from water stress induced by chilling. Melkonian et al. (2004) demonstrated that the partial recovery of maize root L_o under chilling at 5.5 °C stabilised leaf water relations and allowed stomata to reopen. The contribution of the root system could be estimated to account for 44% of the recovery in leaf water potential after 24 h of chilling. They reported that no up-regulation of PIP expression occurred, indicating that recovery probably involved post-translational regulation of aquaporins.

Root hypoxia may also occur during irrigation events (Vartapetian and Jackson, 1997). Hypoxia is more likely to occur in flooded soil than anoxia, but there are fewer studies that have investigated changes in root and cell L_p under hypoxia (Gibbs et al., 1998) than anoxia (Birner and Steudle, 1993; Tournaire-Roux et al., 2003; Zhang and Tyerman, 1991). Under hypoxia it is likely that the cortex of the root will obtain adequate oxygen levels but the stele will become anoxic (Gibbs et al., 1998). Thus the effect on water transport may depend on the sites of membrane-limited flows radially across the root. In both hypoxia and anoxia there is a reduction in root L_p. In the case of anoxia there is a strong reduction in root cortex cell L_p (Tournaire-Roux et al., 2003; Zhang and Tyerman, 1991) consistent with closure of aquaporins also induced by mercury or respiratory inhibitors. Under hypoxia maize roots showed a recovery in root L_p after 4–6 h of treatment (0.05 m*M* O_2). It is not clear if the recovery is related to readjustment of metabolism or regulation of aquaporins as proposed for chilling.

The root L_p varies between species and with environment. These factors impact on the relative importance of the pathways of water movement in the roots. The roots' anatomy is altered with age and stress, and this can either increase or decrease L_p. ABA synthesised in response to stress gives contrasting results in its effect on L_p. Changes in root anatomy, the regulation of aquaporins, and new growth are all important in the uptake of water following rewatering. Potential side effects of irrigation including changes in nutrient concentrations, soil salinity, root temperature and hypoxia may impact on the regulation of aquaporins.

Aquaporins

The existence of channels permeable to water in the membranes of plants, animals, yeast and bacteria has been supported by a large quantity of evidence. The CHIP28 protein expressed in *Xenopus* oocytes increased the osmotic water permeability, which was reversibly inhibited by $HgCl_2$, suggesting that the protein was the first identified water channel (Preston et al., 1992). The protein also reduced the activation energy of water transport. Maurel et al. (1993) were the first to demonstrate the water-transport properties of a plant vacuolar membrane protein, γTIP when expressed in *Xenopus* oocytes. Aquaporins in plants have been extensively reviewed in the last few years and the reader is referred to the following reviews for a more detailed account of the molecular aspects and general physiology and biophysics of aquaporins (Chrispeels et al., 2001; Eckert et al., 1999; Hill et al., 2004; Javot and Maurel, 2002; Johansson et al., 2000; Kjellbom et al., 1999; Maurel, 1997; Maurel et al., 2002; Tyerman et al., 2002)

Aquaporins are members of the MIP group of trans-membrane channels found ubiquitously in all organisms. Johanson et al. (2001) identified 35 MIP-encoding genes in *Arabidopsis thaliana*. At least 31 MIPs have been identified in maize (Chaumont et al. 2001). This family of proteins has been divided into four groups (Johanson et al., 2001) based on sequence homology, and for two groups this seems to match to the membrane location; plasma-membrane intrinsic proteins (PIPs), tonoplast-intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), and the small basic intrinsic proteins (SIPs). The PIPs have been divided into two groups, the PIP1s and PIP2s (Chaumont et al., 2000). The aquaporins consist of 6 membrane-spanning domains.

The amino- and carboxy-termini are located on the cytosolic side of the membrane. Sui et al. (2001) observed that the pore of AQP1 consists of three hydrophilic nodes that bind four water molecules, with the remainder of the pore being hydrophobic. The conserved histidine (182) residue in the pore is essential for the water specificity. Generally the 25–30 kDa polypeptides form tetramers. A number of residues in the protein are conserved in the majority of cases, i.e. the asparagine-proline-alanine (NPA) box. It appears that the protein arose from duplication, as the two halves of the protein share sequence homology, but are orientated in opposite directions in the membrane. The pores of the channels are believed to be narrow so that the water molecules move through in single file. Mercury blocks the water channels by binding to a cysteine residue in the pore.

The PIP1 group generally shows low or no water permeability when expressed in *Xenopus* oocytes, contrasting with the PIP2 group which shows high water permeability (e.g., Fetter et al., 2004). Some members of the PIP1 group (NtAQP1) are also permeable to glycerol (Biela et al., 1999). In fact NtAQP1 has been implicated to carry out substantial water flows in roots (Siefritz et al. 2002) based on the over 50% reduction in root L_p (hydrostatic) in tobacco (*Nicotiana tabacum*) plants with reduced (antisense-induced) expression of NtAQP1. Recently, Uehlein et al. (2003) provided evidence for NtAQP1 to be also involved in CO_2 permeation. This apparent multi-functionality of a single aquaporin is quite remarkable, but perhaps there are additional factors that need to be considered, for example the formation of hetero-tetrameric units with other aquaporins (Fetter et al., 2004) such that the PIP1 may be involved in a regulatory way, or alternatively that the PIP1 is functioning as a sensor (Hill et al., 2004).

Other plant MIPs transport other small solutes such as glycerol and urea, in addition to, or alternatively to water. Nodulin 26 (NOD26), which is targeted to the symbiosome membrane of nitrogen-fixing nodules allows the transport of glycerol and formamide in addition to water (Rivers et al., 1997) and may also be permeable to ammonia (Niemietz and Tyerman, 2000). Recently there have been reports from the groups of Thomas Jahn and Nicolaus von Wiren that some aquaporins are transporting NH_3 (Abstracts of 7th International Symposium on Inorganic Nitrogen Assimilation in Plants, June 23–27, 2004 Wageningen, the Netherlands). Nt-AQP1 and Nt-TIPa transport glycerol, water and urea (Eckert et al., 1999; Gerbeau et al., 1999). Urea transport was also demonstrated for four *Arabidopsis* TIPs (*AtTIP1;1, AtTIP1;2, AtTIP2;1* and *AtTIP4;*1) (Liu et al., 2003). The aquaporins were constitutively expressed in shoots, but were up-regulated in roots under nitrogen deficiency. A maize root PIP (ZmPIP1;5b) was also found to transport urea and water; the transcript was induced by supplying potassium nitrate to nitrogen starved plants (Gaspar et al., 2003).

Niemietz and Tyerman (1997) tested all indicators of water channels in wheat root membrane vesicles, examining the osmotic permeability, sensitivity to temperature and $HgCl_2$, and also the diffusional permeability using D_2O. Osmotic water permeability (P_f) of membrane vesicles was determined using a stopped-flow light-scattering technique, and the evidence suggested that the transport of water was facilitated, rather than by diffusion across the lipid membrane. Niemietz and Tyerman (1997) found that the tonoplast had a higher P_f and lower energy of activation than the plasma-membrane component of vesicles (Wheat data in Figure 2b). The plasma-membrane fraction did have a ratio of greater than one for the ratio of osmotic to diffusional permeability indicating a role for aquaporins rather than diffusion across the lipid bi-layer; however, the aquaporins were insensitive to $HgCl_2$. Recent evidence suggests that the plasma-membrane water permeability is regulated by cytosolic pCa and pH. Higher water permeability for the plasma membrane was observed when isolation of plasma-membrane vesicles was done under conditions that prevents dephosphorylation (Gerbeau et al., 2002). Measurements on plasma-membrane vesicles from *Beta vulgaris* storage roots has revealed very high water permeabilities ($>500\ \mu m\ s^{-1}$, Niemietz and Tyerman, 2002), strongly regulated by pCa and pH (Alleva K, Niemietz C M, Sutka M, Dorr R, Maurel C, Parisi M, Tyerman S D and Amodeo. G, unpublished). Here the water permeability was strongly inhibited by silver (Niemietz and Tyerman, 2002).

Aquaporins may play a role in osmoregulation of cells. There was a high level of expression of

ZmTIP1 mRNA (tonoplast aquaporin of *Zea mays*) in cells involved with intense water or solute transport (Barrieu et al., 1998). For example; root epidermis cells, xylem parenchyma, phloem companion cells, cells that surround phloem strands and basal endosperm transfer cells in developing kernels. The ability to rapidly exchange water between the vacuole and the cytosol enables the cell to respond rapidly to any changes in the osmotic potential of the cytosol, reducing large transient changes in cytosolic volume (Tyerman et al., 1999). The same situation may occur in bacteroid-containing cells of legume nodules. A large volume is occupied in the infected cell by the symbiosomes that contain the bacteroids. The dominant aquaporin on the symbiosome membrane (NOD26) is regulated by osmotic stresses (Guenther et al., 2003; Figure 2b). This buffering function of tonoplast (or symbiosome) aquaporins has been challenged by Hill et al. (2004), based on the argument that external changes in water potential or osmotic potential are rarely a step change, as was modelled by Tyerman et al., (1999), but occur exponentially with time, in which case there is enough time apparently for water flow to equilibrate between cytoplasm and endo-membrane compartments.

Regulation of aquaporin activity

Plant membranes that have been demonstrated to contain functional water channels can show a wide range of water permeabilities (Figures 1 and 2b). This could be due to different intrinsic water transport properties of the single protein, different densities of the channels in the membrane, or different activity states of the channels. One possible method of regulation of aquaporin activity is the phosphorylation of a serine residue. This was demonstrated in spinach leaf plasma membranes by Johansson et al. (1998) using mutants where the putative serine residues were altered to alanine (Ser(6)Ala, Ser(36)Ala, Ser(115)Ala, Ser(188)Ala, Ser(192)Ala, Ser(274)Ala) and with the use of inhibitors of protein kinases and protein phosphatases. The serine residue (Ser-274)

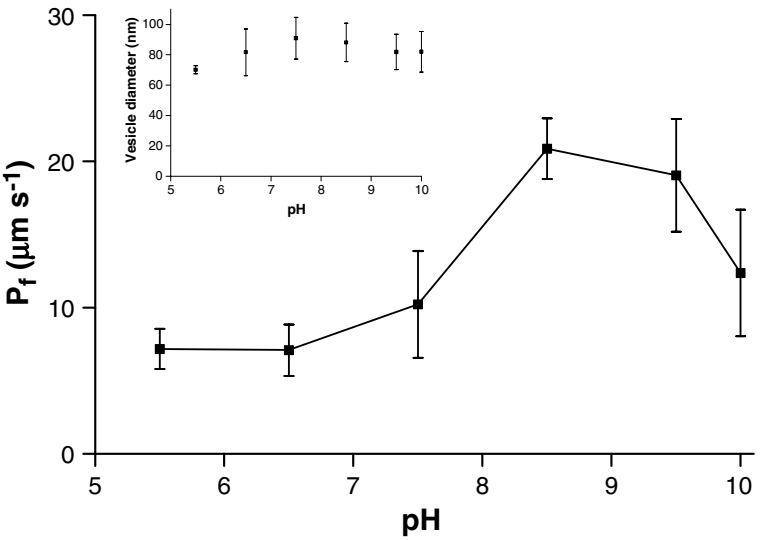

Figure 1. Effect of pH on the water permeability of plasma membrane vesicles isolated from roots of 6 day old seedlings of *Triticum aestivum* cv Krichauff. Plasma membrane vesicles were isolated using two phase partitioning (Niemietz and Tyerman, 1999) using vesicle isolation solutions as used by Gerbeau et al. (2002) to prevent de-phosphorylation of membrane proteins and to maintain free calcium at very low activities. The vesicle diameter determined using a particle size analyser at each pH did not vary significantly (inset) and was similar to the value reported by Niemietz and Tyerman (1997). An osmotic gradient of 100 mosmol kg^{-1} was used to induce vesicle shrinking in a stopped flow spectrofluorimeter. The kinetics of the change in light scattering was used to calculate the P_f (Niemietz and Tyerman, 1997). The treatment pH was imposed on the cytoplasmic face of the plasma membrane. The plants were grown with half strength Hoagland's solution but no difference was observed in P_f between plants grown with 1 m*M* $CaCl_2$ or nutrient solution. Each point is the mean of three determinations on three separate membrane preparations (error bars = SEM).

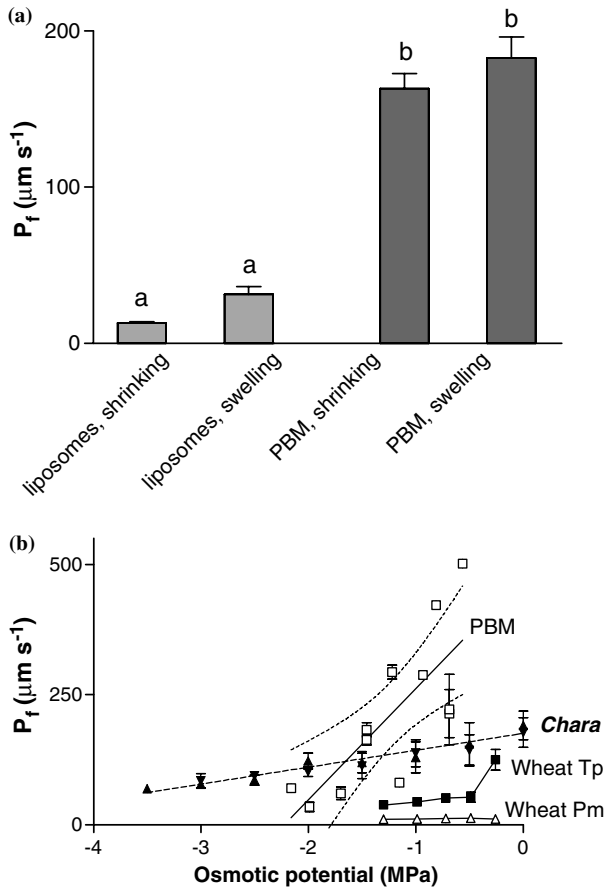

Figure 2. Effect of osmotic potential and direction of water flow on water permeability of various membranes that either contain or do not contain active aquaporins. (a) With the same end point osmotic potential in the bathing medium, Peribacteriod membranes that contain the NOD26 aquaporin do not show any intrinsic rectification of flow. For comparison liposomes produced from soybean lecithin lipid were exposed to the same osmotic conditions to generate swelling or shrinking responses. (b) For membranes that contain active water channels there is a trend for decreased water permeability with decreased osmotic potential of the bathing solution. PBM vesicles that contain NOD26 at high density (open square symbols). *Chara corallina* cells (solid triangle symbols), converted from L_p measured with the pressure probe where osmotic potential was altered with ethanol (Steudle and Tyerman, 1983). Wheat root (*Triticum aestivum* L.) plasma membrane (PM, open triengles) and tonoplast enriched (TP, solid squares) membrane vesicles, where osmotic gradient was altered with sucrose (see Niemietz and Tyerman, 1997). Wheat root and PBM vesicles were obtained by phase partitioning as described in Niemietz and Tyerman (1997, 2002). For PBM vesicles aliquots of the final pellet were equilibrated in HEPES-KOH buffered sucrose solutions (pH 7, 150 mM to 1 *M* sucrose). In a stopped-flow fluorimeter the vesicles were suddenly exposed to osmotic gradients of 100 m*M* sucrose. The time course of vesicle shrinking (in hyper-osmotic solutions) or swelling (in hypo-osmotic solutions) was followed by light scattering. Single exponential fits were used to obtain a rate constant for shrinking and swelling from which P_f was calculated. The P_f values are plotted against the final osmotic potential. For *Chara* and PBM the data could be fit best by a linear regression $P_f = A\Psi\pi + P_{f0}$ where for PBM $A = 214$ μm s^{-1} MPa^{-1} and $P_{f0} = 475$ μm s^{-1} ($r^2 = 0.6$) and for *Chara* A = 33 μm s^{-1} MPa^{-1} and $P_{f0} = 176$ μm s^{-1} ($r^2 = 0.95$). The 95% confidence limits are shown for the peribacteroid membrane regression.

phosphorylated *in vivo* is only found in the PIP2 family of plasma-membrane aquaporins. Johnson and Chrispeels (1992) observed that the phosphorylation of a seed-specific αTIP was by a calcium-dependent protein kinase. Maurel et al. (1995) also showed that phosphorylation of αTIP increased the P_f of *Xenopus* oocytes, and recently NOD26 (a NIP) was shown to exhibit higher P_f in *Xenopus* oocytes when phosphorylated on Ser-262. Phosphorylation was increased *in vivo* under osmotic stress (Guenther et al., 2003). The detection of more proteins than the known number of aquaporin homologues using two-dimensional gel electrophoresis and antibodies

against PIP1;1 and PIP2;2 suggests that modified forms exist in addition to the unmodified forms (Santoni et al., 2003). Many of the modified forms were likely to be phosphorylated, suggested by the lowered iso-electric point. Another possibility is that the antibodies cross-reacted with other aquaporins.

Gerbeau et al. (2002) observed that *Arabidopsis* cell L_p was reduced by 35 and 69% in the presence of magnesium and calcium ions, respectively. This led to the observation that the P_f of the membrane vesicles was also reduced by divalent cations, in particular calcium. The activation energy was increased indicating a reduction in the activity of aquaporins. In addition, acidic pH conditions reduced the permeability reversibly. Cytosolic acidification of root cells caused a 7-fold decrease in cell L_p probably through direct inhibition of PIPs (Tournaire-Roux et al., 2003). Acidification of *Arabidopsis* root cell cytoplasm during anoxia and metabolic inhibition was correlated with reduced whole root L_p (Tournaire-Roux et al., 2003) accounting for the previous observation that anoxia strongly inhibits *Triticum aestivum* root cortex L_p (Zhang and Tyerman, 1991). Both PIP1 and PIP2 aquaporins have a histidine residue (HIS197) that appears to be the sensitive residue to pH (Tournaire-Roux et al., 2003).

For *Triticum aestivum* the P_f of root plasma-membrane vesicles isolated under conditions that protect against de-phosphorylation and calcium (Gerbeau et al., 2002) was no different to the values reported by Niemietz and Tyerman (1999) where protectants were not used and calcium was present at 0.1 m*M* (compare Figure 1 (pH 7) with wheat pm data in Figure 2b noting difference in scale). At pH less than 7.5, P_f was about half that measured above pH 7.5 (Figure 1). In the physiological range of pH (6.5–8.5) the greatest change in P_f would occur, suggesting that this effect is physiologically relevant. Even greater effects have been observed for other species. For *Beta vulgaris* a 100-fold reduction in P_f has been observed at low pH, and there was no effect of a pH gradient across the membrane (Alleva K, Niemietz C M, Sutka M, Dorr R, Maurel C, Parisi M, Tyerman S D and Amodeo. G, unpublished). It seems that cytosolic acidification using weak acids (acetate, propionate, butyrate) at low pH might be a better test of aquaporin activity *in planta* than using mercury.

There is also evidence for mechanosensitive gating of aquaporins. Wan et al. (2004) observed that large pressure pulses (greater than 0.1 MPa) decreased the L_p of maize root cortical cells. The intensity of water flow was suggested to cause a change in the protein's conformation. The inhibition was reversible with time if the pressure pulse was between 0.1 and 0.2 MPa and if the pulse was greater than 0.2 MPa the presence of 500 n*M* ABA reduced the half time (increased L_p). The long half times (low L_p) were not influenced by $HgCl_2$, indicating that the aquaporins were closed.

Aquaporin activity, based on cell L_p in *Chara* was also reduced in the presence of high concentrations of osmotic solutes (Steudle and Tyerman, 1983; Figure 2b), and more strongly with increasing size of these solutes (Ye et al., 2004). Ye et al. (2004) proposed that a *cohesion-tension like* model is operating, but since they were using living cells, metabolic control on the aquaporins cannot be excluded. The model proposes that exclusion of the solutes causes negative pressures within the aquaporin, altering the free energy between states, causing the channel to collapse. This deformation of the channel is reversible. A much stronger dependency of P_f on osmotic potential is observed for peribacteroid membrane (PBM) vesicles containing the aquaporin NOD26 (a NIP) (Figure 2b). In this case the effect of osmotic potential must be direct, since the vesicles used are free of cytosol and are surrounded on both sides of the membrane by similar solutions. This result illustrates the potential complexity of responses of aquaporins to water stress, since for the same aquaporin (NOD26) it was recently demonstrated that water stress *in planta* results in increased phosphorylation of NOD26 which should result *in higher* P_f through the pores (Guenther et al., 2003). Perhaps this response is compensation for the direct inhibitory effect of low water potential on the pore. A membrane fraction from wheat roots enriched in tonoplast that has high water channel activity also shows a reduction in water permeability with an increase in the osmotic gradient (Figure 2b, Niemietz and Tyerman, 1997), although in this case we cannot be certain if it is the magnitude of the gradient or the water potential of the solution that is affecting water channel activity. Plasma-membrane vesicles from wheat roots showed very low water channel

activity and showed no response to osmotic potential (Figure 2b).

Regulation of aquaporin activity may also occur by interactions between different aquaporins in the membrane (Fetter et al., 2004). The co-expression of ZmPIP1;2, which has low activity, with ZmPIP2;1, ZmPIP2;4 or ZmPIP2;5 increased the osmotic water permeability of *Xenopus* oocytes. Fetter et al., (2004) demonstrated a physical interaction between ZmPIP1; 2 and ZmPIP2s.

The redistribution of aquaporins between vesicles and the apical plasma membrane is the mechanism employed in the kidney collecting-duct epithelial cells to regulate water reabsorption in the kidney. Until recently there has been no evidence to indicate that vesicle redistribution of aquaporins is the means of regulating water/solute permeability in plants. Vera-Estrella et al. (2004) have shown that a *Mesembryanthemum crystallinum* TIP (McTIP1;2), which shows high water permeability in *Xenopus* oocytes, is re-localised to other (non-tonoplast) endosomal membrane fractions upon mannitol-induced osmotic stress. This redistribution is prevented by inhibitors of vesicle trafficking. Mannitol-induced redistribution occurred in leaf membranes but not in root membranes although both showed increased density of the TIP1;2 in the tonoplast membrane. Vera-Estrella et al. (2004) also mention (results not reported) that this TIP appears to be permeable to potassium thus adding another dimension to its likely role in osmoregulation.

Finally, a fascinating observation has recently been made on the dynamics of protoplast swelling using careful analysis and experimentation correcting for unstirred layers (Moshellion et al., 2004). A distinct delay of 2–11 s in the beginning of swelling was observed for some protoplasts that could be explained in terms of up-regulation of aquaporin activity from a very low basal membrane water permeability.

Regulation of transcription

The number of aquaporins expressed can also control water transport across membranes. Individual aquaporins can also be expressed in a wide range of tissues or may be tissue specific. It appears that the expression of aquaporins varies diurnally and in response to environmental or developmental influences. For example, the presence of the Casparian strip at the endodermis forces water to leave the apoplast and enter the cell for continued movement to the xylem vessels. Schäffner (1998) observed that the expression of PIP1 aquaporin was greater in the endodermis of the *Arabidopsis* root than in its cortex. Schäffner (1998) also observed high expression levels of PIPs in the stele which would be expected, as the stele is an area of rapid water exchange. In the Norway spruce (*Picea abies*), MIPs were more abundant in the columella cells of the root cap and the meristematic region of the root apex. More distally from the tip the expression was confined to the vascular cylinder and endodermis (Oliviusson et al., 2001). In older roots there was expression in the cells that were forming lateral root primordia. The location of expression of aquaporins cannot always be predicted from its sequence (Maurel et al., 2002). Homologues of various aquaporins appear to be expressed in varied locations depending on the plant species. Eight putative aquaporins have been obtained from a cDNA library produced from the leaves of the grapevine rootstock Richter 110 (*Vitis berlandieri* × *Vitis rupestris*) (Baiges et al., 2001). The highest expression occurred in the roots compared with that in the leaves and shoots of hydroponically grown grapevine. In addition, expression was highest in the youngest tissue.

In the case of water stress there is evidence for both up- and down-regulation of different aquaporin genes. For example, sunflower (*Helianthus annuus*) roots were exposed to air to induce water stress resulting in the mRNA levels of various TIP-like genes varying in their response, one gene increased, another decreased and others did not alter their expression relative to that in the non-stressed conditions (Sarda et al., 1999). The actual function of the aquaporins during water stress of plants is still unclear. Possibilities include down-regulation to prevent water loss to the soil; adjusting the L_p to suit the plant's growth capacity; assist in water movement to critical cells or organs; or assist in the response to a sudden rehydration (Maurel, 1997). The role of aquaporins would depend on the relative importance of apoplastic and cell-to-cell transport during conditions of stress (Schaffner, 1998, Tyerman et al., 1999). Aquaporins would

enable a rapid change in the L_p in response to re-watering compared to changes due to new root growth.

The over-expression of PIP1b (*Arabidopsis* plasma-membrane aquaporin) in transgenic tobacco plants enabled a smaller root mass to support shoot growth (Aharon et al., 2003). This was associated with a greater rate of water use and transpiration rate. Aharon et al. (2003) suggested that this implied that cell-to-cell transport was rate limiting under favourable conditions. However, under conditions of water stress, when irrigation was withheld from 3-week old plants, the transgenic plants wilted faster than the wild-type plants. This is in contrast to the results of Siefritz et al. (2002) who inhibited the expression of the homologous aquaporin NtAQP1 in anti-sense tobacco plants, and observed a reduced resistance to water stress. The water stress was imposed by different methods; Siefritz et al. (2002) irrigated with polyethylene glycol, which would cause a more immediate stress than gradual drying of soil. Drying of the soil had been used initially, but the stress (soil water potential reduced from −0.01 to −0.07 MPa) was insufficient to visibly affect the plants. Another possibility is the function of PIP1b (from *Arabidopsis*) may have been altered when expressed in tobacco. The results of Siefritz et al. (2002) that there was a reduction in root L_p of the anti-sense plants measured with a high-pressure flow meter also supports the importance of cell-to-cell water transport. PIP1b promoter was activated by the presence of ABA (Kaldenhoff et al., 1996). This activation by ABA, which is known to be produced under water stress supports the finding of Siefritz et al. (2002) that PIP1b is important for resistance to water stress.

In *Nicotiana glauca*, a plant adapted to dry conditions, the level of mRNA transcript of MIP2, MIP3 (homologous to TIPs) and MIP4 (homologous to PIPs) in leaves, guard cells, roots and stems declined (40–50% in roots, 80% in stems) when water was withheld from pot-grown plants. In contrast, the level of MIP5 (homologous to PIP) increased 4-fold in the leaves (Smart et al., 2001). In *Nicotinia excelsior* (a drought-resistant species) the levels of mRNA of three aquaporin clones (homologous to PIPs) were up-regulated in the leaves under water stress imposed by withholding water from pot-grown plants (Yamada et al., 1997). Multiple bands were identified following hybridisation for probes specific to each individual MIP indicating that each may represent a few closely related MIPs. The resurrection plant, *Craterostigma plantagineum*, which has good tolerance to water stress, was used to examine the expression of aquaporins in leaves, roots and callus during drying (Mariaux et al., 1998). The roots had a higher level of *Cp-Pipa* (PIP homologue) prior to drying than the leaves. In response to dehydration the level of mRNA increased within 2–4 h by 8-fold in leaves and 6-fold in roots. Between 24 and 72 h there was another smaller peak in expression. Mariaux et al. (1998) observed in callus tissue that some aquaporins were induced during water stress, but via an ABA-independent pathway, whereas other clones were induced by ABA and water stress. The PIP2 genes of radish (*Raphanus sativus*) seedlings appear to be down-regulated by ABA and gibberellic acid, while members of the PIP1 family were not affected (Suga et al., 2002). The down-regulation of PIP2-1 induced by polyethylene glycol and ABA was also observed at the protein level. At the mRNA level the PIP2 family members showed an initial increase and then declined after 1 h of exposure to water stress.

ZmTIP2-3, a tonoplast membrane aquaporin, is expressed in the roots only (Lopez et al., 2004). The 11-day old maize seedlings, grown in hydroponic solution had increased expression of the aquaporin following 8 h under salt (37 m*M* NaCl) and water stress (8%, v/v, polyethylene glycol). The level of the increase was not quantified from the Northern analysis. However, the expression was not altered by the addition of ABA (10 μM).

Javot et al. (2003) inserted *Agrobacterium tumefaciens* T-DNA to ultimately create two *Arabidopsis* mutants of PIP2;2, a plasma-membrane aquaporin, mostly expressed in the roots. The mutants had an L_{pcell} that was 27–28% lower than that of the wild-type cells. The osmotic water conductivity was reduced by 14% suggesting a minor contribution of the aquaporin to water movement during conditions of low transpiration. There was no significant difference in the anatomy of the roots of the mutant and wild-type plants. In contrast Martre et al. (2002)

used *Arabidopsis* plants that were double anti-sense with reduced expression of PIP1 and PIP2, and had an increase in root dry mass presumed to be in compensation for the reduction in root L_p (based on root dry mass). Therefore, the overall plant L_p (based on leaf surface area) was not significantly different from that of the control plants. Kaldenhoff et al. (1998) also observed that an increase in root mass compensated for reduced cell osmotic water permeability in *Arabidopsis* plants with a PIP1b anti-sense construct. During water stress the double anti-sense plants had a greater reduction in leaf water potential by about 0.5 MPa after 8 days, and upon rewatering a reduced recovery of whole plant conductance to about 50% of that for wild type (Martre et al., 2002).

Diurnal fluctuations in the expression of root MIPs have been observed by Yamada et al. (1997), Henzler et al. (1999), Gaspar et al. (2003) and Lopez et al. (2004). Yamada et al. (1997) examined the expression of MIPs obtained from *Nicotiana excelsior*, a plant found in rocky areas of arid central Australia. The levels of mRNA for MIP2 and MIP3 in the leaves only were highest in the morning and declined during the day. The changes correlated with the water potential of the leaves. Carvajal et al. (1996) detected a diurnal cycle in the L_p of *Triticum aestivum* roots. Henzler et al. (1999) also observed a diurnal cycle in the root L_p of *Lotus japonicus*, with the greatest value at noon and the lowest value at the end of the day. There was a 6- to 8-fold change in root L_p for both hydrostatic and osmotic induced water flow. However, there was no cycle observed in the L_p of individual cortical cells. This may be because the diurnal variation could be regulated by the membranes of the endodermal and stellar cells. In addition, the level of mRNA with homology to *AtPIP1* and *AtPIP2* also showed diurnal variation with the transcript level increasing prior to dawn and reaching a maximum 6–8 h into the photoperiod (Henzler et al., 1999). The expression of *ZmTIP2-3* in maize also began to increase just prior to the light period, and was at its greatest after 4 h of light (Lopez et al., 2004). The level of transcript declined at the end of the light period. The diurnal variation did not alter even in continuous darkness.

The expression of aquaporin genes was found to be strongly influenced by salinity, calcium deficiency and potassium deficiency (Maathuis et al., 2003). Salinity induced an initial down-regulation of all aquaporins (PIPs, TIPs, NIPs and SIPs) followed by up-regulation after 5–10 h of various aquaporin isoforms including PIP1;1, PIP2;1 and PIP2;2 (10-fold increase) that paralleled the recovery of fresh weight to dry weight ratio. Calcium deprivation also resulted in a down-regulation of most MIPs that the authors speculated could switch radial water flow across the root to the apoplastic pathway, thereby promoting calcium uptake by the root. Potassium starvation also resulted in changes in MIP transcripts, and this was suggested to be important for osmoregulation during inter- and intra-cellular redistribution of potassium and the fact that potassium is required for turgor generation. Hypoxia (0.5% oxygen) also changed transcript levels of MIPs with transient up-regulation of some TIPs in arabidopsis (Klok et al., 2002). Aquaporins were also prominent amongst those up-regulated in *Arabidopsis* during rehydration after dehydration treatment (wilting on agar plates for 2 h to 50% of initial weight) (Oono et al., 2003). Gene transcripts induced by water stress and chilling were examined by Seki et al. (2001). Although aquaporins were up-regulated by water stress, there was no clear increase in expression of the same genes with chilling. Recently the *Arabidopsis* root transcriptome was analysed using serial analysis of gene expression (Fizames et al., 2004). Differentially expressed genes were examined between roots grown with different nitrogen sources. Several root specific aquaporin genes (PIP2;2, PIP1;2. PIP2;1, TIP2;3) were up-regulated when plants were grown with ammonium nitrate relative to nitrate as the nitrogen source. Based on the Tag frequency in the NH_4NO_3 library relative to the frequency in the NO_3 library PIP2;2 was elevated 2 fold, PIP1;2 many fold (not detected in NO_3 library), PIP2;1 by 2-fold, and TIP2;3 by 4-fold.

Concluding remarks

Wetting and drying cycles that occur during irrigation and rain-fed situations have been shown

to alter the anatomy and subsequent L_p of roots of a number of species, in particular desert plants subjected to infrequent rainfall events. The association between ABA and root L_p appears quite variable, with contrasting results between species and also possibly depending on the initial water flux. Aquaporins are also likely to be involved in the changes in root L_p. Additionally, some aquaporins are induced by ABA. The actual role of aquaporins during water deficits remains unclear, in particular due to the variable response of aquaporin gene expression, with some transcript levels increasing while other genes are down-regulated. Some aquaporins may be involved in the adjustment of the plant L_p; others may assist in water movement to critical cells or organs of the plant; while other aquaporins may be involved in the plant's response to re-hydration (Maurel, 1997). The inhibitory effect of anoxia and hypoxia on aquaporins may also be relevant to situations where concentrated delivery of water occurs from the emitters of drip-irrigation systems. Waterlogging even for a short period will reduce the roots' capacity to take up water and may reduce the efficiency of water uptake by the plant. The reason why aquaporins are turned off under cytosolic acidification, as occurs during anoxia and other stresses (salinity?) has not been addressed in the literature, but one possibility may be that it is a mechanism to divert water flow from roots in unfavourable regions of the soil to those in more favourable regions. The association between increased aquaporin activity and uptake of anion macronutrients is interesting and may indicate a link between active transport and aquaporin activity. It remains to be seen if aquaporins are up-regulated in root cells when extra demand for water uptake is placed on a particular region of the root system. Shoot-to-root signals are also likely to be important in matching root L_o to overall transpirational demand (Dodd, 2005).

Acknowledgments

We thank the Australian Research Council for financial support to CMN and SDT and the CRC viticulture for support to RV. Many thanks to Wendy Sullivan for expert assistance.

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Plant and Soil (2005) 274:163–174
DOI 10.1007/s11104-004-0262-z

Mechanisms of plant resistance to metal and metalloid ions and potential biotechnological applications

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Received 25 November 2003. Accepted in revised form 4 May 2004

Abstract

Metal and metalloid resistances in plant species and genotypes/accessions are becoming increasingly better understood at the molecular and physiological level. Much of the recent focus into metal resistances has been on hyperaccumulators as these are excellent systems to study resistances due to their very abnormal metal(loid) physiology and because of their biotechnological potential. Advances into the mechanistic basis of metal(loid) resistances have been made through the investigation of metal(loid) transporters, the construction of mutants with altered metal(loid) transport and metabolism, a better understanding of the genetic basis of resistance and hyperaccumulation and investigations into the role of metal(loid) ion chelators. This review highlights these recent advances.

Introduction

Research into metal and metalloid ion resistance of plant species was started by evolutionary ecologists who were interested in the rapid speed of colonisation of newly created metalliferrous environments such as mine spoils (Bradshaw et al., 1989). The genetics of metal(loid) ion resistance were generally found to be simple, with crosses between resistant and non-resistant plants showing that resistance is normally under major gene control (Macnair, 1993). In the past, experiments to find the physiological basis of metal(loid) resistance have been somewhat inconclusive. Analytical and molecular advances over the past five years have revolutionised our understanding of the physiological and biochemical mechanisms of metal and metalloid resistances. It is these recent advances that will be the focus of this review.

Plant species or genotypes/accessions inhabiting metal-contaminated environments have considerably lower sensitivity to the toxicants present within these soils compared to their counterparts inhabiting non-contaminated environments (Macnair, 1993). The genes for metal resistance are generally at low frequency in 'uncontaminated' populations, consistent with the stochastic mutation rate (Bradshaw et al., 1989). When contaminated environments arise due to mining or smelting activities, genotypes with resistance genes establish on the sites, giving a characteristic metal(loid)-resistant flora. Some of the plant species occupying naturally metalliferrous soils, such as those formed on serpentine geology, are the same as those species typically found on anthropogenically created metalliferrous soil, while others are endemic to naturally metal-enriched soils (Kruckeberg and Kruckeberg, 1989).

The start of any physiological investigation into the phenomenon of metal(loid) resistances in plants is to establish whether the plants are more insensitive to the metals of interest or not, usually investigated by short term root elongation tests in the presence and absence of the toxicant (Macnair, 1993). From this simple phenotypic test it can be established if the plant is more resistant than the wild-type population, or less commonly

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that the wild-type and contaminated-soil population have the same degree of resistance, but with this resistance being greater than that 'normally' observed in plants growing on uncontaminated soil. For example, *Calluna vulgaris* populations from uncontaminated and metal(loid)-contaminated soils have the same degree of metal resistance for copper, but in both cases higher than in most other species (Bradley et al., 1981). Endemic metalliferrous plants, that have, by definition, no uncontaminated counterpart, are termed metallophytes (Kruckeberg and Kruckeberg, 1989). Species endemic to metalliferrous soils have reduced sensitivities to the metals present in those soils compared with species from uncontaminated environments.

There has been much interest in the possibility that one particular mechanism of detoxification may give resistance to two or more metal(loid) ions, i.e. does evolution in soils high in copper (II) give rise to zinc (II) resistance? This phenomenon is called co-tolerance (co-resistance) (Macnair, 1993). Generally, when co-resistance has been tested, resistance mechanisms appear to be highly specific to a particular metal(loid) ion.

The search for the mechanistic basis of metal(loid) resistance usually starts by analysing the plants' ability to assimilate the metal(oid) ions of interest. Two clear strategies have been documented. Normally, eco-types adapted to metalliferrous environments take up less of the toxicant than their counterpart non-resistant ecotypes. This is called exclusion or avoidance, where the plant reduces its exposure to the metal(loid) ions. The other main strategy is hyperaccumulation (Ernst, 2000), but this is much less common than exclusion. The massive translocation of metal(loid)s to plant shoots in hyperaccumulating plants is of considerable interest to biotechnologists. Hyperaccumulators may be used to remediate metal(loid)-contaminated soils (Lasat, 2002).

Hyperaccumulation

Hyperaccumulation is a sub-class of metal resistance, as discussed above, where high tissue concentrations of metal(loid)s are found in above-ground tissues (Baker and Whiting, 2002). Hyperaccumulation has been reported for lead, nickel, cobalt, copper, zinc, cadmium, selenium and arsenic amongst others. Hyperaccumulation strategies are less common than exclusion strategies, and generally, hyperaccumulator plants are restricted to metalliferrous soils (Kruckeberg and Kruckeberg, 1989), with the notable exception of plants with arsenic hyperaccumulation (Meharg, 2002).

The definition of what constitutes a hyperaccumulator is difficult because the shoot concentration of a particular element that is classed as hyperaccumulation differs between elements (Baker and Whiting, 2002). Metal levels in shoot tissues should be considerably higher than metal levels in the soil. In defining hyperaccumulation, it must be made clear that the hyperaccumulation strategy should be evaluated under controlled laboratory conditions. There are many reports of hyperaccumulators in the literature that are incorrect, as the studies have just conducted geochemical surveys of plants on metalliferrous soils, disregarding the fact that soil dust contamination of the shoots will greatly confound results (Meharg, 2003). In any case, a working definition of hyperaccumulation should be that shoot levels need to exceed soil levels.

The fundamental reasons of why, and how, plants hyperaccumulate metal(loid)s is poorly understood. The most commonly postulated hypothesis regarding the reason why plants hyperaccumulate is for plant protection, to deter herbivores by making leaves unpalatable or toxic, or by reducing pathogenic infection. A number of ecological studies show contrasting results, some showing deterrence of insect herbivory and fungal pathogenesis due to selenium accumulation in *Brassica juncea* (Hanson et al., 2003), while others find no effects such as zinc not deterring insect herbivores (Huitson and Macnair, 2003). Other hypotheses concerning why plants hyperaccumulate metal(loid)s are thin on the ground.

Many questions concerning hyperaccumulation of metal(loid)s by plants still need to be resolved. How do plant roots mobilise high concentrations of what are generally relatively immobile ions from soils? Exudation of organic acids to complex ions or to free them from counter ions in soil, or the involvement of rhizosphere micro-organisms in mobilisation (Lasat, 2002) and enhanced plasmalemma-transport capabilities (Lasat et al., 1996) have all been investigated. Experiments with the cadmium hyperaccumulator *Thlaspi caerulescens* show that it is no more efficient at mobilising non-labile or poorly labile cadmium

from soils than the non-accumulator *Lepidium heterophullum* (Hutchinson et al., 2000). The results of this study also suggest that there are limits to how effectively hyperaccumulators can clean up contaminated soils, as a fraction of the metals of concern will remain unavailable, at least in the short-term to medium-term, to plants. In a very elegant study Whiting et al. (2000) investigated the effect of a localised supply of zinc and cadmium added to soil. They found that roots of *T. caerulescens* actively concentrated root growth in the metal-enriched zones, suggesting that the plants can sense the metals, and that high metal concentrations in the soil are a trigger to proliferate roots.

An ever-increasing number of ferns, normally from the *Pteris* genus, have been found to hyperaccumulate arsenic (Meharg, 2003). These fern species are not endemic to arsenic-enriched soils, and grow vigorously. They are also, of necessity, arsenic tolerant. Other hyperaccumulators such as the zinc and cadmium hyperaccumulator *T. caerulescens* grow on metal-contaminated sites and non-contaminated sites, although they have a limited ecological and geographical range (Assuncao et al., 2003; Dubois et al., 2003; Frerot et al., 2003). Interestingly, both Assuncao et al. (2003) and Frerot et al. (2003) found that populations from non-metal-enriched populations hyperaccumulated more zinc, but had lower resistance than populations from metal-enriched soils. Similar results were found for zinc resistance in the hyper-accumulator *Arabidopsis halleri* (Bert et al., 2000). These studies show that resistance and hyperaccumulation may be independent characters, as also suggested earlier by Macnair et al. (1999) investigating crosses between hyperaccumulating *A. halleri* and non-hyperaccumulating *A. petraea*. Macnair et al. (1999) concluded that resistance to zinc was controlled by a single gene, while hyperaccumulation of zinc was under the regulation of a small number of genes. Populations from non-metal-enriched sites will be exposed to much lower levels of zinc and cadmium, and do not need to express the same degree of resistance to these metals as populations from enriched soils. If hyperaccumulation has an ecological role in *T. caerulescens*, populations from non-enriched soils would have to be more efficient hyperaccumulators compared with populations from metal-enriched environments to obtain high shoot metal(loid) concentrations. Furthermore, genetic studies into *A. halleri* crosses with the tolerant and non-hyperaccumulating related species *A. lyrata* found that cadmium resistance and hyperaccumulation were independent characters, and that cadmium and zinc may show co-resistance and hyperaccumulation (Bert et al., 2003), i.e. the plant treats cadmium and zinc the same with respect to the mechanism(s) of resistance and mechanism(s) of hyperaccumulation. Such co-resistances are postulated to occur because the cadmium and zinc ions are thought to be chemically similar with respect to metal transporters or enzymes. Even though cadmium has a larger ionic radius (as it has a much higher mass), both cadmium and zinc have a charge of $2+$. As metal transporters are charge dependent, a transition metal with similar charge may enter the cell through the same mechanisms as an analogous ion. There has been much interest in such co-tolerances as plants on contaminated sites often are resistant to more than one metal ion. However, in most cases the actual mechanism of tolerance are independent, and co-resistances are rarer than thought (Macnair, 1993).

Macnair et al. (1999) postulated that metal(loid) resistance must have evolved in plants before hyperaccumulation evolved, and, therefore, the genetic basis of hyperaccumulation must involve more than one gene.

Resistance mechanisms

Essential trace micronutrients, such as copper, nickel and zinc, are toxic to plants once exposure concentrations reach certain thresholds. Transition metal ions, at high enough concentrations, will cause a wide range of cellular processes to be disrupted, as these compounds will interact with many biomolecules. Many transition metal ions are also redox-active under cellular conditions, giving rise to oxidative damage. Thus, it is essential for the plant to tightly regulate the cytoplasmic concentrations of essential micronutrients (Meharg, 1994). Homeostasis is achieved through regulation of ion uptake, potentially through ion efflux, detoxification through complexation or transformation, intracellular compartmentalisation such as vacuolar storage, and intercellular transport to cells that have limited metabolic

function. To achieve metal resistance it is generally thought that some of these processes must be adapted in resistant ecotypes compared to sensitive ones.

For non-essential elements, including transition metals such as cadmium and silver and the metalloids arsenic, antimony, lead, tin and mercury, maintaining low cytoplasmic concentrations is also essential. These elements enter cells as analogues of essential ions, or through passive diffusion (Meharg, 1993). If a particular ion is not taken up into cells in any appreciable quantity, it may still exert toxicity through disrupting external membrane function (Meharg 1993). Resistance to non-essential elements, as for essential elements, must involve adaptation to normal physiological function (Clemens, 2001).

From knowledge of a metal(loid) chemistry and how these compounds are likely to interact with constituents of cells, certain physiological processes have been targeted for investigation to identify where resistance adaptations occur. Research into the mechanisms of metal(loid) resistance have focussed on six main areas (a) uptake kinetics, (b) metabolism, (c) complexation, (d) redox stress, (e) sub-cellular localisation and (f) intra-cellular localisation.

Uptake kinetics

Surprisingly, most plants do not alter uptake kinetics to achieve metal resistance, although this would seem a simple way to achieve resistance as it automatically reduces exposure of many cells within the plant to the toxicant. There is only one element, arsenic, to date, for which alteration of uptake kinetics is widely deployed as a resistance mechanism (Meharg and Hartley-Whitaker, 2002). Arsenate is a phosphate analogue, and is taken up into plant cells via both high-and low-affinity phosphate co-transport systems (Meharg and Hartley-Whitaker, 2002). In resistant plants the high-affinity uptake system is suppressed, greatly reducing arsenate uptake. This suppression has been found in a range of species that exhibit arsenic resistance, such as *Holcus lanatus, Agrostis cappillaris, Deschampsia cespitosa* and *Silene vulgaris* (Meharg and Hartley-Whitaker, 2002). The mechanism of adaptation (suppressed arsenate/phosphate uptake), in the most-studied arsenate-resistant species *H. lanatus*, co-segregates with resistance (as determined by experiments measuring root elongation) in the progeny of crosses between a resistant and a sensitive genotype, showing that the resistance was conferred by suppression of arsenate/phosphate uptake (Meharg and Macnair, 1992). The F2 segregated 3:1 resistant:sensitive, showing that resistance is under the control of a single dominant gene.

Although arsenate uptake is considerably less in resistant genotypes compared with sensitive genotypes, resistant plants can still take up considerable quantities of arsenic over their life-cycle. Thus, the adaptive mechanism of resistance does not work in isolation; it still requires the plant to internally detoxify arsenate through metabolism and complexation. Metabolism and complexation of arsenate, for example to arsenite or arsenite-phytochelatin (PC) complexes, is constitutive to both arsenate-resistant and -sensitive phenotypes of *H. lanatus* (Hartley-Whitaker et al., 2001). It is essential in investigating mechanisms of plant metal(loid) resistance to distinguish between adaptive and constitutive mechanisms of resistance (Meharg, 1994), as will be discussed later.

Reduction in uptake has been screened as a mechanism of resistance for other metal(loid)s (Meharg, 1993), although nothing as clear-cut as for arsenate resistance has been observed. Unlike arsenate which is an analogue of the macronutrient phosphate, most other trace metal ions of interest are inherently taken into cells at slow rates, and given that their transporters are under feed-back regulation, the subtle differences in influx between resistant and sensitive plants, if they exist, will be difficult to detect.

With the advance of molecular techniques, and the identification of transition metal transporters, the role of such transporters in resistance can now be investigated. Evidence suggests that these transporters may have a role in metal ion resistance adaptation, particularly for hyperaccumulators.

A cation-diffusion facilitator (CDF) that transports zinc, ZATlp, has been identified in *A. thaliana* (Van der Zaal, 1999) and heterologously expressed in *Escherichia coli* (Bloss et al., 2003). Zinc transporters have also been cloned and isolated from the zinc hyperaccumulator *T. caerulescens* (Assuncao et al., 2001, Pence et al, 2000). Three genes, ZTP1 (which have high homology with the *A. thaliana* ZAT gene), ZNT1

and ZNT2 all show enhanced expression in *T. caerulescens* compared with a related non-accumulator *T. arvense*. ZNT1 and ZNT2 are expressed under zinc deficiency in *T. arvense*, but are non-responsive to zinc in *T. caerulescens*, showing these transporters are constitutively expressed in the hyperaccumulator. *T. caerulescens* shows differential accumulation and resistance to zinc dependent on the origin of the population. ZNT1 and ZNT2 are mainly expressed in roots, and ZTP1 in the shoots, suggesting that the transporters have different roles for zinc acquisition and transport in the plant. Populations *T. caerulescens* from zinc and cadmium rich calamine soils are exposed to higher zinc levels than populations from serpentine soils and are more resistant to zinc. Calamine populations have higher expression of the shoot-expressed transporter ZTP1 which may have a role in increased resistance in this ecotype.

Influx experiments using cadmium show that different populations of *T. caerulescens* have greatly differing root uptake kinetics, but zinc transport was similar (Lombi et al., 2001). This suggests variation in transport capacities within hyperaccumulator populations, and that, although cadmium and zinc are both hyperaccumulated by *T. caerulescens*, there are, at least, some differences in the mechanisms by which the plant processes cadmium and zinc.

With the identification of metal transporters, the roles of these transporters in metal resistance can be identified. For example Cobbett et al. (2003) reviews 8 potential copper- and zinc-transporting ATPases in *A. thaliana*, yet only one protein RAN1, so far, has function been identified.

Metabolism

Once a metal(loid) ion enters into the cell's cytosol, its speciation may remain unaltered within the cell, it may have its speciation altered simply by the change in pH between the cytosol and soil solution, it may be metabolised enzymatically, it may have its redox state changed chemically, or it may be complexed by chelators or by cell macro-molecules (Figure 1). How a metal(loid) ion speciation is altered within a cell depends, of course, on the chemistry on the ion itself. For example, Zn^{2+} is redox stable whereas Cu^{2+} is readily reduced to Cu^{+} under cellular conditions.

Ions found in aerobic soil solution which are more redox active than zinc may be reduced in the cytoplasm, either chemically, through the action of anti-oxidants such as glutathione and ascorbic acid; or enzymatically, for example by arsenate reductases, as yet only identified in micro-organisms (Dhanker et al., 2000; Meharg and Hartley-Whitaker, 2002). The ions Cu^{2+}, Fe^{3+}, and AsO_4^{3-} are readily reduced to Cu^{+}, Fe^{2+}, and AsO_3^{3-}, respectively, in the cytoplasm, with redox cycling between the reduced and oxidised forms causing considerable cellular stress (Hartley-Whitaker et al., 2001). Note that glutathione and ascorbic acid can act as the terminal electron donors in enzymatically mediated reactions. This stress is through the action of reactive oxygen species (free radicals), which cause a host of cellular damage, including the well studied damage to membrane lipids (Hartley-Whitaker and Meharg, 2002). Mechanisms for protecting the plant from oxidative stress such as superoxide dismutases have been investigated with respect to metal(loid) resistance, and although essential for helping to protect cells from redox-active metal(loid) ions, such mechanisms are found to be constitutive in both resistant and sensitive plants (Hartley-Whitaker and Meharg, 2002; Hartley-Whitaker et al., 2001).

For metalloids, conversion of inorganic species into organic species is also feasible. Pickering et al. (2003) reported the synthesis of Se-methylseleno-cysteine at high concentrations in young leaves of the selenium hyperaccumulator *Astragalus bisulcatus*. In older leaves, inorganic selenate tends to dominate, even though the enzyme to produce seleno-cysteine is present. This switch in speciation may reflect the importance of younger leaves in whole plant functioning compared with that of older leaves.

Although organic arsenicals have been identified in plant tissues, it is not clear whether these species arise in tissues by uptake from soil, where they are synthesised by the soil micro-flora, or whether these compounds are produced *in planta*. Organic arsenicals are less phytotoxic than inorganic species (Meharg and Hartley-Whitaker, 2002). Metabolism of arsenic, certainly in marine plants, is a mechanism of detoxification.

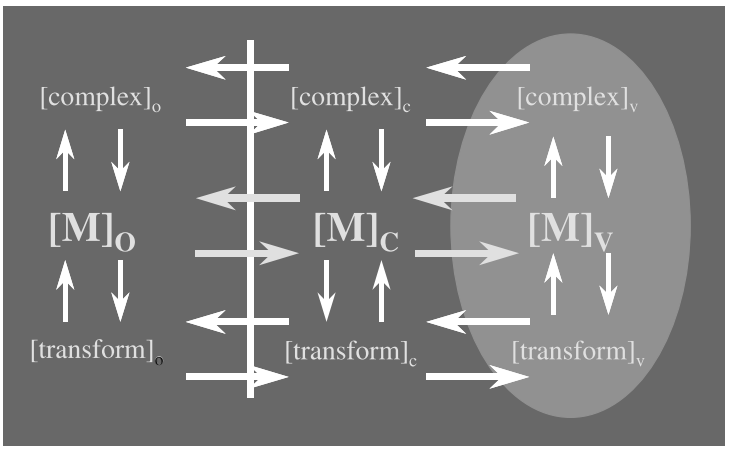

Figure 1. The fate of a metal(loid) ion (M) in the soil (O) - cytoplasm (C) - vacuole (V) continuum. Transformations may occur chemically or by metabolism. Soil micro-organisms, plant released extracellular enzymes, plant released complexing agents and nutritionally driven changes in rhizosphere pH will effect speciation in the rhizosphere soil solution. Once inside the plant both chemical and enzymatic reactions will regulate speciation.

Complexation

The role of organic chelating agents has been widely investigated in metal(loid) detoxification in plants. Certain metal ions such as Zn^{2+}, Mn^{2+}, Fe^{3+} and $A1^{3+}$, have a high affinity for oxygen ligands, and it is thought that dicarboxylic acids in the cytosol have a role in chelating these ions (Sarrat et al., 2002). Similarly, the oxy-ions AsO_3^{3-} and $SeO_2^{2-}$and the cations Cu^{2+}, Cd^{2+} and Hg^{2+}, all have high affinities for sulfur (Cobbett, 2000). On exposure of plant roots to these ions, synthesis of cysteine rich phytochelatins (PCs) is induced, and it is thought that PCs have a vital role in complexing these ions in plant tissues (Cobbett, 2000).

There is evidence that metals with affinity for oxygen are coordinated with dicarboxylic acids within plant cells. X-ray absorption studies on the zinc hyperaccumulator A. *halleri* showed that zinc in aerial parts was predominantly octahedrally coordinated with malate (Sarret et al., 2000). A similar study using *T. caerulescens* showed again that zinc was predominantly bound by carboxylic acids in above-ground tissues (Salt et al., 1999). However, zinc was predominantly complexed with histidine in the roots, and was present as free Zn^{2+} in the xylem sap. It is becoming apparent that, as ions move through plants, their speciation can change dramatically; specifically regulated by the cellular compartment they are in.

There has been much investigation into the role of PCs in metal(loid) resistance. The role of PCs has been found to be constitutive rather than inducible (Ebbs et al., 2002; Schat et al., 2002). PCs are equally important for cytoplasmic homeostasis of metal ions in resistant and sensitive species. This was illustrated for both cadmium and arsenic by addition of the inhibiter buthionine sulfoximine (BSO) to the bathing solutions of plant roots; differences in resistance between the contrasting phenotypes were eliminated (Hartley-Whitaker et al, 2002; Schat et al, 2002). So, although PCs are not involved in inducible resistances, without them all plants would be even more sensitive to metal(loids). Inducible and constitutive mechanisms involved in metal(loid) ion biochemistry must be considered together (Meharg, 1994).

Experiments with *Brassica juncea* found that xylem sap from Indian mustard contained only inorganic arsenic, with no evidence of PC complexation, in contrast to leaves and root where the arsenic was present as a sulfur-co-ordinated complex (Pickering et al, 2000).

Direct measurement of actual PC-metal(loid) complexes in plant cells is limited, and little is known about the stoichiometry of complexation. X-ray absorption spectroscopy (XAS) evidence

shows that arsenic is complexed with sulfur in Indian mustard, presumably as PCs (Pickering et al., 2000), while a range of crude chromatography experiments suggest that PCs are associated with copper, mercury, cadmium and arsenic (Raab et al., 2003). A recent chromatographic study by Raab et al. (2003) using HPLC interfaced with parallel elemental (ICP-MS) and organic carbon (ESI-MS) detectors has shown, for the first time, unequivocal complexation of a metal(loid) with PCs. They showed the stoichiometry of As-PC interaction in arsenate-resistant ferns and grasses. Importantly this study showed that only a fraction of cellular arsenic is PC complexed, 1% in ferns and 13% in the grass *H. lanatus*. Furthermore, they discovered for the first time mixed PC-glutathione complexes, where $As.PC_2.GS$ is much more stable than $AsPC_2$ or $As(PC_2)_2$.

Unlike Pickering et al.'s (2000) study, most of the arsenic in *H. lanatus* and the fern *Pteris cretica* is in non-complexed inorganic forms (Rabb et al., 2003), As free inorganic forms are highly reactive with biomolecules, these are likely sub-compartmentalised within cells, probably in the vacuole.

Metalothioneins (MTs), sulfur-rich proteins, may also have a role in plant defence against ions with a strong affinity for sulfur, as MTs have been isolated from both metal-resistant and non-resistant plants (Ma et al, 2003; Van Hoof et al., 2001). However, the role of MTs in this regard is better understood in animals, where they are considered PC analogues. A potential role of MTs in conferring enhanced resistance to plants that have, an as yet unidentified inducible resistance mechanism to copper, has been identified by Van Hoof et al. (2001). They found that the MT-like gene SvMT2b from *Silene vulgaris* had significantly enhanced transcript levels in copper-resistant populations compared with sensitive populations that segregated with a 3:1 ratio in crosses. The expression of SvMT2b did not co-segregate with resistance, and therefore is not the inducible gene for resistance. Thus SvMT2b is presumably modifying resistance in a resistant population. A MT gene has also been characterised in a lead-zinc-cadmium-copper-resistant genotype of the grass *Festuca rubra* (Ma et al., 2003). On challenge with Cu^{2+}, expression of the gene was enhanced.

In the nickel hyperaccumulators of the *Alyssum* genus and *Thlaspi goesingense*, evidence suggests that nickel is complexed with histidine (Kramer et al., 1996, 1997, 2000). There is not enough histidine produced in these species to complex all the nickel in the tissues. This suggests that the role of histidine in this species is transient, such as trafficking through the cytosol or longer-distance translocation within the plant. Nicotianamine has recently been shown to chelate nickel in *T. goesingense* (Vacchina et al., 2003).

The studies on arsenic, nickel and zinc suggest that metal(loid)-chelating agents have specific roles in the plant's processing of metals, rather than just complexing all the metal present. Perhaps the metals are complexed in the cytosol or moved around the plant as complexes, until they are deposited in compartments such as the vacuole. That is, complexes are inactivating toxic species for the purposes of transport within the cell or in the plant.

Advances in analytical technologies now mean that it is possible to observe metal complexes in plant extracts using combined inductively coupled plasma (ICP)-MS and electrospray ionisation (ESI)-MS interfaced with HPLC (Raab et al., 2004; Vacchina et al., 2003). Raab et al. (2004) were able to identify intact As-PC complexes in extracts of *H. lanatus* and *P. cretica*, identifying, for the first time, mixed arsenie-glutathione-PC complexes. Vacchina et al. (2003) identified a new class of potential metal-complexing agents in plants, discovering that nickel was complexed with nicotinamine in the hyperaccumulator *T. carulescens*. With wider deployment of such analytical tools it is likely that a new understanding in complexation of metals *in planta* will be unravelled.

Redox stress

As discussed above, ions that undergo redox changes in cellular environments cause oxidative damage to cells, primarily through the action of free radicals. Up-regulation of enzymes that counter-act oxidative stress such as SOD is a potential mechanism of tolerance. However, there is little evidence for any role of such enzymes in inducible tolerances; their role is constitutive (Hartley-Whitaker et al., 2001). Again, it is likely that without constitutive enzymes that counteract

oxidative stress, adaptive mechanisms would not function.

The response of enzymatic systems to metal(loid) ions may expose the plant to enhanced oxidative stress damage. Glutathione is the precursor of PCs, which are induced on exposure to a wide range of metal(loid) ions. Glutathione is itself thought to be in the front line of plant defences to counteract oxidative stress as it is a reducing agent. A number of studies suggest that glutathione depletion during PC production may expose plant cells to enhanced oxidative stress, such as in *A. thaliana* modified to over- express PCs where this over-expression leads, counter-intuitively, to enhanced cadmium sensitivity due to glutathione depletion (Lee et al., 2003). Interestingly, A. *thaliana* and *Nicotiana tabacum* modified with an *E. coli* arsenate reductase gene, arsC, had enhanced cadmium resistance (Dhanker et al., 2003). The authors proposed three non-exclusive potential mechanisms to explain their results: (a) that the reductase reduces Cd^{2+} to elemental cadmium, which is relatively inert, (b) that the ArsC enzyme sequesters Cd^{2+}through binding to the cysteine rich region of this enzyme (which the authors think unlikely) and (c) that cellular toxicity to metals is normally associated with glutathione depletion, and therefore enhanced glutathione production will counteract toxicity. The authors suggest that as glutathione is required for the function of ArsC, glutathione depletion will induce PC synthesis, and therefore, enhanced PC-Cd complexation. This seems unlikely as simple exposure to Cd induces PC synthesis. To effectively counteract toxic metal(loid) ions, biochemical processes must interact in such a way that a particular response to the imposed stress does not lead to knock-on effects elsewhere.

Sub-cellular localisation

Sub-cellular localisation in the vacuole may hold the key to understanding metal tolerances in general (Persans et al., 2001). However, it is difficult to actually prove sub-cellular location, and the tonoplast processes involved in maintaining high vacuolar metal(loid) concentrations are not well understood.

Evidence for vacuolar location of metal(loid) ions comes from x-ray emmission studies (Kupper et al., 2000), radioactive tracer studies (Lasat et al., 1998), and recent molecular investigations (Persans et al., 2001). Persans et al. (2001) have identified a putative tonoplast metal-ion-transport protein in the nickel hyperaccumulator *Thlaspi goesingense*. These metal-transport proteins, TgMTPs, have been heterologously expressed in yeast *Schizosaccharomyces pombe* were they confer enhanced resistance, TgMTPltl for cadmium, cobalt and zinc, and TgMTPlt2 for nickel (Persans et al., 2001). TgMTPs are highly expressed in *T. goesingense* compared with the nonhyperaccumulators *A. thaliana, Thlaspi arvense* and *Brassica juncea*. The overexpression of ZAT1 in *A. thaliana* resulted in zinc tolerance (van der Zaal et al, 1999). As ZAT and MTP are orthologous or strongly homologous, this adds evidence for a putative role of this transporter in zinc tolerance. Cellular localisation of a range of metals determined using X-ray techniques suggest differential cellular localisation of elements, providing evidence that some toxic ions may be stored in the cytoplasm (Kupper et al., 2000). Most of these studies have concentrated on hyperaccumulators where there are very high levels of the elements of interest in the plant tissues. For resistant non-hyperaccumulators the story is not as clear.

Some studies suggest the existence of metal ion – complex transporting cassettes in the tonoplast of some species, indicating that metal ions such as cadmium may be translocated into the vacuole as complexed PCs, organic acids or metallo-thioneins (Cobbett, 2000).

The most-illuminating study conducted to date on the role of tonoplast metal transporters with respect to metal resistance was Chardonnens et al.'s (1999) study *on Silene vulgaris*. They where able to show that isolated vacuolar vesicles of zinc-resistant *S. vulgaris* accumulated more zinc than a sensitive ecotype. These differences in uptake across tonoplast vesicles segregated with resistance in F3 homozygous resistant and sensitive plants, providing strong evidence that vacuolar localisation of zinc is the level at which the mechanism of zinc resistance is operating. They also found that free zinc was translocated across the tonoplast, rather than dicarboxylic-acid-complexed zinc.

Inter-cellular localisation

Different cells may have different metal-storage capabilities, based on the degree of vacuolisation,

and the other processes that the cell must maintain. Kupper et al. (2000) have shown that zinc and cadmium were mainly stored in shoot mesophyll cells, as opposed to epidermal cells in the hyperaecumulator *Arabidopsis halleri*.

Phytoremediation

There is considerable interest using hyperaccumulators to phytoremediate metal-contaminated sites (McGrath and Zhao, 2003). Hyperaccumulators mobilise metals from soils and then transfer them to above-ground biomass, where the metals can be easily harvested, and fed into metal-waste-recycling streams. However, most hyperaccumulators are small individuals, and are slow-growing, because they come from metalliferrous soils that are low in nutrients and where they have little competition (Lambers and Poorter, 1992). The exception to hyperaccumulators having small biomasses and natural restriction to metalliferrous soils are arsenic hyperaccumulating ferns (Ma et al., 2001; Meharg, 2002).

If the genes involved in hyperaccumulation can be isolated and characterised, transferring these genes into other plants may be the way to create the 'ideal' phytoremediator. Alternatively, it may be possible to construct plants that hyperaccumulate by transforming them to express proteins involved in metal sequestration and complexation (Dhankher et al., 2002; Kramer and Chardonnens, 2001). Also, by constructing or generating mutants with characterised genetic modifications with respect to the wild-type, the role of enzymes encoded by these genes in metal resistance can be investigated.

Thomas et al. (2003) have introduced the yeast metallo-thioneins gene CUP1 into tobacco *(Nicotiana tabacum)* plants. The transformed tobacco accumulated 2 to 3 times as much copper as the wild-type when grown on contaminated soils. Dhanker et al. (2002) constructed A. *thaliana* that contained the *E. coli* arsC genes that encode arsenate reductase and g-glutamyl cysteine synthase (γ-ECS), the later is the enzyme that synthesises PCs. These two genes were placed in A. *thaliana* on strong constitutive promoters. Transformed plants had much greater arsenate resistance than the wild-type, and accumulated 4 to 17 times more arsenic on a fresh mass basis than the wild-type.

B. juncea was genetically modified to overexpress an *E. coli* gene gsh1, which codes for γ-ECS (Zhu et al., 1999). The modified plant accumulated up to 90% more cadmium than wild-type and was more cadmium resistant than the wild-type.

A. thaliana has been transformed to contain bacterial mercury resistance genes merA and merB. merB is an organic-mercurial lyase, which breaks down methyl-mercury bonds; merA is a mercuric reductase (Bizily et al., 2000). Transformed A. *thaliana* could withstand 50-fold higher mercury concentrations compared with the wild-type.

Screening of mutants in model plant species such as A. *thaliana* has also proved a fruitful means of investigating metal(loid) resistances (Navarro et al. 1999). Cadmium-resistant A. *thaliana* mutants have been identified which restrict cadmium accumulation (Navarro et al., 1999). Similar screening has identified lead-accumulating *B. juncea* mutants (Schulman et al., 1999).

Arsenate resistance in A. *thaliana* is enhanced in a mutant that has enhanced phosphate acquisition (Lee et al., 2003). As phosphate and arsenate are analogues, the toxicity of arsenate is intimately linked with plant phosphate nutrition (Meharg and Hartley-Whitaker, 2003). Meharg et al. (1994) were able to show that sensitive *H. lanatus* can be made arsenate resistant by pre-exposing it to high levels of phosphorus. Similarly, resistant *H. lanatus* could be made more resistant by phosphorus pre-exposure. The effect of phosphorus nutrition on arsenate is twofold. Firstly, high plant phosphorus status leads to a down-regulation of the arsenate/phosphate plasma-lemma transporters. Secondly, high cellular phosphate levels will result in greater competition with arsenate for biochemical processes where arsenate substitutes for phosphate.

There appears to be potential for developing phytoremediating plants through genetic constructs. How well these constructs will perform against plants selected over the epochs to grow on contaminated soils is another matter. Hyperaccumulators hold the key to the ideal phytoremediator.

Conclusions

Molecular advances and increasingly sophisticated analytical technologies are gaining considerable new insights into metal resistance in plants. The roles of metal and metalloid ion transporters in enhanced uptake and vacuolar localisation reveal that these processes are key to understanding resistances. Similarly, speciation using x-ray spectroscopy and HPLC with combined ICP-MS and ESI-MS detection reveals true speciation in plant tissues. Following the expression of MT and PC genes and determining the localisation of their expression enables the role of these chelators to be defined. The construction of mutants to over-express proteins, or to have transgenic proteins introduced, leads to a more detailed understanding of the role of these proteins in 'normal' and resistant plant function, and ultimately may lead the way to developing plants that improve phytoremediation of contaminated sites.

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Plant and Soil (2005) 274:175–195
DOI 10.1007/s11104-004-1158-7

The physiology, genetics and molecular biology of plant aluminum resistance and toxicity

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Received 7 January 2004. Accepted in revised form 5 February 2004

Key words: aluminum resistance, aluminum toxicity, anion channel, organic acid exudation

Abstract

Aluminum (Al) toxicity is the primary factor limiting crop production on acidic soils (pH values of 5 or below), and because 50% of the world's potentially arable lands are acidic, Al toxicity is a very important limitation to worldwide crop production. This review examines our current understanding of mechanisms of Al toxicity, as well as the physiological, genetic and molecular basis for Al resistance. Al resistance can be achieved by mechanisms that facilitate Al exclusion from the root apex (Al exclusion) and/or by mechanisms that confer the ability of plants to tolerate Al in the plant symplasm (Al tolerance). Compelling evidence has been presented in the literature for a resistance mechanism based on exclusion of Al due to Al-activated carboxylate release from the growing root tip. More recently, researchers have provided support for an additional Al-resistance mechanism involving internal detoxification of Al with carboxylate ligands (deprotonated organic acids) and the sequestration of the Al-carboxylate complexes in the vacuole. This is a field that is entering a phase of new discovery, as researchers are on the verge of identifying some of the genes that contribute to Al resistance in plants. The identification and characterization of Al resistance genes will not only greatly advance our understanding of Al-resistance mechanisms, but more importantly, will be the source of new molecular resources that researchers will use to develop improved crops better suited for cultivation on acid soils.

Introduction

Aluminum (Al) toxicity is the primary factor limiting crop production on strongly acidic soils. At soil pH values at or below 5, toxic forms of Al are solubilized into the soil solution, inhibiting root growth and function, and thus reducing crop yields. It has been estimated that over 50% of the world's potentially arable lands are acidic (von Uexküll and Mutert, 1995; Bot et.al., 2000); hence, Al toxicity is a very important worldwide limitation to crop production. Furthermore, up to 60% of the acid soils in the world occur in developing countries, where food production is critical. Breeding crops with increased Al resistance has been a successful and active area of research; however, the underlying molecular, genetic and physiological bases are still not well understood. Because of the agronomic importance of this problem, this is an area that has attracted significant interest from a number of molecular biology and physiology laboratories around the world. Despite the interest from many researchers, no Al resistance genes have yet been cloned from any plant. However, recent progress by a number of researchers has set the stage for the identification and characterization both of the genes and associated physiological mechanisms that contribute to Al resistance in important crop species grown on acid soils. This

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should, in turn, provide the necessary molecular tools to address a worldwide agronomic problem that is only exceeded by drought stress with regards to abiotic limitations to crop production (von Uexküll and Mutert, 1995).

Physiological mechanisms of aluminum toxicity

Aluminum in soils is present as insoluble alumino-silicates and oxides. As the soil pH drops below 5, the octahedral hexahydrate $Al(H_2O)_6^{3+}$, more commonly referred to as Al^{3+}, is solubilized into the soil solution. This form of Al appears to be the most important rhizotoxic Al species (Kinraide, 1991; Kin-raide and Parker, 1989, 1990). Al interferes with a wide range of physical and cellular processes. Potentially, Al toxicity could result from complex Al interactions with apoplastic (cell wall), plasma membrane, and symplastic (cytosol) targets. Given the vast literature and the diverse experimental approaches employed in studying Al toxicity, it is difficult to reach a consensus on the timing for Al toxicity as well as the cellular processes targeted by Al. For instance, while some Al-toxic symptoms and responses are detectable within seconds to minutes after exposure to Al, others are only discernible after long-term (hours to days) exposure. The direct association of long-term responses with mechanisms of Al toxicity should be interpreted cautiously as these may not be the result of a direct disturbance of a given pathway by Al, but could rather be the result of a general homeostasis disturbance on an unrelated physiological pathway triggered by an earlier Al-toxicity event.

Inhibition of root growth: The earliest Al-toxicity response.

Given the above, it is not surprising that a significant part of the research on Al toxicity has focused on the most rapid effects of Al on plant function. Root growth inhibition upon exposure to Al has been used extensively as a measurement of Al toxicity (Foy, 1988), as the primary and earliest symptom of Al toxicity is a rapid (beginning within minutes) inhibition of root growth (Kollmeier et al., 2000; Ryan et al., 1993; Sivaguru and Horst 1998; Sivaguru et al., 1999). Detailed spatial studies have indicated that within the root, the root apex, and more specifically the distal part of the transition zone within the apex, is the primary target of Al toxicity (Sivaguru and Horst, 1998). Within this root zone, recent studies have indicated that some Al can enter the cytosol of cells within minutes following Al exposure (Silva et al., 2000; Taylor et al., 2000; Vazquez et al., 1999). Consequently, although a large fraction of the Al interacts with apoplastic targets, a small fraction enters the symplasm and interacts with symplastic targets. The promptness of the root growth inhibition upon exposure to Al indicates that Al quickly disrupts root cell expansion and elongation, prior to inhibiting cell division (Frantzios et al., 2001; Wallace and Anderson, 1984). Prolonged exposures lead to Al interactions with the root cell nuclei, resulting in disruption of cell division and the cytoskeleton (Silva et al., 2000).

Al toxicity is associated with gross changes in root morphology (Ciamporova, 2002). Briefly, Al toxicity results in inhibited root elongation, which yields swollen root apices and poor or no root-hair development. This extensive root damage results in a reduced and damaged root system and limited water and mineral nutrient uptake (see, for example, Barcelo and Poschenrieder, 2002; Jones and Kochian, 1995). The degree of toxicity reported in the literature varies widely depending on the plant species, growth conditions, Al concentrations, and the duration of the exposure. Thus, given the complexity of the many cellular processes involved in root growth inhibition, the precise Al toxicity targets in this complex chain of events remain elusive.

Researchers have begun to dissect out and identify diverse Al targets in different pathways associated with root growth. Because Al is so reactive, there are many potential sites for injury, including: (A) the cell wall, (B) the plasma membrane, (C) signal-transduction pathways, (D) the root cytoskeleton, and (E) DNA/nuclei.

(A) The Cell Wall. X-ray microanalysis and secondary ion mass spectroanalysis have indicated that a significant fraction of Al in roots is associated with apoplastic binding sites, predominantly in walls of cells of the root periphery (Vazquez et al., 1999). The net negative charge of the cell wall determines its cation exchange

capacity (CEC), and consequently the degree to which Al interacts with the cell wall. Among the many components of the cell-wall network, pectins have been proposed to be a critical site for Al-cell-wall interactions (Blamley et al., 1993). Al interactions lead to the displacement of other cations (e.g., Ca^{2+}) fundamental for cell-wall stability (Mat-sumoto et al., 1977; Rincón and Gonzales, 1992; Schmohl and Horst, 2000; Tabuchi and Matsumoto, 2001), Consequently, the strong and rapid binding of Al can alter cell-wall structural and mechanical properties, making it more rigid, leading to a decrease in the mechanical extensibility of the cell wall required for normal cell expansion.

(B) The Plasma Membrane. Given its physicochemical properties, $A1^{3+}$ can interact strongly with the negatively charged plasma-membrane surface (Akeson and Munns, 1989; Kinraide et al., 1992, 1994, 1998). As Al has a more than 500-fold greater affinity for the choline head of phosphatildylcholine, a-lipid constituent of the plasma membrane, than other cations such as Ca^{2+} have, $A1^{3+}$ can displace other cations that may form bridges between the phospholipid head groups of the membrane bilayer (Akeson et al., 1989; Akeson and Munns, 1989). As a consequence, the phospholipid packing and fluidity of the membrane is altered. In addition, Al interactions with the plasma membrane lead to screening and neutralization of the charges at the surface of the plasma membrane that can alter the activities of ions near the plasma-membrane surface. Thus, Al interactions at the plasma membrane can modify the structure of the plasma membrane as well as the ionic environment near the surface of the cell; both can lead to disturbances of ion-transport processes, which can perturb cellular homeostasis.

Callose (β-l,3-glucane) synthesis (synthesized by β-l,3-glucane synthetase) on the plasma membrane is also quickly activated upon exposure to Al. Thus, callose accumulation in the apoplast has also been used as a measure of early symptoms of Al toxicity (Horst et al., 1997; Massot et al., 1999). Since callose synthesis depends on the presence of Ca^{2+}, it has been suggested that Al displacement of Ca^{2+} from the membrane surface may increase the apoplastic Ca^{2+} pool required to stimulate callose synthesis. Under Al stress, callose accumulation may lead to further cellular damage by inhibiting intercellular transport through plasmodesmatal connections (Sivaguru et al., 2000).

As mentioned above, Al binding to plasma membrane phospholipids surrounding transmembrane transporters may induce local charge disturbances, and alter local ion concentrations, thus affecting ion movement to binding sites in membrane-transport proteins. One of the most noticeable consequences of root Al exposure is an almost instantaneous depolarization of the plasma membrane (Lindberg et al., 1991; Papernik and Kochian, 1997). This change in the trans-plasma membrane electrochemical potential may be due to both direct and indirect interactions of Al with a number of different ion transport pathways (Miyasaka et al.,1989).

Plasma membrane H^+-ATPase. Al can significantly inhibit the activity of the plasma-membrane H^+-ATPase, impeding formation and maintenance of the trans-membrane H^+ gradient. Al-induced inhibition of H^+-ATPase activity and consequent disruption of the H^+ gradient has been reported both *in vitro* (e.g., membrane vesicle studies) and in intact roots of several plant species (Ahn et al., 2001, 2002; Ryan et al., 1992). The transmembrane H^+ gradient serves as the major driving force for secondary ion transport processes. Consequently, Al disruption of the H^+ gradient could indirectly alter the ionic status and ion homeostasis of root cells.

Inhibition of cation uptake and Al blockade of channel proteins. Exposure to Al can inhibit the uptake of many cations including Ca^{2+}, Mg^{2+}, K^+, and $NH^+{}_4$ (Huang et al., 1992; Lazof et al., 1994; Nichol et al., 1993; Rengel and Elliott, 1992; Ryan and Kochian, 1993). Although it has long been accepted that Al directly blocks root-cell ion transport proteins, it was not until fairly recently that evidence in support of such direct interactions has been presented. For example, a number of studies have shown that Al exposure strongly inhibits Ca^{2+} fluxes across the plasma membrane of root cells (Huang et al., 1992; Rengel and Elliott, 1992). Electrophysiological approaches were subsequently used to demonstrate that $A1^{3+}$ interacts directly with several different plasma-membrane channel proteins, blocking the uptake of ions such as K^+

and Ca^{2+} (Gassmann and Schroeder, 1994; Piñeros and Kochian, 2001; Piñeros and Tester, 1995). In addition to directly altering ion permeation through channels, extracellular Al can also modulate the transporter's activity via changes in the membrane potential. For example, Al-induced membrane depolarizations can alter voltage-dependent Ca^{2+} channel transport by indirectly modulating and shifting the activation thresholds of distinct transport pathways, such as hyperpolarization-activated (Kiegle et al., 2000; Very and Davies, 2000) and depolarization-activated (Piñeros and Tester, 1997; Thion et al., 1996; Thuleau et al., 1994) Ca^{2+} channels.

(C) Al Effects on Signal-Transduction Pathways.

Disruption of cytosolic Ca^{2+} and H^{+} activity. Al interactions with signal-transduction pathways, in particular disruption of intracellular Ca^{2+} and pH homeostasis, have been proposed to play crucial roles in Al toxicity. Several studies have shown that Al exposure can alter cytosolic Ca^{2+} and pH levels (Jones et al., 1998a, b; Lindberg and Strid, 1997; Ma et al., 2002b; Rengel, 1992; Zhang and Rengel, 1999). Al can also interact with and inhibit the enzyme phospholipase C of the phosphoinositide pathway associated with Ca^{2+} signaling (Jones and Kochian, 1995; Jones and Kochian, 1997). The Al-induced disruption of ion fluxes described above could directly lead to changes in cytosolic ion activities (e.g., Ca^{2+} homeostasis) as well as ion-dependent signaling pathways (e.g., inhibition of Ca^{2+}-dependent enzymes such as phospholipase C) which would ultimately reflect in any of the physiological and morphological changes described above. This is an interesting and potentially important research area regarding mechanisms of Al toxicity, and although there is some evidence in support of an association of Al-induced root growth inhibition with changes in a complex network of responses involving a signal transduction root cells, this is still a topic that is poorly understood and requires more research. For a recent review on Al disruption of Ca^{2+} homeostasis, see Rengel and Zhang (2003).

Oxidative Stress. Reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide that result from photosynthesis and oxidative metabolism can be involved in a number of stress responses (Bowler et al., 1992; Foyer et al., 1994). It has been shown that Al exposure is associated with peroxidative damage of membrane lipids due to the stress-related increase in the production of highly toxic oxygen free radicals (Cakmak and Horst, 1991). However, it appears that lipid peroxidation is only enhanced after a prolonged exposure to Al (24 h or more). Thus, although Al-induced lipid peroxidation does not occur rapidly enough to be an initial mechanism of Al toxicity (Horst et al., 1992; Yamamoto et al., 2001), Al-induced ROS generation and associated mitochondrial dysfunction could still play a more general role in Al inhibition of root growth (Yamamoto et al., 2002).

(D) The Root Cytoskeleton. Because of the central importance of cytoskeletal components (microtubules and microfilaments) in cell division and expansion of a growing root, several laboratories have investigated the cytoskeleton as a potential cytosolic target for Al toxicity. Al could disrupt cytoskeletal dynamics either via a direct interaction with cytoskeletal elements (i.e., microtubules and actin filaments) or indirectly, via alteration of signaling cascades such as cytosolic Ca^{2+} levels that are involved in cytoskeletal stabilization. The orientation of the cytoskeleton provides a template both for cell division and cell-wall biosynthesis (Sivaguru et al., 1999). For example, cortical microtubules are involved in the orientation of cellulose microfibrils, and as such, proper orientation of micotubules is a prerequisite for normal cell expansion. It has been well documented that Al exposure can disrupt both the organization of microtubules and microfilaments in root cells (Alessa and Oliveira, 2001; Blancaflor et al., 1998; Frantzios et al., 2000, 2001; Grabski et al., 1998; Horst et al., 1999; Sasaki et al., 1997a, b; Schwarzerova et al., 2002; Sivaguru et al., 1999, 2003b). For example, exposure to Al results in the disruption and reorganization of cortical microtubules. Likewise, Al induced a significant increase in the tension of the actin filaments of soybean (*Glycine max*) cells (Grabski and Schindler, 1995). Such Al-induced cellular structural changes are likely to result in, and underlie, the morphological changes and structural malformations observed in Al-stressed roots.

(E) DNA/nuclei. Prolonged exposures can lead to Al interactions with structures within the

nucleus, detrimentally affecting DNA composition, chromatin structure, and template activity (Matsumoto, 1991; Sampson et al., 1965; Silva et al., 2000). The presence of Al at the surface of the nucleus can potentially lead to microtubule binding at the membrane surface during the G2 phase of the cell cycle, as well as protein recognition, binding, and transport into the nucleus (Franklin and Cande, 1999; Smith and Raikhel, 1999). These types of interactions of Al with the nucleus can result in the disruption of the cytoskeleton and cell division processes.

The above putative mechanisms of Al toxieity are summarized in the model shown in Figure 1 (left side) except for Al interactions with the cell wall that were not included for reasons of visual clarity.

Physiological mechanisms of Al resistance

Research from a number of laboratories has made it clear that Al resistance can either be mediated via exclusion of Al from the root apex or via intracellular tolerance of Al transported into the plant symplasm. There has been considerable evidence presented in the literature for an Al-exclusion mechanism based on carboxylate exudation from the root apex. More recently, evidence has been presented for an internal tolerance mechanism based on chelation and detoxification of Al in the symplast with carboxylate anions. A summary and overview of both types of resistance mechanisms is considered here, as well as speculation about other possible Al-resistance mechanisms. For recent reviews of this topic, the reader is directed to Barcelo and Poschenrieder (2002), Garvin and Carver (2003), Kochian et al. (2004), Kochian and Jones (1997), Ma and Furukawa (2003), Ma et al. (2001), Ma (2000) and Matsumoto (2000).

Al Exclusion via Root Carboxylate Exudation

The first evidence in the literature for this resistance mechanism came from Miyasaka et al. (1991) who showed in long-term studies that an Al-resistant cultivar of snapbean (*Phaseolus vulgaris)* excreted eight-fold more citrate from the roots than did an Al-sensitive genotype. Citrate is a very potent chelator of Al^{3+}, and it appears that roots do not take up Al-carboxylate complexes. This is supported by the observations in wheat (*Triticum aestivum)* that Al-resistant genotypes release malate, and accumulate significantly less Al in the root apex (but not the mature root regions) compared with Al-sensitive genotypes (Delhaize et al., 1993a; Rincon and Gonzales, 1992; Tice et al., 1992). The seminal work on this resistance mechanism came from Delhaize and Ryan and coworkers who showed, using near isogenic lines (NIL) of wheat differing at a single Al-resistance locus: Al very rapidly activates malate release (within minutes); Al-activated malate release is localized very specifically to the first few millimeters of the root apex of the tolerant NIL (Delhaize et al., 1993a, b; Ryan et al., 1995a, b). Since these initial reports, high levels of Al-activated release of carboxylates have been correlated with Al resistance in a large number of plant species, as summarized in Table 1. When all the evidence in support of Al-activated root carboxylate release as a major resistance mechanism is examined, a very strong case in support of this concept is seen. Some of the major aspects of this resistance mechanism include:

- A correlation between Al resistance and Al-activated carboxylate release in many plant species (Table 1);
- Al-carboxylate complexes are not transported into roots or across membranes (Akeson and Munns, 1990; Shi and Haug, 1990);
- Al resistance cosegregates with Al-induced malate release in wheat and *Arabidopsis* (Delhaize et al., 1993a, b; Hoekenga et al., 2003);
- Activation of carboxyle release is triggered specifically by exogenous Al^{3+} (Ryan et al., 1995a) (although some lanthanide cations can act as Al^{3+} analogs in this response; see Kataoka et al. 2002);
- The rates of Al-activated carboxyate release are dose-dependent on the Al activity in the rhizosphere (Delhaize et al., 1993b; Ma et al., 1997a; Piñeros et al., 2002);
- Overexpression of genes encoding enzymes involved in organic acid synthesis, such as citrate synthase and malate dehydrogenase can, in some cases, result in enhanced Al resistance (de la Fuente et al., 1997; Koyama et al., 2000; Tesfaye et al., 2001);

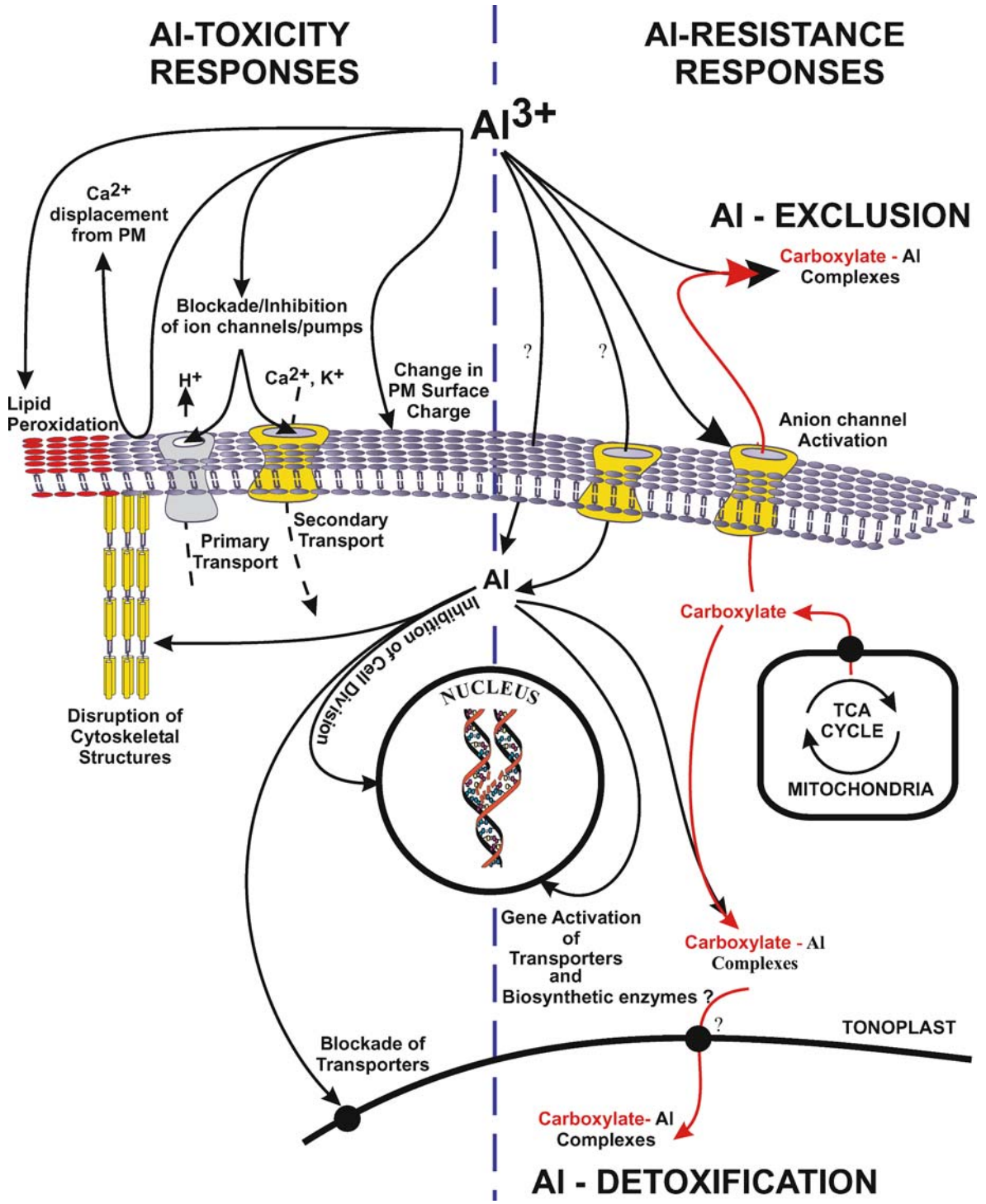

Figure 1. Possible mechanisms of Al toxicity and Al resistance in plants. Al toxicity targets described in the text are illustrated on the left side of the diagram. For clarity, the interactions of Al with the cell wall were not shown. On the right side, Al-resistance mechanisms (Al exclusion and internal Al detoxification) are based on the formation of Al complexes with carboxylates. The Al-exclusion mechanism involves the release of carboxylate anions via an Al-gated anion channel at the plasma membrane. The internal Al-detoxification mechanism involves chelation of cytosolic Al by carboxylate anions with the subsequent sequestration into the vacuole via unknown transporters.

Table 1. Plant species exhibiting Al-activated root carboxylate exudation that is correlated with Al resistance.

Organic Acid Released	Plant Species (common name)	Genotype	Reference
Citrate	*Cassia tora* (sickle senna)		Ishikawa et al. 2000; Ma et al. 1997a
Citrate	*Galium saxatile* (heath bedstraw)		Schöttelndreier et al. 2001
Citrate	*Glycine max* (soybean)	PI 416937	Silva et al. 2001
Citrate	*Glycine max* (soybean)	Suzunari	Yang et al. 2000
Citrate	*Miscanthus sinensis* and *Miscanthus sacchariflorus*		Kayama 2001
Citrate	*Nicotiana tabacum* (tobacco)		Delhaize et al. 2001
Citrate	*Oryza sativa* (rice)		Ishikawa et al. 2000; Ma et al.2002a
Citrate	*Sorghum bicolor* (sorghum)	SC283 and derived NILs	Magalhaes 2002
Citrate	*Zea mays* (maize)	ATP-Y	Kollmeier et al. 2001
Citrate	*Zea mays* (maize)	Cateto-Colombia	Piñeros et al. 2002
Citrate	*Zea mays* (maize)	DK789	Ishikawa et al. 2000
Citrate	*Zea mays* (maize)	IAC-TAIUBA	Jorge and Arruda 1997
Citrate	*Zea mays* (maize)	SA3	Pellet et al. 1995
Citrate, & Malate	*Avena sativa* (oat)		Zheng et al. 1998a
Citrate, &Malate	*Brassica napus* (rape)		Zheng et al. 1998a
Citrate, & Malate	*Helianthus annuus*(sunflower)	Saber et al. 1999	
Citrate, & Malate	*Raphanus sativus* (radish)		Zheng et al. 1998a
Citrate, & Malate	*Secale cereale* (rye)		Li et al. 2000
Citrate, & Malate	*Triticale ssp* (triticale)		Ma et al. 2000
Citrate, & Oxalate	*Zea mays* (maize)	Sikuani	Kidd et al. 2001
Malate	*Arabidopsis thaliana*	Landsberg erecta, Columbia, derived RILs	Hoekenga et al. 2003
Malate	*Triticum aestivum* (wheat)	Atlas 66	Huang et al. 1996; Pellet et al. 1996
Malate	*Triiicum aestivum* (wheat)	Chinese Spring & derived ditelosomic lines	Papernik et al. 2001
Malate	*Triticum aestivum* (wheat)	Kitakami B	Ishikawa et al. 2000
Malate	*Triticum aestivum* (wheat)	Line ET3	Ryan et al. 1995a Delhaize et al. 1993a; Delhaize et al. 1993b
Oxalate	*Colocasia esculenta* (taro)		Ma and Miyasaka 1998
Oxalate	*Fagopyrum esculentum* (buckwheat)		Ma et al. 1997b; Zheng et al. 1998a;b

- An Al-gated anion channel in maize (*Zea mays*) and wheat root tip protoplasts has been identified via electrophysiological experiments, and exhibits the properties necessary for it to be the transporter mediating Al-activated carboxylate release (Piñeros and Kochian, 2001; Piñeros et al., 2002; Ryan et al., 1997; Zhang et al., 2001).

An interesting possible feature of this mechanism is whether it is inducible at the level of gene expression. A number of researchers have assumed that Al-resistance genes and proteins are inducible by Al exposure, and this has been the impetus for some of the molecular studies discussed later. There appears to be evidence in support of an Al-inducible resistance mechanism in some plant species such as rye (*Secale cereale*), triticale (*Triticale ssp.*), and *Cassia tora* (sickle senna), where a lag in Al-activated carboxylate exudation is seen, and the rate of exudation increases over the first 12–24 hrs of Al exposure (Li et al., 2000; Ma et al., 1997a).

However, in other species exemplified by wheat, root malate exudation is very rapidly activated by Al exposure, and the rate of malate efflux does not appear to increase over time. Therefore, in species like wheat Al apparently activates an already expressed carboxylate transporter, and gene activation does not seem to play a role.

In species where the rate of carboxylate exudation apparently increases with time, it is possible that induction of Al-resistance genes contributes to this increased capacity. However, it is still not clear which part of the ligand-release pathway is being induced. The possibilities include: (1) an increased abundance or activity of a plasma-membrane carboxylate transporter; (2) an increased rate of carboxylate synthesis, driven by an increase in abundance or activity of enzymes involved in carboxylate synthesis; and (3) an increased availability of carboxylate ligands for transport, perhaps through altering internal carboxylate compartmentation within cells. To date, no strong evidence exists for a role of any of the enzymes catalyzing carboxylate synthesis and metabolism (PEP carboxylase, malate dehydrogenase, citrate synthase, isocitrate dehydrogenase) in this inductive response. For example, Al activates an up to ten-fold increase in citrate and malate exudation in rye and triticale (with exudation rates being higher in the Al-resistant cultivars), with little or no change in the *in vitro* activities of PEP carboxylase, isocitrate dehydrogenase, malate dehydrogenase, and citrate synthase in the root tips of both Al-resistant and -sensitive cultivars (Hayes and Ma, 2003; Li et al., 2000).

A point that is often ignored regarding this pattern of increasing rates of carboxylate exudation is that this should result in a measurable increase in Al resistance. It is surprising that this has not been addressed in plant species such as rye, where the noticeable induction and time-dependent increase in carboxylate release has been observed. Research in our laboratory on Al resistance in sorghum (*Sorghum bicolor*) has found that Al-activated root citrate exudation correlates closely with Al resistance between two cultivars differing in Al resistance (Magalhaes, 2002). We have found that in sorghum, longer exposures to Al (5–6 days) result in a significant increase in Al resistance compared with measurements of root growth after 24 to 48 hrs of Al exposure. However, even though Al resistance increases over a 6 day Al exposure in sorghum, the Al-activated root citrate exudation actually exhibits a slight *decrease*, suggesting that some other process is induced to cause this increase in Al resistance.

What is the Carboxylate Transporter?

In many plant species, Al exposure rapidly activates the exudation of carboxylate anions, and the release seems to be specific for one or two carboxylate species from a cytoplasm that contains a number of different carboxylate species. Thus, it seems clear that an important part of this Al-resistance mechanism is the activation of a particular carboxylate transporter that presumably resides in the root-cell plasma membrane. In wheat, Al activates malate release almost instantly, suggesting that transport is the limiting step (Osawa and Matsumoto, 2001; Ryan et al., 1995a). It also appears that in wheat increased carboxylate synthesis is not involved in the malate exudation response, as no differences in root tip malate concentration or in PEP carboxylase or malate dehydrogenase activity in Al-resistant versus sensitive genotypes have been observed, even though Al exposure activates a large and continuous efflux of malate in the Al-resistant genotype (Delhaize et al., 1993b; Ryan et al., 1995a, b).

The thermodynamic conditions for carboxylate transport from the cytosol to the external solution suggest that ion channels could be the primary transporter involved in this resistance response. The organic acids in the cytosol exist primarily as anions, and due to the large negative-inside transmembrane electrical potential in plant cells, there is a very strong gradient directed out of the cell for anions. Thus, an anion channel that opens upon exposure to Al would be sufficient to mediate this transport. Anion channels that are specifically activated by extracellular Al^{3+} have recently been identified using the patch-clamp technique with protoplasts isolated from root tips of Al-resistant wheat (Ryan et al., 1997; Zhang et al., 2001) and maize (Kollmeier et al., 2001; Piñeros and Kochian, 2001; Piñeros et al., 2002). In wheat, Ryan et al. (1997) have shown that Al^{3+} activates an inward Cl^- current (indicative of Cl^- efflux; Cl^- was the

only anion in the patch pipette) across the plasma membrane of wheat root protoplasts from the Al-resistant line; as long as Al was maintained in the external solution, this channel remained open. The transport properties for this channel are similar to that exhibited by intact wheat roots for Al-activated malate release. These researchers have also shown that this anion channel can transport malate, and the channel is more active and open more frequently in the presence of Al in root tip protoplasts from the Al-resistant wheat genotype compared with those from the sensitive one. Taken together, these results suggest this anion channel is involved in wheat Al resistance.

A similar anion channel has been identified in root tip cells from Al-resistant maize where Al-activated root citrate release is correlated with resistance (Piñeros and Kochian, 2001; Piñeros et al., 2002). The most important discovery from these studies was that the anion channel could be activated in isolated plasma-membrane patches, where the anion channel is operating in isolation from cytosolic factors. As shown in Figure 2, both whole cells, from maize root tips, and excised membrane patches isolated in the absence of Al, were electrically quiet. When both the whole cells and isolated membranes were exposed to extracellular Al^{3+}, the inward anion current indicative of anion efflux was activated. It was

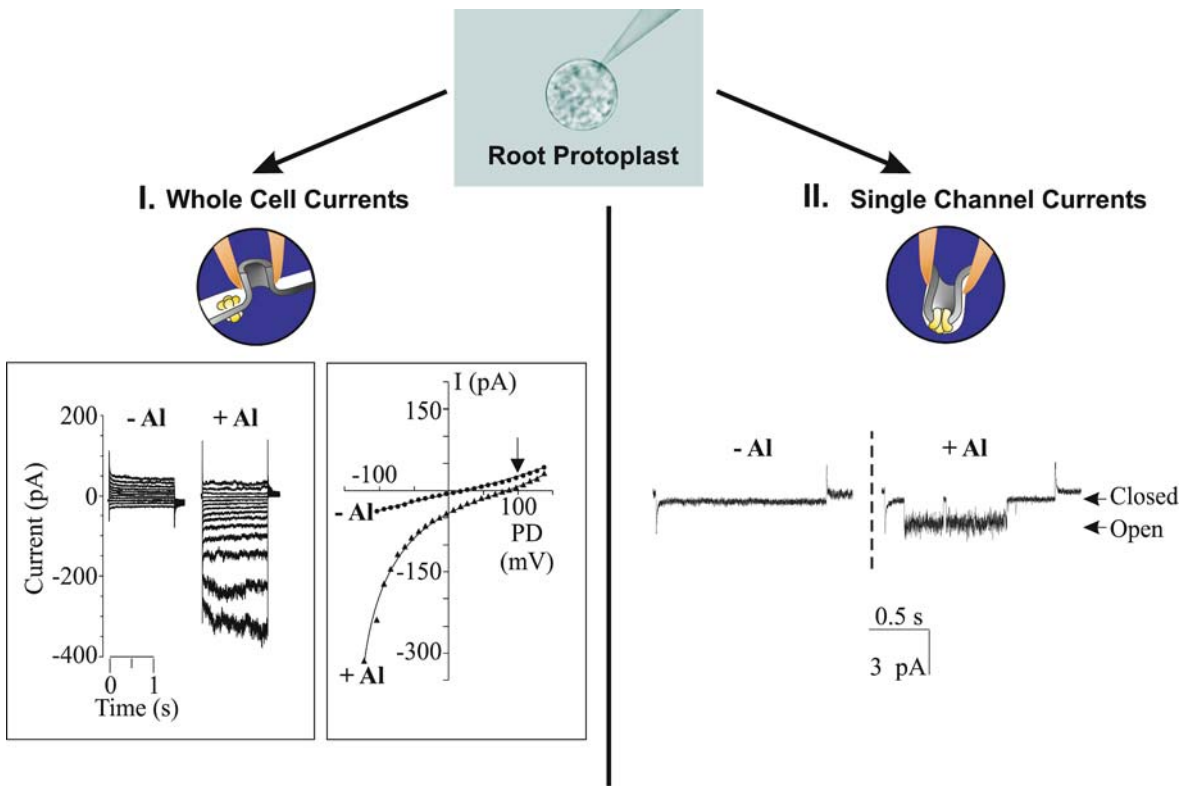

Figure 2. Al-activated anion channel in the plasma membrane of root cells from Al-resistant maize. The patch-clamp technique was employed to record macroscopic currents (whole cell currents on the left) or to study the transporter activity in isolated membrane patches (single channel currents on the right). I. Al activates an inward plasma membrane whole cell anion current (anion efflux). Whole-cell currents were elicited at holding potentials clamped in 10 mV increments. The bath contained 1 mM Cl^- (pH 4.0) minus (left traces) or plus (right traces) 50 μM Al^{3+}. Right panel: Current-voltage relationship for the currents shown on the left. The arrow indicates the Cl^- theoretical reversal potential. II. Al can activate single anion channels in excised membrane patches. A negative voltage potential was employed to test for single-channel activity in outside-out membrane patches excised in the absence of extracellular Al^{3+} (the resulting trace is shown on the left side in II. Subsequently the membrane patch was exposed to extracellular Al^{3+} (50 μM), and the same voltage protocol was employed. The single trace is shown on the right panel. Arrows on the right indicate the closed and open states of the channel.

not determined whether the anion channel could also transport citrate or other organic acids, as Cl^- was used as the primary anion. However, in another study on Al-activated anion channels in protoplasts from root tips of a different Al-resistant line, an Al-activated anion channel could mediate the transport of Cl^-, $malate^{2-}$, and $citrate^{3-}$ (Kollmeier et al., 2001). Thus, the features needed for Al activation of the anion channel are either contained within the channel protein itself, or are close by in the membrane (e.g., an associated membrane receptor). As depicted in the model summarizing Al resistance mechanisms (Figure 1), and described in detail in a previous review of root carboxylate exudation by Ryan et al. (2001), there are three possible ways that Al could activate a plasma-membrane anion channel involved in carboxylate exudation: (1) Al^{3+} might directly bind to and activate the channel; (2) Al^{3+} might bind to a separate but closely associated membrane receptor, which in turn, activates the channel; or (3) Al^{3+} activates the channel indirectly through a signal cascade that could involve cytosolic components. The findings in Al-resistant maize suggest possibility 1 or 2 as the most likely scenario.

Several families of anion channels have been identified in plants and other organisms, with much of the work coming from animal studies. The two most prominent families include the CLC (Cl channel) family, and a subset of the ATP-binding cassette (ABC) protein superfamily (Barbier-Brygoo et al., 2000). ABC proteins comprise a very large family of transporters that bind ATP during the transport of a wide range of organic and inorganic solutes. One subgroup of ABC transporters includes the cystic fibrosis transmembrane regulator (CFTR) in mammalian cell membranes which has been shown to be a Cl^- channel (Anderson et al., 1991). In yeast, another ABC protein, Pdrl2, mediates carboxylate efflux (Piper et al., 1998). There is no strong evidence that the carboxylate transporter involved in Al resistance is a member of the CLC family. However, there is some circumstantial evidence suggesting it could be an ABC transporter. The anion channel characterized in Al resistant wheat and maize shares a number of transport similarities with an anion channel in the guard-cell plasma membrane, the 'slow' anion channel (for slow inactivation), which also mediates the sustained release of anions (Leonhardt et al., 1999; Schroeder et al., 1993). It has been suggested that the slow anion channel in guard cells could be a member of the ABC family, based on its sensitivity to the ABC transporter antagonist, diphenylamine-2-carboxylic acid (DPC). In an electrophysiological investigation of the Al-activated anion channel in Al-resistant wheat roots (Zhang et al., 2001), DPC inhibited this anion channel, and also inhibited the Al-activated malate efflux from intact wheat roots.

However, some very recent work suggests that at least in wheat, the Al-activated malate transporter may actually be a novel type of membrane transporter. Research in Matsumoto's laboratory has recently described a wheat gene that appears to encode the root tip Al-activated malate transporter (Sasaki et al., 2003). These researchers have found that this gene is expressed more strongly in the root tip of the Al-resistant near-isogenic line (NIL) compared with the sensitive NIL, and appears to encode a novel membrane protein. Heterologous expression of this gene, named *ALMT1* for Al-activated malate transporter, resulted in Al-activated malate exudation in *Xenopus* oocytes, as well as in roots of transgenic rice (*Oryza sativa)* seedlings and tobacco (*Nicotiana tabacum)* suspension cells. Furthermore, expression of *ALMT1* increased Al resistance in tobacco suspension cells. This finding may represent the identification of the first major Al-resistance gene in plants.

Al resistance mechanism involving internal detoxification

Several researchers have recently identified a second Al resistance mechanism that is based on the complexation and detoxification of Al after it enters the plant. This discovery has come from research focusing on plants that can accumulate Al to high levels in the shoot. For example, *Hydrangea macrophylla* is an ornamental plant whose sepals turn from red to blue when the soil is acidified; this color change is due to Al accumulation in the sepals resulting in the formation of a blue complex of Al with two compounds, delphinidin-3-glucoside and 3-caffeolylquinic acid (Takeda et al., 1985). *Hydrangea macrophylla* can accumulate more than 3000 μg Al g^{-1} dry weight

in its leaves (Ma et al., 1997c); Ma and colleagues showed that the Al in the leaves exists primarily as a 1:1 Al-citrate complex. This type of complex should bind Al very tightly in a cytosol with a pH of around 7, and should protect the cytosol against Al injury. Ma and colleagues also studied a second Al accumulator, buckwheat (*Fagopyrum esculentum*). A portion of the Al resistance in buckwheat is due to Al-activated oxalate exudation from the root apex (exclusion) (Zheng et al., 1998b). However, buckwheat also accumulates Al to very high levels in its leaves, as high as 15, 000 μg Al g^{-1} dry weight when the plant is grown on acid soils (tolerance) (Ma et al., 2001). Most of the Al in both roots and leaves was complexed with oxalate in a 1:3 Al-oxalate complex (Ma et al., 1998). Subsequently, they showed that the Al being transported to the shoot in the xylem sap is complexed with citrate, and not oxalate (Ma and Hiradate, 2000). These findings suggest that the Al undergoes a ligand exchange from oxalate to citrate when it is transported into the xylem, and is exchanged back with oxalate when in the leaves. Leaf compartmental analysis showed that 80% of the Al in buckwheat leaves was stored in vacuoles as a 1:3 Al-oxalate complex (Shen et al., 2002). On the right side of Figure 1, where the different possible Al-resistance mechanisms are depicted, this internal detoxification mechanism is shown to involve Al chelation in the cytosol and subsequent storage of the Al-carboxylate complex in the vacuole. The tonoplast-localized mechanisms mediating the transport of Al into the vacuole, as well as the nature of its substrate (i.e., free Al versus Al-carboxylate complexes) remain unknown.

Genetic and molecular aspects of aluminum resistance

Increasing Al resistance has been a goal for plant scientists for many years, as this should lead to increased crop production on acid soils (see Garvin and Carver, 2003; Hede et al., 2001, for recent reviews). The majority of plant breeding attention has been focused on the economically important grasses (e.g., wheat, rice, maize), and thus most of the studies on the inheritance of Al resistance have been performed in these species. In the Triticeae, Al resistance has relatively simple, or qualitative, inheritance, such that one major gene explains the majority of Al resistance observed. By contrast, rice and maize have complex, quantitative inheritance of Al resistance, such that several genes are required to explain a plurality of Al resistance differences. More recently, genetic experiments have been conducted in model plants, such as *Arabidopsis thaliana*, to identify genes important for Al resistance. In spite of this body of work, Al-resistance genes have yet to be cloned from any species, with the exception *of ALMT1* from wheat (Sasaki et al., 2004). This situation is sure to change in the coming years, with the utilization of genome sequence analyses from rice and *Arabidopsis* and the application of genomics-based approaches to gene discovery.

Genetics of Al resistance: cases of simple inheritance

The inheritance of Al resistance in wheat (*Triticum aestivum*) has been studied longer and, perhaps, more completely than in any other plant species. Crop improvement programs in Brazil and the US led to the development of excellent cultivars (cv. BH1146 and Atlas66, respectively), which have subsequently been well studied in both field and laboratory conditions. In many crosses between these elite Al-resistant cultivars and Al-sensitive varieties, Al resistance is apparently conferred by a single, dominant locus; in other crosses, segregation patterns suggest two loci are responsible for resistance (Garvin and Carver, 2003). One of these loci has been mapped to the long-arm of chromosome 4D, sometimes called Alt_{BH} or *Alt2* (Milla and Gustafson, 2001). The existence of other loci important for Al resistance elsewhere in the genome can further be inferred from the study of varieties that contain chromosomal deletions. Additional locations have been identified through the comparison of nullisomics, ditelosomics or lines with small segmental deletions that have diminished resistance to Al relative to their euploid progenitors (Aniol and Gustafson, 1984; McKendry et al., 1996; Papernik et al., 2001). One can imagine that most cellular processes will have multiple components, such that mutations or deletions in any single part could compromise the entire mechanism. The

importance of these additional genetic factors beyond those located on 4DL (e.g., 5 AS, 7AS) have been difficult to evaluate, as genetic experiments have yet to implicate these regions as containing resistance genes by cosegregation analysis.

It has been well established that Al resistance in wheat is highly correlated with an Al-activated release of malate. In a seminal study, Ryan et al. (1995b) quantified both relative root length and malate release in thirty-six cultivars to assess Al resistance. A correlation analysis of these two parameters demonstrated that 84% of the variance was explained such that differences observed in relative root length (RRL) were largely explained by the quantity of malate released. This high degree of agreement suggests that differences within a single physiological mechanism are responsible for the majority of differences in Al resistance between wheat cultivars. Taken together with the observations regarding cosegregation analysis, it is possible that a very few regulatory loci are responsible for the differences in Al resistance reported in wheat. This is not to say that malate release is the only Al-resistance mechanism in wheat; merely that malate release is only Al-resistance pathway to exhibit polymorphism among wheat cultivars studied to date.

Rye (*Secale cereale*) is generally regarded as having excellent resistance to abiotic stressors, including Al, superior to that observed in its close relative wheat (Aniol and Gustafson, 1984). Unlike wheat, rye is self-incompatible, and thus an obligate out-crossing species. This may help explain why cosegregation experiments in rye generally detect a greater number of Al resistance loci than are detected in wheat (Aniol and Gustafson, 1984; Gallego and Benito, 1997; Hede et al., 2001), Like wheat, the long-arm of chromosome 4 contains a major Al-resistance locus, called *Alt3* (Gallego and Benito, 1997). An improved map estimate for *Alt3* demonstrated tight linkage with markers linked to Alt_{BH}, advancing the suggestion that homeologous loci act as Al resistance genes in both species (Miftahudin et al., 2002). A second resistance locus (*Alt1*) has been mapped to a small interval on the short-arm of chromosome 6 (Gallego et al., 1998). Unfortunately, the molecular markers closely linked to *Alt1* have not been used for additional mapping studies or otherwise incorporated into a consensus rye genetic map, such that fine-scale comparative mapping is not possible.

Barley (*Hordeum vulgare*), a third member of the Triticeae tribe, also contains a major Al resistance locus, *Alp*, on the long-arm of chromosome 4 (Minella and Sorrells, 1992). Like *Alt3* in rye, the *Alp* locus is linked to markers useful for following Alt_{BH} in wheat (Tang et al., 2000) (Figure 3). Unlike rye or wheat, barley is very sensitive to Al (Minella and Sorrells, 1992). If *Alp, Alt3* and Alt_{BH} were truly orthologous loci, one would expect that an analysis of the protein sequences should reveal a great deal about how this wide range of tolerance phenotypes is achieved.

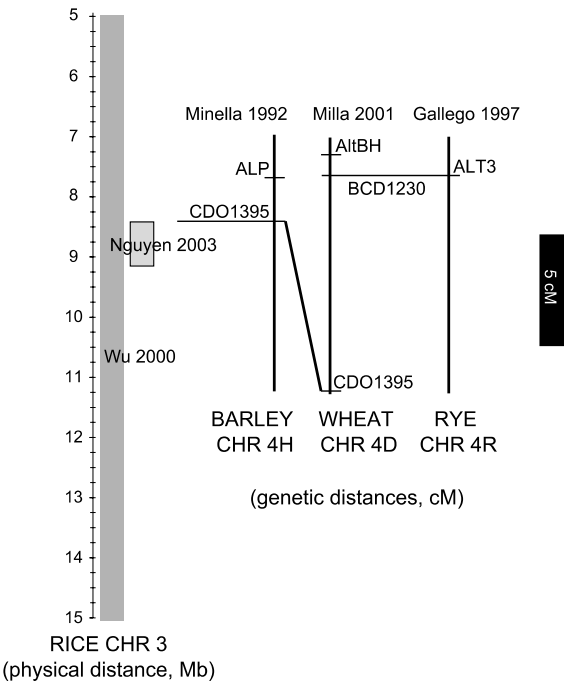

Figure 3. Comparative mapping of Al-resistance loci between Triticeae and rice. Loci located to rice linkage block 3C. Flanking genetic markers for the QTLs identified in rice (Nguyen et al., 2003; Wu et al., 2000) were located on the rice chromosome 3 pseudo-molecule using Gramene. The CDO1395 marker defined one QTL boundary (Nguyen et al., 2003) and served to link this region of the rice genome to the corresponding region in genomes of species in the Triticeae, Genetic distances between linked markers and Al-resistance genes in the Triticeae (Gallego et al., 1997; Milla et al., 2001; Minella et al., 1992) are shown. The 5 cM genetic distance scale bar was set using the physical to genetic distance ratio from rice over this interval (approx. 0.36 Mb/1 cM).

Al resistance genetics in sorghum (*Sorghum bicolor L.* Moench) has only very recently received any attention. Sorghum is closely related to maize, possesses the second smallest genome among cultivated grasses (after rice), and exhibits a wide phenotypic range for biotic and abiotic stress resistance making this warm-weather grass an attractive model experimental system (Mullet et al., 2001), Recent investigations into the inheritance of Al resistance indicate that, like wheat, rye and barley, sorghum exhibits a simple pattern of inheritance with a single locus explaining the majority of differences observed among genotypes (Magalhaes, 2002; Magalhaes et al., 2003). Unlike the Triticeae, the Alt_{SB} locus in sorghum is neither located in the homeologous chromosomal location to *Alp, Alt3* and Alt_{BH}, nor is it linked to the shared set of RFLPs and SSRs. The phenomenology of Al resistance is also somewhat different in sorghum, relative to the Triticeae, as the resistance response appears to be inducible and take days to fully manifest (Magalhaes, 2002). The combination of genetic and physiological data suggests that sorghum utilizes a different pathway to achieve Al resistance than the mechanism characterized in wheat and its relatives.

Genetics of Al Resistance: Cases of Complex Inheritance

Rice (*Oryza sativa)* has been the subject of the largest number of quantitative trait locus (QTL) mapping experiments to identify the basis of Al resistance (Ma et al., 2002a; Nguyen et al., 2001, 2002, 2003; Wu et al., 2000). These studies used ten different parents, including improved *indica* and *japonica* cultivars, and a wild relative, *Oryza rufipogon*. Twenty-seven QTLs important for Al resistance, as estimated by relative root growth, were identified in the five studies. Given the conservation of location for Al resistance loci among the Triticeae, one wonders if an ortho-logous locus to $Alp/Alt3/Alt_{BH}$ plays a similar role in rice. Although a portion of rice chromosome 3 (rice linkage block 3C) is homeologous to Triticeae 4L (Gale and Devos, 1998), and genetic markers linked to Al-resistance loci are shared between rice, wheat, and barley (Figure 3) (Nguyen et al., 2003), the Al resistance locus on rice 3 is not the primary one for resistance in rice. Instead, a locus on rice 1 typically explains the largest percentage of Al resistance in the mapping populations studied. Rice 1 was identified by all five studies as important for Al resistance (Figure 4A). Interestingly, the rice 1 QTL is in a region homeologous (rice linkage block 1B) to the portion of sorghum linkage group G that contains Alt_{SB} (Figure 4A). Further work will be necessary to evaluate whether orthologous loci are at work in both sorghum and rice, or if the apparent linkage is merely serendipitous.

Two other chromosomal regions were repeatedly identified among rice mapping populations as important for Al resistance. An interval on rice 9 was identified by three studies; in each case, the *indica* parent provided the sensitive allele (Figure 5A) (Nguyen et al., 2002, 2003; Wu et al., 2000). The indica parent again provided the sensitive alleles for a common QTL on rice 8, although this interval was identified by only two studies (Figure 5B) (Nguyen et al., 2002, 2003).

Like wheat, maize (*Zea mays* spp. *mays*) has long been the subject of breeding programs that seek to increase Al resistance or understand the basis for it (Magnavaca et al., 1987; Sawazaki and Furlani, 1987). Some investigators concluded that Al resistance was a qualitative trait, although these studies utilized either small mapping populations (<100 F2 individuals) or nearly identical mapping parents (a resistant inbred and a sensitive somaclonal variant) (Rhue et al., 1978; Sibov et al., 1999). The majority of investigators have concluded that Al resistance is a quantitative trait, based on the segregation analysis of large F2:3 populations or recombinant inbred lines (Giaveno et al., 2001; Magnavaca et al., 1987; Ninamango-Cardenas et al., 2003; Sawazaki and Furlani, 1987). Only one QTL mapping study has been published to date; five genomic regions were identified as important for Al resistance (Ninamango-Cardenas et al., 2003). When we conducted an *in silico* comparative mapping analysis of these regions using the Gramene database (Ware et al., 2002), markers flanking two of these regions in maize could be located to regions containing Al resistance loci in other grasses. The markers that flank a principal maize QTL region (bin 6.05) can be landed to rice chromosome 5, in the same vicinity of a QTL identified by Nguyen et al. (2001) (Figure 4B). Perhaps more intriguing is the fact that the third most important QTL from the maize

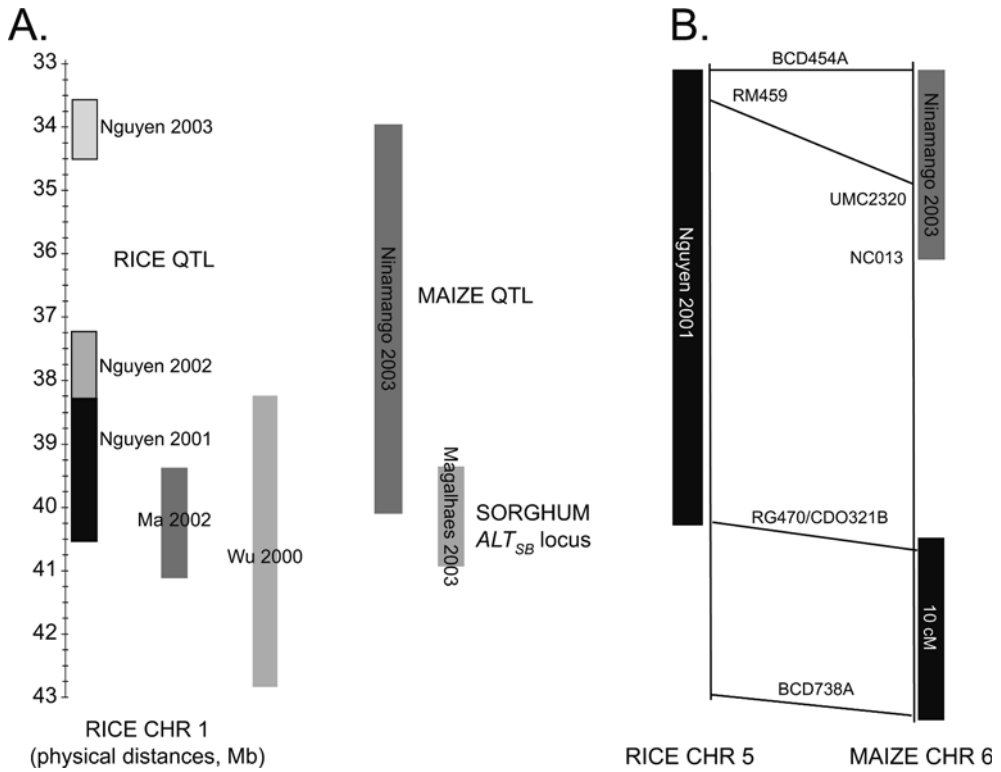

Figure 4. Comparative mapping of Al-resistance loci between rice, maize and sorghum. A) Loci located to rice linkage block 1B. Fanking genetic markers for the QTLs identified in rice (Ma et al., 2002a; Nguyen et al., 2001, 2002, 2003; Wu et al., 2000), maize (Ninamango-Cardenas et al., 2003), and sorghum (Magalhaes et al., 2003) were located on the rice chromosome 1 pseudomolecule using Gramene. The gray bars indicate QTL confidence intervals; distances are in Mb. B) Loci located to rice linkage block 5B. The BCD454A marker defined one boundary for a QTL in rice (Nguyen et al., 2001) and mapped very close to the boundary for a maize QTL (Ninamango-Cardenas et al., 2003). Three additional markers mapped in both rice and maize over this region are shown to demonstrate the extent of synteny.

study (bin 8.07) falls within a region of the maize genome homeologous to rice chromosome 1 and sorghum linkage group G, the chromosomal segment identified by six different studies (Figure 4A). As the physical map of maize improves, it should be possible to locate the other maize QTLs to their related chromosomal regions in rice, to identify possible orthologous tolerance loci. In any event, there is a great deal of exciting comparative mapping to be done in the grasses, to investigate the role of putative orthologs in Al resistance.

Two studies offer insight into the basis for Al resistance in the model species *Arabidopsis thaliana* (Hoekenga et al., 2003; Kobayashi and Koyama, 2002). This pair of studies utilized the same Lands-berg *erecta* X Columbia RIL mapping population. In both studies, the principal QTL was found at the top of chromosome 1 and explained approximately 30% of the variance observed; however, the locations for all of the other putative QTLs were in complete disagreement between studies. This outcome is likely due to the affect of the growth conditions on root growth in each study. Kobayashi and Koyama (2002) used a low ionic strength hydroponic growth condition at pH 5.0, while Hoekenga et al. (2003) used higher ionic strength nutrient solution and Al concentration in gelled (semi-solid) growth media. As any phenotype is the result of both genetic and environmental effects, the differences in growth conditions likely illuminated factors important for the resistance of low ionic strength and/or pH, in addition to the intended target of Al tolerance.

Molecular Biology of Al Resistance

Genomics-based inquires are, by their nature, multidisciplinary endeavors that integrate several lines of inquiry. Based on the physiological char-

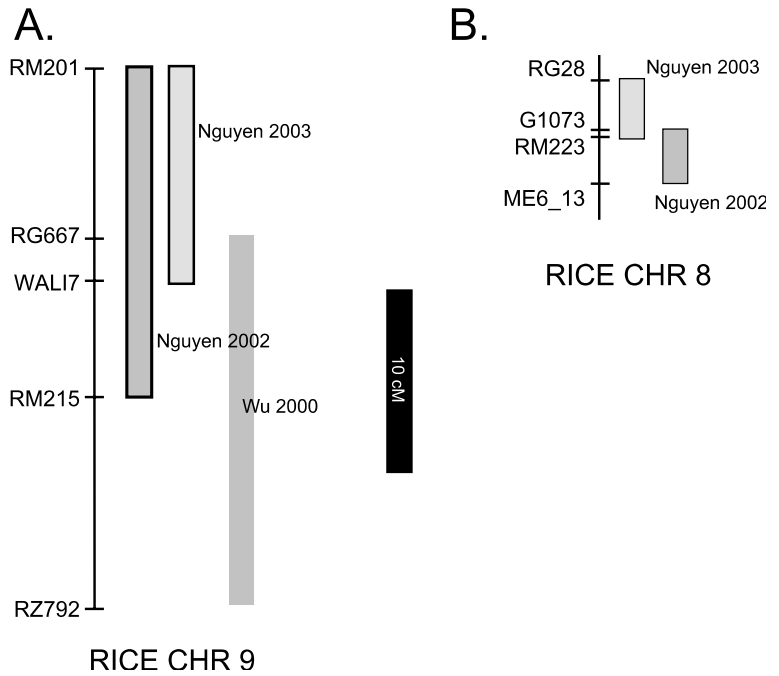

Figure 5. Comparative mapping of A1-resistance loci among rice varieties. The gray bars indicate QTL confidence intervals; distances are in cM, where the scale is shared in both panels. (A) Loci located to rice linkage block 9. Three rice QTLs were identified in the same interval of rice chromosome 9 (Nguyen et al., 2002, 2003; Wu et al., 2000). (B) Loci located to rice linkage block 8. Two rice QTLs were identified in the same interval of rice chromosome 9 (Nguyen et al., 2002, 2003).

acterizations of Al resistance and toxicity, it is clear that carboxylate release and internal detoxification are key mechanisms in Al resistance. This prior knowledge facilitates the analysis of large-scale gene ('microarrays') or protein ('proteomics') expression datasets, as candidate Al-resistance factors should make themselves obvious from the collected data. Furthermore, Al resistance may be an inducible process, (e.g., Li et al., 2000; Magalhaes, 2002), such that profiling gene or protein expression through a time course of Al toxicity may be useful. Unfortunately, the modern techniques of microarray or proteomic analysis have yet to be fully applied to Al resistance questions; one can assume this will change in the near future.

Gene-expression profiling

The primary strategy employed to estimate genome-wide changes in gene expression during Al stress has been to construct subtractive cDNA libraries. Libraries have been constructed from sugarcane Saccharum spp. (hybrid cv. N19), tobacco, *Arabidopsis*, rye and wheat to identify genes more highly expressed in Al-stressed roots (Drummond et al., 2001; Ezaki et al., 1995; 1996; Milla et al., 2002; Richards et al., 1998; Watt, 2003). Candidate genes identified in this manner are then typically assessed using transgenic plants; transformants are subsequently challenged with Al, and their resistance evaluated (Ezaki et al., 2000, 2001; Sivaguru et al., 2003a). Ezaki et al. (2000) had only modest success in increasing the Al resistance of *Arabidopsis* with their transgenes, with gains of 50% or less (from 60% inhibition of root growth in untransformed to 40% inhibition in transgenics). An-ionic peroxidase from tobacco produced the largest and most consistent increase in Al resistance in these experiments, presumably by increasing the capacity of the plant to cope with reactive oxygen species. Basu et al. (2001) produced a similar result working in *Brassica napus*, where they over-expressed a manganese superoxide dismutase and observed a modest gain in Al resistance. These results reinforced the importance of free radical quenching as an Al-resistance mechanism. However, truly novel

mechanisms of Al resistance have yet to be identified using this strategy.

Only one microarray experiment has been published on the effects of Al stress on gene expression (Hoekenga et al., 2003). This experiment was limited in scope, due to the fact it was conducted with the *Arabidopsis* Functional Genomics Consortium (AFGC), utilizing an 8000 feature array and a single, replicated hybridization. One of the benefits of the (now defunct) AFGC program is that all of the data generated are searchable via The *Arabidopsis* Information Resource (TAIR) website (Finkelstei et al., 2002; http://www.Arabidopsis.org). As these data were up-loaded to the public website shortly after the microarray experiments were complete, other workers in the Al resistance field gained access to the gene expression profiling data. In this manner, Schultz et al. (2002) were able to identify that the Al treatment differentially affected members of the arabinogalactan protein (AGP) family, which is their study area. AGPs are cell-wall localized proteins thought to be important for growth and development, but poorly characterized. Al stress induced two members and repressed two others of the nineteen-member gene family. AGP2 was demonstrated to be Al inducible using Northern Blot hybridization, reaching maximal expression after 8 h of exposure (Schultz et al., 2002). Thus, a novel facet of Al-stress response was identified, although it is still unknown what role AGP2 does play in modulating cell wall architecture or structure.

Concluding remarks

The considerable interest in mechanisms of plant Al resistance and toxicity exhibited by researchers around the world has resulted in an increased understanding of the mechanistic basis for these complex topics. This is particularly true for investigations into the molecular, genetic and physiological mechanisms of Al resistance. Al-resistant genotypes in many plant species appear to employ Al-activated carboxylate release as a resistance mechanism based on exclusion of Al from the growing root tip. Furthermore, we now have a better understanding of many of the physiological features of this resistance mechanism. However the research has also resulted in many questions that remain unanswered. For example, why do some species release malate, others citrate, and still others oxalate in response to Al stress? How do roots perceive the Al signal and transduce this signal to activate the processes involved in resistance? What other Al-resistance mechanisms are employed by different plant species? We are just beginning to understand a second Al-resistance mechanism involving internal detoxification of Al with carboxylate ligands and the sequestration of the Al-carboxylate complexes in the vacuole, and it is likely that other additional resistance mechanisms exist. Finally, we are poised to discover the genes that underlie Al resistance mechanisms. The identification and characterization of Al resistance genes will not only greatly advance our understanding of the mechanistic functioning of these processes, but, more importantly, will be the source of new molecular resources that researchers will use to develop improved crops better suited for cultivation on the acid soils that comprise such a large fraction of the world's lands.

Acknowledgements

The authors want to acknowledge the financial support for their own research on acid soil tolerance from the USDA-CSREES National Research Initiative Competitive Grants Program (USDA-NRI Grants #00–35100–9280, #01–35301–10647, and #02–35100–12058), the McKnight Foundation Collaborative Crop Research Program, and from the USDA Agricultural Research Service.

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Plant and Soil (2004) 254: 197–214
DOI 10.1007/s11104-004-1650-0

Acclimation to soil flooding – sensing and signal-transduction

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Received 2 April 2004. Accepted in revised form 26 July 2004

Key words: acclimation, adventitious roots, aerenchyma, ethylene, oxygen sensing, soil flooding, waterlogging

Abstract

Flooding results in major changes in the soil environment. The slow diffusion rate of gases in water limits the oxygen supply, which affects aerobic root respiration as well as many (bio)geochemical processes in the soil. Plants from habitats subject to flooding have developed several ways to acclimate to these growth-inhibiting conditions, ranging from pathways that enable anaerobic metabolism to specific morphological and anatomical structures that prevent oxygen shortage. In order to acclimate in a timely manner, it is crucial that a flooding event is accurately sensed by the plant. Sensing may largely occur in two ways: by the decrease of oxygen concentration, and by an increase in ethylene. Although ethylene sensing is now well understood, progress in unraveling the sensing of oxygen has been made only recently. With respect to the signal-transduction pathways, two types of acclimation have received most attention. Aerenchyma formation, to promote gas diffusion through the roots, seems largely under control of ethylene, whereas adventitious root development appears to be induced by an interaction between ethylene and auxin. Parts of these pathways have been described for a range of species, but a complete overview is not yet available. The use of molecular-genetic approaches may fill the gaps in our knowledge, but a lack of suitable model species may hamper further progress.

Introduction

Flooding of the soil, also called waterlogging, can have a tremendous impact on the growth and survival of plants, and thereby on agricultural as well as natural ecosystems. In the last decades considerable progress has been made in our understanding of the mechanisms that enable certain plant species and cultivars to withstand periods with excess soil water, or even complete submergence. Much of the research has been carried out with crop plant species, such as rice (*Oryza sativa*), maize (*Zea mays*) and sunflower (*Helianthus annuus*), but also wild species originating from wetland habitats have been used, mostly for comparative studies (e.g., Justin and Armstrong, 1987; Laan et al., 1989; Smirnoff and Crawford, 1983; Visser et al., 1996a).

The main effect of soil flooding is a considerable slow-down of the exchange of oxygen, as gas diffusion rates are four orders of magnitude slower in water than in air (Jackson, 1985). Consequently, flooded soils rapidly develop anoxic conditions at depths greater than a few centimetres, as the demand by aerobic respiration of soil organisms greatly exceeds the influx of oxygen from the atmosphere. Shortage of oxygen is obviously detrimental to the development of root systems, and those roots whose aerobic metabolism entirely depends on oxygen from the soil will cease growth, and may eventually die (Bradford and Yang, 1981; Drew, 1997). Additionally, anoxic soils may accumulate phytotoxic products from microbial reduction processes (such as hydrogen sulphide, Fe^{2+} and Mn^{+}; Ernst, 1990; Laanbroek, 1990; Lamers et al., 1998; Ponnamperuma, 1984), and the gaseous plant hormone ethylene (Smits and Scott-Russell, 1969; Campbell and Moreau, 1979; Visser et al., 1996b),

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which can strongly inhibit root growth (Konings and Jackson, 1979; Visser et al., 1997).

In-depth discussions of the large variety of adaptations that have evolved in plant species prone to soil flooding, have been presented in recent reviews, such as publications on root function, physiology and biochemistry (Jackson and Ricard, 2003), aerenchyma development (Jackson and Armstrong, 1999; Evans, 2003), gas diffusion in plants (Colmer, 2003), root-to-shoot signalling (Jackson, 2002), changes in gene expression (Subbaiah and Sachs, 2003) and root metabolism (Drew, 1997; Geigenberger, 2003; Gibbs and Greenway, 2003a, b). In the current review we will focus on how plants sense the changed conditions of a waterlogged soil. Much of this is still unclear and speculative, but recently progress has been made. Furthermore, we will discuss the two best described signal-transduction pathways leading to morphological and anatomical acclimation to soil flooding; namely, those resulting in aerenchyma formation and in adventitious rooting. Finally, we conclude with a brief summary of the most prominent advances and our view on the future development of the field.

Sensing of flooded conditions

Signals

Plants use external and internal signals to sense changes in the environment, such as shifts from aerial to aquatic. These signals are often the first step in transduction cascades leading to rapid down-regulation of metabolic pathways to decrease oxygen consumption, and, in the long-term, to morphological adjustments such as aerenchyma development and fast shoot elongation to increase oxygen entry (Geigenberger, 2003).

The two internal gaseous signals, oxygen and ethylene, are frequently associated with the responses of plants or plant parts surrounded by water. Submergence has a dramatic effect on the endogenous concentrations of these two components due to the very slow diffusion of gases in water compared to that in air (Jackson, 1985). In non-photosynthesising organs such as roots, oxygen levels will rapidly decline due to continuous oxygen consumption in respiration, and the very slow delivery of aerial oxygen to the root. Ethylene, on the other hand, will accumulate to physiologically active levels in submerged tissues, due to production in almost every organ and hampered diffusion to the atmosphere (Voesenek and Blom, 1999). However, a prerequisite for continued ethylene production is the presence of at least some molecular oxygen, since conversion of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) to ethylene, catalysed by ACC oxidase, uses molecular oxygen as a co-substrate (Kende, 1993).

Ethylene

Elevated ethylene levels are important for the induction of morphological and anatomical traits upon soil flooding, such as formation of aerenchyma and adventitious roots. During the last decade enormous progress has been made in disentangling the ethylene-response pathway from hormone perception at membrane structures to transcriptional regulation in the nucleus. This was mainly achieved with a genetic and molecular analysis of *Arabidopsis* mutants disturbed in ethylene perception and signalling.

In plants, ethylene is perceived by a family of receptor molecules located in the endoplasmic reticulum (ER) (Chen et al., 2002). These receptor molecules share strong homology with bacterial two-component regulators (Chang et al., 1993), and are by default functionally active and are switched to an off-state by ethylene binding to the N-terminal transmembrane part of the dimerized molecule. Ethylene receptors form a complex with a protein called CONSTITUTIVE TRIPLE RESPONSE (CTR). CTR proteins are activated by association with the receptors at the ER and repress downstream ethylene responses. In the presence of ethylene, the ethylene receptor proteins presumably undergo conformation changes that inactivate them. Under these conditions CTR is released from the ER and also becomes inactivated (Gao et al., 2003). Consequently, this derepression will result in ethylene responses. Further downstream ethylene signals are transduced via several positive regulators (e.g., ETHYLENE INSENSITIVE2 (EIN2), EIN5 and EIN6) ending with the transcription factors EIN3 and EIN3-like (EIL) (Guo and Ecker, 2003). An immediate target for EIN3 is the ethylene-responsive gene *Ethylene Response Factor1* (*ERF1*). The ERF1 protein belongs to the family of so called ETHYLENE RESPONSE ELEMENT BINDING PROTEINS (EREBPs) transcription factors. These EREBPs play an important role in controlling expression of ethylene target genes (Solano et al., 1998).

For all known ethylene responses the obligatory components of the signal transduction cascade range from the receptor molecules to the transcription factor

family EIN3/EIL. The branch point resulting in the wide array of ethylene responses lies downstream of EIN3/EIL (Guo and Ecker, 2003).

Oxygen

Oxygen is another internal gas signal that changes dramatically upon submergence. The stress induced by low oxygen concentrations is not restricted to flooding environments, but can occur in tissues characterized by fast metabolic rates (e.g., root meristems, phloem tissue). In these tissues the rate of oxygen delivery via diffusion is too slow to keep pace with its consumption (Geigenberger, 2003).

It is important to distinguish real anoxic conditions from those where cytochrome oxidase activity is not limited by oxygen, but where the oxygen levels are below ambient. Falling oxygen levels are sensed in plants, and lead to a fast inhibition of respiration, a lowering of the adenylate status, and a down-regulation of the TCA cycle and glycolysis (Geigenberger, 2003). These changes are consistent with the down-regulation of genes that encode enzymes involved in the biosynthesis of cell walls, lipids and flavanoids, defense responses and protein degradation in *Arabidopsis* roots exposed to low oxygen (Klok et al., 2002). This inhibition of biosynthetic fluxes and metabolic rates occurs even at oxygen levels that are much higher than the K_m of cytochrome oxidase and alternative oxidase (AOX), indicating that it is very likely that oxygen sensing operates independently of the electron transport chain. However, it cannot be ruled out that diffusion limitation in bulky tissues might, in fact, mean that a proportion of the cells are exposed to oxygen levels below the K_m of cytochrome oxidase and AOX. The observed metabolic shift is assumed to be adaptive, since it decreases oxygen consumption and saves ATP, and thus delays the onset of anoxia (Geigenberger, 2003). In a micro-array study with *Arabidopsis* roots exposed to low oxygen it became evident that next to a set of down-regulated genes, several genes were up-regulated. These up-regulated genes belong to three categories: (i) genes involved in ethanolic and lactic fermentation, (ii) genes that potentially play a role in post-anoxia injury, and (iii) genes related to ethylene synthesis, ethylene signalling, programmed cell death and cell-wall loosening (Klok et al., 2002). The first two classes could be interpreted as pre-adaptive genes that are expressed to continue energy production in subsequent anoxic periods, and that enhance survival in post-flood phases in which oxygen re-enters the plant. The third category of genes is strongly associated with aerenchyma formation which leads to a more long-term acclimation to enhance entry of oxygen.

Thus, in order to timely respond to decreasing oxygen levels, plants need to sense oxygen concentrations that are between the K_m of cytochrome oxidase and AOX and normoxia. This oxygen-sensing system in higher plants operates independently of changes in energy metabolism. This contention is supported by findings that induction of the *ADH1* gene by low oxygen concentration cannot be mimicked by respiratory inhibitors (Bucher et al., 1994). One of the first detectable changes upon oxygen deprivation is an elevation of cytosolic Ca^{2+} (Subbaiah et al., 1994), probably caused by calcium mobilization from mitochondria (Subbaiah et al., 1998), suggesting that mitochondria are at the centre of oxygen sensing (Subbaiah and Sachs, 2003). Several low-oxygen-induced genes are characterized by an anaerobic response element in their promotor (Klok et al., 2002). The transcription factor AtMYB2, induced by low oxygen, binds to this promoter, and can activate *ADH1* promotor activity (Hoeren et al., 1998).

Oxygen-sensing systems have long been known for prokaryotes (reviewed by Bunn and Poyton, 1996). A well-studied example in this respect, found in various bacteria, is the oxygen-sensing protein FixL. It contains a histidine kinase domain belonging to the class of two-component regulatory systems and a heme-binding sensory domain that shares homology with the PAS domain superfamily (Taylor and Zhulin, 1999). Under well-aerated conditions FixL is oxygenated, and kinase activity is turned off. When FixL is deoxygenated during falling oxygen levels it autophosphorylates at a histidine. The subsequent transfer of this phosphoryl group to the transcription factor FixJ triggers a cascade of gene expression (Gong et al., 1998).

Hemoglobins

During recent years much progress has been made on the role of hemoglobin proteins during hypoxia stress. Genes encoding for non-symbiotic class 1 hemoglobins or stress-induced hemoglobins are expressed during low-oxygen conditions (Klok et al., 2002), and upon exposure to elevated levels of nitrate and sucrose (Dordas et al., 2003). These hemoglobin-encoding genes, in contrast to the *ADH* gene, are also induced by respiratory chain inhibitors, indicating

that oxygen affects hemoglobin expression indirectly (Nie and Hill, 1997). Hemoglobin proteins reversibly bind oxygen, and are characterized by very low dissociation constants. Consequently, hemoglobins remain oxygenated at oxygen levels far below those that induce so-called anaerobic responses (Sowa et al., 1998). This chemical property and their indirect response to low oxygen make it very unlikely that hemoglobins act as an oxygen carrier, store or sensor. However, it seems that they do have an important role in hypoxia tolerance of higher plants as illustrated below. Transgenic *Arabidopsis* plants overexpressing a class 1 hemoglobin (GLB1) are more tolerant towards severe hypoxia, and plants overexpressing a mutated *glb1* gene resulting in a protein that has a strongly reduced affinity for oxygen are as susceptible to hypoxia as wild-type plants (Hunt et al., 2002). Similar results were obtained in a system with cell cultures of maize that constitutively express barley hemoglobin in either sense or antisense orientation (Sowa et al., 1998). Furthermore, *GLB1* overexpression in *Arabidopsis* could phenocopy low-oxygen pretreatments in terms of survival (Hunt et al., 2002).

Interestingly, hemoglobins also appear in rapidly growing tissues (e.g., root tips of germinating seeds), and they improve early growth in *Arabidopsis*, even under normoxic culture conditions (Hunt et al., 2002; Dordas et al., 2003). This occurrence probably reflects the presence of localized low oxygen tensions often observed in densely packed organs such as meristems.

Second messengers

Hemoglobin up-regulation is not directly controlled by low oxygen, but presumably a consequence of reduced ATP levels (Nie and Hill, 1997). This stresses the importance of second messengers in the regulation of low-oxygen responses. Very recently, the decline in cytosolic pH, probably caused by lactate fermentation, was identified as a second messenger controlling water permeability of *Arabidopsis* root cells exposed to anoxia (Tournaire-Roux et al., 2003). In a set of elegant experiments, Tournaire-Roux and colleagues (2003) demonstrated that the decline in hydraulic conductivity in roots during anoxia was related to a hampered water influx through plasma-membrane intrinsic proteins (PIPs) induced by cytosolic acidification. A particular histidine at position 197 of the aquaporin molecule was responsible for cytosolic pH sensing, and thus for gating these water-channel proteins.

Next to Ca^{2+} and pH, hydrogen peroxide (H_2O_2) was very recently identified as a second messenger in responses to low oxygen (Baxter-Burrell et al., 2002). In *Arabidopsis*, exposure to low oxygen increases the production of H_2O_2 co-ordinately with a substantial increase in ADH activity. The production of H_2O_2 is fine-tuned by two proteins, Rop and RopGAP4, in which RopGAP4 negatively regulates Rop and Rop has a positive regulatory impact on RopGAP4. It is of utmost importance that this Rop rheostat controls the levels of H_2O_2 in plants cells precisely, since levels that are too high may trigger formation of reactive oxygen species that may induce cell death, whereas levels that are too low prevent the expression of adaptive genes (e.g., *ADH*) that improve survival during low-oxygen stress (Baxter-Burrell et al., 2002).

Signal transduction – hormones and further down-stream components

Aerenchyma formation

Aerenchyma is the specialised tissue in petioles, stems and roots consisting of longitudinal gas-filled channels, which may result from various anatomical patterns of cellular configuration (Smirnoff and Crawford, 1983; Justin and Armstrong, 1987; Visser et al., 2000). A common feature of aerenchyma is that it forms a network of gas-filled spaces between the cells that interconnects most parts of the (partly) submerged plant with the atmosphere, and thereby is able to deliver oxygen almost throughout the plant (Armstrong, 1979). Conversely, gases produced by the soil or plant may be vented through these channels to the atmosphere (Colmer, 2003).

Schizogenous versus lysigenous aerenchyma

In general, two types of aerenchyma can be distinguished, although intermediate forms do occur. The first type, which is most abundant in roots and rhizomes, is initiated by the death of cells in the cortex, resulting in gas-filled voids between the living cells that remain. This type is named lysigenous aerenchyma, after the lysis of cells that precedes gas space development (Figure 1A). The second type is schizogenous aerenchyma, which forms through the separation of cells from each other in an early stage of development (Figure 1B, C). Schizogenous aerenchyma may also be found in roots, but more often in the stems and petioles of wetland plants. In both aerenchyma types, there is a large variation

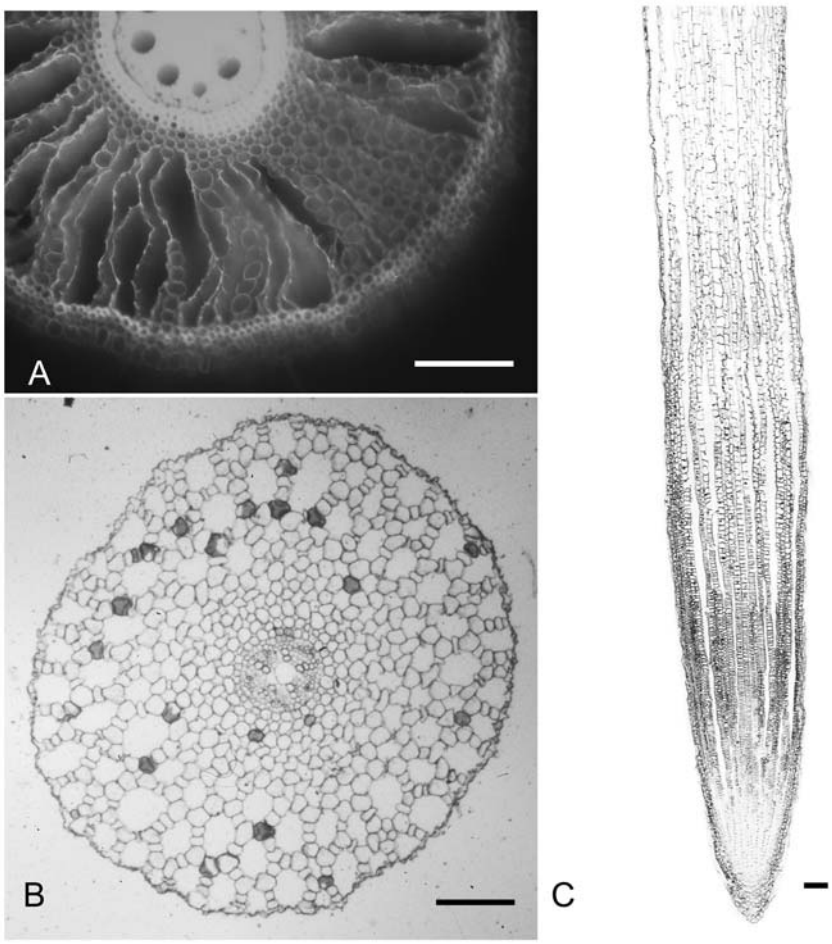

Figure 1. A. Cross-section of the aerenchymatous root of a hydroponically grown *Juncus effusus* plant. Large lysigenous aerenchyma has developed in the cortical parenchyma. B. Cross-section of an adventitious root of a hydroponically grown *Rumex palustris* plant. C. Longitudinal section of a similar root. The aerenchyma forms in a schizogenous way by directional division and separation of the cortical cells (B) and develops close to the root apex (C). Scale bars indicate 0.2 mm.

in the exact configuration of the cells and cell remnants, as shown by Justin and Armstrong (1987) in their extensive screening of root anatomy of wetland, intermediate and non-wetland species.

Schizogenous aerenchyma seems a more or less constitutive feature within a given root, and does not change in those roots that are already present at the onset of soil flooding. Instead, new roots may develop that contain a larger amount of schizogenous aerenchyma (Laan et al., 1989; Visser et al., 1996a). In contrast, lysigenous aerenchyma can develop in both mature and in newly-developing roots (although older wheat roots were not capable to form aerenchyma (Thomson et al., 1990)), and its presence often depends on environmental stimuli. Many crop species, such as wheat (*Triticum aestivum* – Huang et al., 1994; Boru et al., 2003; Malik et al. 2003), barley (*Hordeum vulgare* - Bryant, 1934; Garthwaite et al., 2003), maize (McPherson, 1939; Drew et al., 1979; Konings and Verschuren, 1980), sunflower (Kawase, 1979), and rice (Jackson et al., 1985b), form lysigenous aerenchyma. This is probably the reason why so little research has been done on the regulation of schizogenous aerenchyma formation (Jackson and Armstrong, 1999), whereas the inducible signal-transduction pathway of lysigenous aerenchyma has gained far more attention, particularly by using maize root aerenchyma as a model.

Hypoxia and ethylene

The first evidence for a role of ethylene in the induction of aerenchyma in tissues of waterlogged plants

was derived from studies on sunflower. However, soon after the discovery that treatment of sunflower with ethylene resulted in similar aerenchyma formation in the hypocotyl as did soil flooding (Kawase, 1974), Drew et al. (1979) reported that root aerenchyma could be evoked by treating maize with ethylene. Also, inhibition of ethylene action by silver ions inhibited aerenchyma formation in waterlogged maize plants (Drew et al., 1981). The latter species proved to be a suitable model to investigate lysigenous aerenchyma formation, and subsequent research was done to find the triggers that set off the lysis of cells. One would expect that accumulation of ethylene due to the physical entrapment by the surrounding water-saturated soil suffices to induce aerenchyma. Nevertheless, the prevalent low oxygen concentrations further stimulate ethylene production (Jackson et al., 1985a), thereby adding to the build-up of ethylene concentrations. Ethylene levels exceeding 0.5 μL L^{-1} are usually sufficient to evoke the maximum response in treated roots.

Much of the role that ethylene plays has been elucidated since these early observations (Figure 2; Drew et al., 2000). He et al. (1994) described an increase in the root tips of maize of the enzyme that is largely responsible for the rate of ethylene biosynthesis, i.e. ACC-synthase, as a response to low oxygen concentrations. This response was not present when oxygen was completely absent (accomplished by flushing with nitrogen gas), possibly because strict anoxia largely prevents protein synthesis. Application of either low oxygen or increased ethylene concentrations resulted in an increase of the cellulase activity in the root apex (He et al., 1994), which therefore likely contributes to cell-wall break-down, being the last step in lysigenous aerenchyma formation. Blocking the activity of ACC-synthase with a specific inhibitor (aminoethoxyvinylglycine, AVG) suppressed cellulase activity to the constitutive control levels, providing further proof that ethylene is the plant hormone responsible for controlling this part of aerenchyma development (He et al., 1994).

Down-stream parts of the signal transduction pathway

Earlier processes in the chain of events leading to death of cells predestined to become aerenchymatous spaces are still largely unknown. A first attempt to elucidate these was the application of inhibitors and elicitors of programmed cell death (PCD) that were previously used successfully in animal systems or with other PCD processes in plants (He et al., 1996b). K-252a, a substance that inhibits protein kinases and protein kinase C in particular, inhibited both cellulase activity and aerenchyma formation under low oxygen concentrations, suggesting a role for protein kinase C in the induction of aerenchyma. On the other hand, specific inhibition of protein phosphatases 1 and 2A by applying okadaic acid (Cohen et al., 1990) is supposed to enhance protein phosphorylation and proved to promote cellulase activity and aerenchyma formation (He et al., 1996b). Similarly, GTPγS, which is capable of locking G-proteins in the active state, increased the volume of aerenchyma under normoxic conditions, whereas its analogue GDPβS, which inactivates the proteins, did not have effect on the aerenchyma content, neither under normoxic nor under low-oxygen conditions. Apart from protein phosphorylation processes and G-proteins, also inositol phospholipids appear to be involved, since neomycin, which interferes with the binding of inositol phospholipids with the plasma membrane, almost fully prevented low-oxygen-induced aerenchyma formation (He et al., 1996b).

Manipulation of cytoplasmic calcium concentrations and fluxes also proved successful in changing the capacity to form aerenchyma (He et al., 1996b). Both thapsigargin and caffeine increase intracellular calcium concentrations, by blocking Ca^{2+}-ATPase activity in the ER, and opening Ca^{2+}-channels, respectively. Either substance resulted in an increase in aerenchyma content in normoxic roots, and in a faster progress of PCD in oxygen-deficient roots. Lowering Ca^{2+} levels with EGTA (a Ca^{2+}-chelator) or ruthenium red (which blocks Ca^{2+}-channels) inhibited cell lysis. Ruthenium red also binds to calmodulin (CM), thereby preventing Ca-CM dependent protein activity, and a similar effect could be expected from $W-7$ ($N-$[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide), which inhibits CaCM-mediated ion-channel function. Again, the latter substance prevented aerenchyma formation. These results make it highly likely that an influx of Ca^{2+} into the cytoplasm is a necessary step in the process leading to cell death (see also Figure 2).

Programmed cell death

The chemical compounds mentioned above have originally been successfully applied in studies of apoptosis in animal cells, and the similarity in responses between apoptosis in these systems and aerenchyma formation suggested that the regulation of these processes

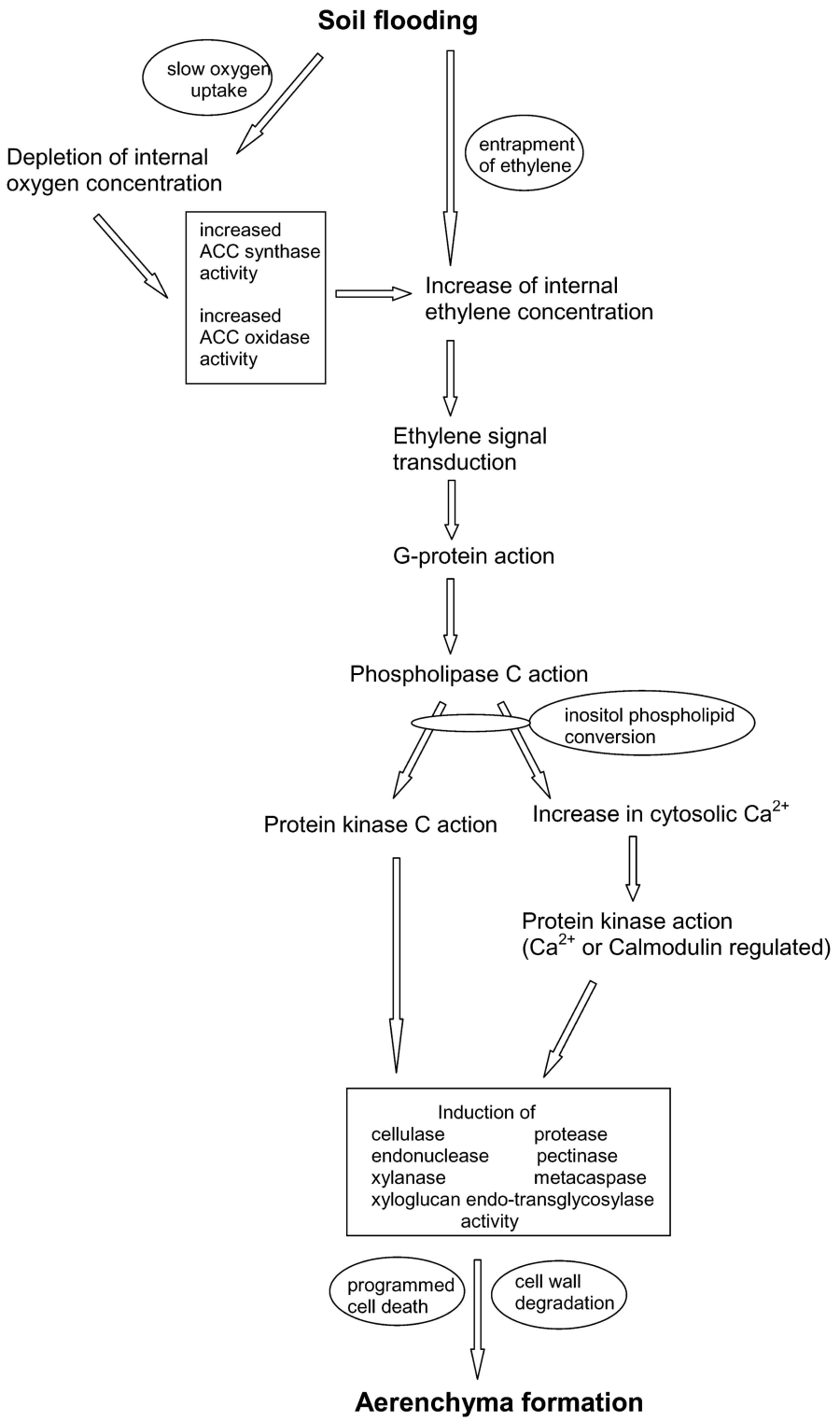

Figure 2. Schematic view of the processes likely involved in lysigenous aerenchyma formation. Drawn according the data and schemes shown and reviewed in Drew et al. (2000), Gunawardena et al. (2001), Woltering et al. (2002), Aschi-Smiti et al. (2003) and Bragina et al. (2003). Most of the data originated from studies on maize (*Zea mays*).

may be similar at the cellular level (Pennell and Lamb, 1997; Buckner et al., 1998). However, more detailed studies changed this view somewhat. Campbell and Drew (1983) already observed some early events at the cytoplasmic level in maize root cells that underwent programmed cell death, but only more advanced techniques made it possible to search for the specific changes involved in apoptosis.

TUNEL-staining of fragmented nuclear DNA showed that already in 0.5-day-old ethylene-treated cortical root cells endonuclease activity increased, which was confirmed by increased laddering of genomic DNA on an agarose gel (Gunawardena et al., 2001). Apoptotic ultrastructural changes of the cells, such as chromatin condensation and its relocation to the nuclear periphery, could be detected after one day by electron microscopy. Changes in the plasma membrane, differences in the staining of vacuoles and cytoplasmic content (of which the meaning was not known), and the occurrence of large numbers of vesicles preceded the visual changes in the nucleus. Plasmodesmata then became very distinct in 1.5-day-old tissues, and apoptotic body-like structures containing entire cell organelles appeared, which were possibly involved in the hydrolysis of cell components. Although there are quite a few similarities, it is evident that there are also clear differences between apoptosis in animal cells and the programmed cell death during aerenchyma formation. For example, macrophage digestion does not take place at the final stages in the plant tissue, as it does in animal cells, and also the sequence of nuclear and other cytosolic events is different, as the latter do not precede nuclear changes in animal apoptosis (Gunawardena et al., 2001). Apparently, aerenchyma formation forms a class of programmed cell death of its own.

Although in animals a group of proteins named caspases form an intrinsic element of the cascade of events in programmed cell death, until recently no direct evidence was found for the presence of functional homologues of these enzymes in plants (Woltering et al., 2002). However, indirect evidence suggested that caspase-like activity is needed for programmed cell death in plants (Woltering et al., 2002), and metacaspases have now been identified that may serve as such in plants (Uren et al., 2000; Hoeberichts et al., 2003).

Cell-wall-degrading enzymes

The final step in aerenchyma formation is the breakdown of cell walls, which ultimately creates the voids needed for gas diffusion. Conditions of low oxygen and high ethylene concentrations may induce a steep increase in cellulase activity within three days (He et al., 1994). This was confirmed by Bragina et al. (2003), who also found increased levels of pectinase and xylanase activity. Additionally, a structural homologue of xyloglucan endo-transglycosylase (XET) was found to be induced by hypoxia (Saab and Sachs, 1996). This combination of enzymes enables a stepwise degradation of the cells and resorption of their structural components.

It is interesting that quite often radial files of cells remain unaffected by the lytical process (Justin and Armstrong, 1987), and also the presence of a developing lateral root may inhibit cell break-down. Apart from this, programmed cell death usually does not affect the inner- and outermost parenchyma layers of the cortex. The reason of this 'immunity' of cells to the action of ethylene or to components more downstream of ethylene is unknown. Also it is not clear why in many species mainly the tangential cell walls collapse, whereas the majority of radial walls remain intact (*e.g.*, in Poaceae); conversely, in Juncaceae and Cyperaceae most tangential walls remain, while a large part of the radial walls disappear, resulting in so called 'spider's web' aerenchyma (Smirnoff and Crawford, 1983). This directional targeting of cells and cell-wall material requires a very specific distribution of signals, or of the sensitivities to these signals, and much remains to be elucidated about how these patterns develop.

Other growth regulators

Some attention has been paid to a possible role of polyamines in aerenchyma formation in maize roots, because spermine and spermidine are biosynthesised from the same precursor as ethylene (*S*-adenosylmethionine), and a decline of these polyamines may be involved in the rise of ethylene production during hypoxia (Jackson and Hall, 1993). However, no proof could be found for such an involvement, since these polyamine levels did not decrease in hypoxic maize roots, and addition of putrescine, a precursor of spermine and spermidine, inhibited rather than increased aerenchyma formation (Jackson and Hall, 1993).

Auxin has been studied as a potential regulator of aerenchyma content as well (Konings and De Wolf, 1984; Justin and Armstrong, 1991a). The first study indicated an inhibition of aerenchyma formation in maize roots by the synthetic auxin 1-naphthalene

acetic acid (1-NAA), but, after adjustment of the data for the profound effects of auxin on the root growth rate, Justin and Armstrong (1991a) found a small stimulation, which they attributed to auxin-induced ethylene production. Gibberellin (GA_3) and kinetin (a cytokinin) also promoted the formation of aerenchyma in maize (Konings and De Wolf, 1984), which may again, in the case of GA_3, be caused by ethylene action, since the effect was counteracted by the simultaneous application of ethylene inhibitors. In the same study, abscisic acid (ABA) had a negative effect on aerenchyma, but none of these effects have been followed up in further research.

Other plant species

The hormonal regulation of aerenchyma formation in maize has at least partially been validated for several other plant species. Aerenchyma formation in crop species such as sunflower, bean (*Phaseolus vulgaris*), tomato (*Lycopersicon esculentum* – Kawase, 1981) and wheat (Wiengweera et al., 1997) and in tree species like *Pinus serotina* (Topa and McLeod, 1988) seems under the control of low oxygen concentrations and ethylene, although silver did not affect aerenchyma formation in barley (Larsen et al., 1986). In *Trifolium subterraneum* a change in protease composition was shown following hypoxia, which correlated with cell lysis during root aerenchyma development and may point to the expression of a specific set of lysis-involved protease genes (Aschi-Smiti et al., 2003). However, the lack of model species that develop inducible aerenchyma and are also suitable for molecular-genetic studies seems to have hindered further progress in the signal transduction chain down-stream of ethylene.

Constitutive versus inducible aerenchyma

Many wetland plant species display a considerably high porosity in their roots even under well-aerated conditions (Justin and Armstrong, 1987). This is due to the presence of aerenchyma whose development is apparently not dependent on low oxygen concentrations or ethylene accumulation. It is as yet unclear how the onset of programmed cell death is initiated in these examples of constitutive aerenchyma, as until now only two studies dealt with this question, and their conclusions are contradictory. Jackson et al. (1985b) used inhibitors of ethylene in two cultivars of rice, of which one formed partly constitutive and partly inducible aerenchyma, whereas the other only developed constitutive aerenchyma. In the latter cultivar aerenchyma formation was not affected by ethylene inhibition, which led the authors to conclude that ethylene does not play a role in constitutive aerenchyma development. In response to this paper, Justin and Armstrong (1991b) partly repeated the experiments, but corrected the data for the negative effect of the treatments on the elongation rate of the aerenchymatous roots. With this correction, they concluded that ethylene did play a role. Most likely the root cortical cells had a much higher sensitivity to ethylene than in plant species with inducible aerenchyma, making them responsive to the low internal ethylene concentrations that prevail in a non-flooded root system. It would be interesting to conduct similar experiments with plant species whose root elongation is less sensitive to ethylene. This would prevent confounding of the results by side effects of ethylene. Until then, it remains to be seen whether these conclusions are broadly valid for other wetland plants with constitutive aerenchyma.

Factors other than flooding that induce aerenchyma

Next to oxygen shortage, also nutrient deficiency may lead to programmed cell death in the root cortex. Konings and Verschuren (1980) observed the development of aerenchyma in maize roots when these were grown in aerated but nitrogen-deficient nutrient solution, and Smirnoff and Crawford (1983) found that treatment of *Nardus stricta* plants in sand culture with low concentrations of nutrient solution increased root porosity to equally high levels as in waterlogged plants. Studies with maize later confirmed that both low concentrations of nitrate and low levels of phosphorous nutrition induced aerenchyma in the roots, and that this was accompanied by lower activities of ACC-synthase and ACC-oxidase, lower ACC levels and, therefore, lower ethylene-production rates (Drew et al., 1989). This apparent contrast to the response of the roots to hypoxia, where ethylene production increases and can be causally linked to programmed cell death (Jackson et al., 1985a; Atwell et al., 1988), could be explained by subsequent work by Drew and co-workers. Although ethylene biosynthesis slowed down upon nutrient deficiency, this was counteracted by a strong increase in the sensitivity of the cortical tissues to ethylene, so that the threshold leading to aerenchyma development was exceeded (He et al., 1992).

Recently, the promoting effect of low mineral nitrogen on ethylene sensitivity has been confirmed in a study where ethylene interacted with the release of herbivore-induced volatiles, such as indole and

sesquiterpenes (Schmelz et al., 2003). Analogously, low phosphorus increased the sensitivity of root elongation in *Arabidopsis thaliana* to ethylene (Ma et al., 2003). Aerenchyma formation does not appear to be exclusively induced by low phosphorus and nitrogen, since Bouranis et al. (2003) found similar responses in sulphate-starved maize plants.

The aerenchyma in nutrient-starved plants seems to have an identical structure as that in roots of waterlogged plants, and the acting signal-transduction pathway is probably the same, except for the part upstream of ethylene perception. However, it is conceivable that the increased ethylene sensitivity of phosphorus- and nitrogen-depleted plants serves a different goal than aerenchyma formation per se, e.g., a change in root topology (Borch et al., 1999) or the formation of root hairs (although the latter is unlikely given the evidence provided by Schmidt and Schikora (2001) that low iron but not low phosphorus induces root hairs via the ethylene-perception pathway). On the other hand, Fan et al. (2003) measured respiration rates of phosphorus-starved roots, which were lower per volume root due to the lower number of cells after aerenchyma developed. Combined with the assumption that cell components are being resorbed during cell lysis, this would imply a lower investment of construction and maintenance costs per unit root length, which would in turn add to the capacity of the plant to explore the soil for sources of phosphorous.

Finally, soil compaction can also lead to formation of aerenchymatous tissues, which develop independently from low oxygen concentrations (He et al., 1996a). Again, ethylene plays a key role in the process, possibly by increased biosynthesis rates due to the pressure exerted on the root tip and by ethylene accumulation resulting from the increased gas diffusion resistance in the rhizosphere. It is, however, unlikely that aerenchyma is a favourable structure under conditions of high soil strength. Studies on *Rumex* and *Plantago* species indicated that aerenchymatous roots grew in a highly distorted way when encountering compacted soil (Engelaar et al., 1993b).

Adventitious root development

Flooding often causes malfunctioning of roots formed prior to flooding, even in wetland species (Justin and Armstrong, 1987; Visser et al., 1996a). This may eventually lead to the death of a considerable part of the root system, and a fast replacement by well-adapted adventitious roots that contain more aerenchyma than the original roots (Table 1, Figure 3).

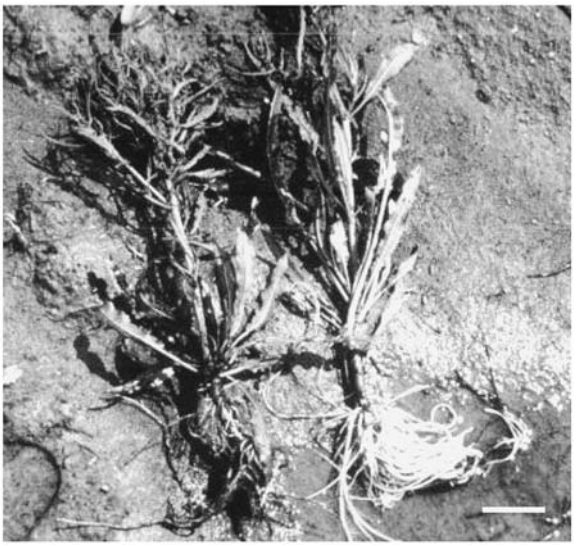

Figure 3. Root systems of intact *Rumex palustris* plants excavated from a floodplain near Nijmegen, the Netherlands. Left: a plant from a site with the groundwater table at 0.15 m below the soil surface. Right: a plant from a soil-flooded site. Notice the abundant development of thick, white adventitious roots in the soil-flooded plant, as compared to the thinner, brownish primary root system of the drained plant. Scale bar indicates 0.1 m.

There is some controversy about the exact definition of the term adventitious root, as many authors use it specifically for shoot-derived roots (i.e. roots that originate from shoot tissue), whereas others argue that roots that develop from older parts of the root system, where normally new root development would not take place, should also be considered adventitious roots (Barlow, 1994). In the current context, we will adopt the second definition, since there seems to be no functional difference between the roots that develop from the base of the stem and those that develop just centimetres lower on the upper part of the taproot (Visser et al., 1996a).

Kramer (1951) recognised that the recovery of tomato plants from the initial growth reduction imposed by soil flooding coincided with the formation of new adventitious roots. Since then, it has been well established that adventitious root development contributes greatly to the tolerance of a plant to poorly aerated soils, and the biomass of roots formed by a flooded plant usually correlates well with its resistance to waterlogging, as has been shown for closely related species of several genera (*Rumex* –Laan et al.,

Table 1. Fresh mass of primary and adventitious roots from hydroponic *Rumex palustris* plants of different age, before and after 1-week growth on stagnant, oxygen-deficient agar solution (for methods see Visser et al., 1996a). SE in brackets; $n = 6$

Age of the plant at start of treatment	Root fresh mass (g)			
	At the start of treatment		After 1 week on stagnant agar	
	Primary roots	Adventitious roots	Primary roots	Adventitious roots
2.5 weeks	0.01 (0.00)	0.00 (0.00)	0.13 (0.02)	0.08 (0.02)
3.5 weeks	0.19 (0.01)	0.00 (0.00)	0.39 (0.05)	0.77 (0.09)
4.5 weeks	2.14 (0.11)	0.04 (0.01)	1.54 (0.18)	2.26 (0.23)
5.5 weeks	5.63 (0.54)	0.07 (0.02)	4.35 (0.82)	2.82 (0.46)

1989; Visser et al., 1996a; *Pinus* – Topa and McLeod, 1986b; *Hordeum* – Garthwaite et al., 2003; various other genera – Justin and Armstrong, 1987). Flooding-induced adventitious roots are usually thick and have a relatively low degree of branching, whereas much of the cortical cell layers are occupied by schizogenous or lysigenous aerenchyma. Certain groups of plant species form adventitious roots in the natural course of root development (i.e. also without soil flooding), but even in these cases adventitious root formation is favoured above primary root development when the root system is flooded (McDonald et al., 2001a).

A major role for auxin

Adventitious roots are not only formed by waterlogged plants, but are also essential in the regeneration process of shoot cuttings, a common propagation technique in horticulture. From these studies we know since long that auxins are essential regulators in the re-differentiation of shoot cells into root-forming meristems, and Phillips (1964) was the first to experimentally link auxin action to the onset of flooding-induced adventitious root formation. The results of this study indicated that auxin accumulated at the base of the shoot of soil-flooded sunflower plants as a result of the oxygen deficiency of the roots, like suggested before by Kramer (1951). Since IAA is transported in a polar fashion by carrier proteins that are ATPase-dependent (reviewed by Palme and Gälweiler, 1999), hypoxia-induced low levels of ATP may disturb active auxin movement in the root, thereby causing auxin that is transported downward from the shoot to accumulate at the boundary of oxygen-deficient and normoxic plant tissues. Similar to auxin application or removal of the root system, such flooding-induced accumulation could then lead to or enhance adventitious root formation at the base of the shoot and upper part of the tap root. More recent work on sunflower has shown that auxin is indeed crucial to adventitious root formation in both shoot cuttings of this species (e.g., Fabijan et al., 1981; Liu and Reid, 1992; Oliver et al., 1994) and in flooded plants (Wample and Reid, 1979). Auxin is also important for adventitious rooting in other species that were given a soil flooding treatment, such as *Rumex palustris* and *R. thyrsiflorus* (Visser et al., 1995), *Acer negundo* (Yamamoto and Kozlowski, 1987) and tobacco (*Nicotiana tabacum* – McDonald and Visser , 2003). Flooding-induced root formation could experimentally be inhibited by specifically blocking auxin transport in the shoot with N-1-naphtylphtalamic acid (NPA) (Visser et al., 1995; McDonald and Visser 2003) or by applying competitors for auxin-binding sites (Visser et al., 1995). This clearly indicates that auxin is an important component in this acclimation process, at least in dicotyledonous species. Whether this also applies to monocotyledonous species is not clear, since experimental evidence is scarce. Lorbiecke and Sauter (1999) did not find an effect of applied auxin on adventitious root formation in rice (except via ethylene, see below), whereas Zhou et al. (2003) claimed that endogenous auxin is critical for adventitious root development in the same species.

Involvement of ethylene

Not only auxin but also ethylene can induce adventitious roots, although in cuttings the response seems to depend on the plant species. For instance, ethylene inhibited adventitious root formation in pea (*Pisum sativum*) cuttings (Nordström and Eliasson, 1984) and *Prunus avium* explants (Biondi et al., 1990), whereas it stimulated root initiation in cuttings of *Picea abies* (Bollmark and Eliasson, 1990). Also, experimental conditions seem to matter, as contrasting results were found in various studies on mung bean (*Vigna radiata*

- e.g., Robbins et al., 1983 vs. Geneve and Heuser, 1983). More recently, a study of the rooting capacity of two ethylene-insensitive genotypes, i.e. the tomato mutant *never ripe* and the transgenic petunia (*Petunia x hybrida*) line 44568, showed that both intact ethylene-insensitive plants and their vegetative cuttings produced far less adventitious roots than did their respective wild-types (Clark et al., 1999). Since these plants have a defective ethylene receptor, they were not very responsive to the application of the precursor of ethylene, ACC. However, more surprising was the lack of response of ethylene-insensitive plants to indole-butyric acid (IBA), whereas in wild-type plants this auxin increased adventitious root formation if applied in moderately high concentrations. Apparently, ethylene is in these species indispensable for normal adventitious root development, even when rooting is evoked by auxin application.

During soil-flooded conditions, ethylene concentrations in the root system may increase up to several μL L^{-1}, as shown for *Rumex* plants (Visser et al., 1996b). These concentrations proved to be sufficient to induce adventitious root formation, even when plants were not flooded, whereas flooded plants with impaired ethylene production (by the use of inhibitors) produced fewer roots than normally under flooded conditions (Visser et al., 1996b). A similarly important role for ethylene has been found for rice (Bleecker et al., 1987), maize (Drew et al., 1979), and tobacco (McDonald and Visser, 2003). Studies with sunflower (Wample and Reid, 1979) and *Acer negundo* (Yamamoto and Kozlowski, 1987), however, attributed a less essential role to ethylene with respect to flooding-induced adventitious root formation.

One way ethylene may promote adventitious root development was elucidated by Mergemann and Sauter (2000). Similar to its role in aerenchyma formation, ethylene seemed involved in programmed cell death, in this case of the epidermal layer of rice nodal initiation sites of adventitious roots, thereby providing unhampered outgrowth of the root primordia.

A positive effect of ethylene on adventitious root development under soil-flooded conditions seems, however, contradictory to its usual effect on root growth, as many studies report a strong inhibition of root elongation by increased ethylene (e.g., Konings and Jackson, 1979; Etherington 1983; Visser et al. 1997). Possibly, internal ethylene concentrations can be kept sufficiently low in extending adventitious roots by the ventilating aerenchyma that is often present in such flooding-induced roots.

Interactions between auxin and ethylene

Auxin and ethylene action are not entirely independent of each other; increases in the action of one of these hormones may cause a change in the action of the other. The best known effect is that auxin generally increases the production of ethylene (e.g., Imaseki et al., 1977), but the opposite is also possible. Increased ethylene concentrations may affect the polar transport of auxin in a positive (Beyer and Morgan, 1969; Suttle, 1988) or negative way (Musgrave and Walters, 1973), thereby changing the delivery rate and concentrations at the shoot base. Perhaps more importantly, ethylene may increase the sensitivity of plant tissues to auxin, such as demonstrated in pea coleoptiles (Bertell et al., 1990) and sunflower hypocotyls (Liu and Reid, 1992). In the latter study, applied ethylene stimulated auxin-dependent root formation at the base of hypocotyl cuttings without increasing auxin concentrations. A very similar mechanism appeared to induce adventitious roots in flooded *Rumex palustris* (Figure 4; Visser et al., 1996c). Although auxin was clearly needed for an effective rooting response, shown by increased adventitious root formation upon auxin supply and decreased rooting in the presence of competitive or transport inhibitors (Visser et al., 1995; Visser et al., 1996c), there was no change in endogenous free IAA concentration when plants were waterlogged. Ethylene-induced root formation in the same species appeared auxin-dependent, whereas auxin-induced adventitious root formation was not affected if ethylene production was blocked, indicating that in these plants ethylene accumulation probably led to an increase in sensitivity of the root-forming tissue to auxin, which then triggered adventitious root development (Visser et al., 1996c). This contrasts with the increased levels of auxin found by Phillips (1964) and Wample and Reid (1979) in flooded sunflower plants, which may have been the result of oxygen deficiency or increased ethylene concentrations in the root system, both capable of impairing the auxin-transport system. In ethylene-insensitive transgenic tobacco, the number of adventitious roots induced by flooding was less than half that of wild-type plants, and this effect could not be fully restored by auxin application (McDonald and Visser, 2003). On the other hand, treatment of wild-type plants with NPA, an inhibitor of the auxin-efflux carrier that is part of the polar transport mechanism, decreased adventitious root formation to the level of ethylene-insensitive plants (McDonald and Visser, 2003). The results of this study suggest again interaction of auxin and ethylene in

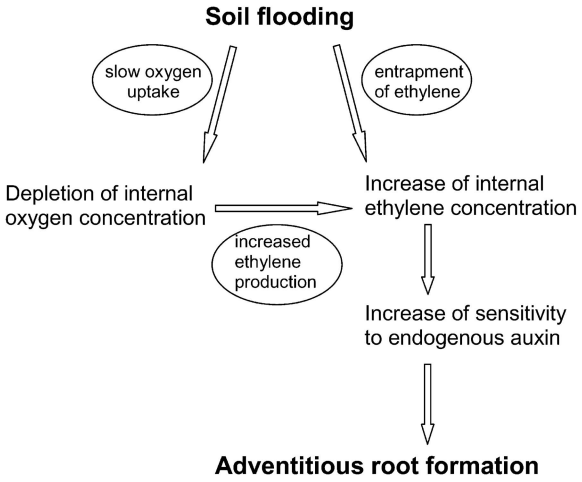

Figure 4. Schematic action scheme of the induction of adventitious root development in *Rumex palustris*. Drawn according the data shown in Visser et al. (1995, 1996b, c).

controlling flooding-induced adventitious root formation. Apparently, multiple signal-transduction pathways are possible, with varying roles for ethylene and auxin, depending on the species, and probably also on the exact environmental conditions, which makes it difficult to arrive at a unifying model on auxin and ethylene action in flooding-induced adventitious root formation.

Other hormones

There is very little evidence for a possible role for hormones other than auxin and ethylene in flooding-induced adventitious root formation. Application of cytokinin and gibberellin to deepwater rice did induce some roots, but not nearly as many as did ethylene (Lorbiecke and Sauter, 1999). It is conceivable that these treatments may have evoked some extra ethylene production. Earlier, Suge (1985) found in the same species a synergistic effect of gibberellin, when applied together with ethylene, but this could not be reproduced in a later study (Lorbiecke and Sauter 1999). It cannot be ruled out that other hormones do play a role in other species, particularly when these do not constitutively produce adventitious root primordia, such as rice does, but there is as yet no information available.

Genes involved in flooding-induced adventitious root formation

Surprisingly little is known about the genes that regulate the initiation and development of adventitious root primordia during soil flooding. In deepwater rice, Lorbiecke and Sauter (1999) distinguish four stages of adventitious root development: (i) an initiation phase, followed by (ii) development into a root primordium, and then subsequent (iii) arrest of growth until an appropriate stimulus causes (iv) further development and emergence of the root from the stem epidermis. The first three stages seem part of the constitutive development course of the rice stem nodes. The final phase is under control of flooding and ethylene, and emergence is apparent within 10 h after reception of a stimulus. This emergence of adventitious roots is preceded by the expression of a number of genes involved in regulating the cell cycle, such as the mitotic cyclin *cycB2;2*, which is a marker for dividing cells. Most of these genes are also expressed during flooding-induced internode growth, and are most likely involved in the transition of cells from one phase of the cell cycle to the following. One gene appeared to be expressed specifically in developing roots, and not in the stem intercalary meristem, i.e., *cdc2Os-1*, and may therefore be part of a root-specific signal-transduction pathway that is triggered by ethylene (Lorbiecke and Sauter, 1999).

Summary and perspectives

The responses of plants to soil flooding are diverse, but mostly focused on surviving the deleterious effects of oxygen deficiency imposed by the flood water. In the short term, anaerobic metabolism may partly overcome the low energy production under anaerobiosis. However, if plants are subjected to long-term flooding (i.e., weeks or months), a more structural solution is provided by restoring the oxygen supply to the submerged plant parts. Formation of aerenchyma and new roots containing this specialised tissue are two means of morphological acclimation that fulfil this task.

The timing of acclimation strongly depends on what signals are involved in sensing the environmental stress. Waterlogging and submergence in higher plants is sensed through changed concentrations of at least two signal molecules: ethylene and oxygen. Ethylene perception and transduction has been the subject of intense study during the last decade, and the molecule forms a very reliable detection system for submerged plant organs that contain some oxygen. It triggers important anatomical and morphological modifications (e.g., aerenchyma formation, adventitious roots, stimulated shoot elongation) that improve the oxygen status of submerged organs.

Virtually nothing is known about the sensing mechanism for oxygen in higher plants, although systems homologous to those described for various prokaryotes are likely. Cleverly designed screens, preferably for *Arabidopsis thaliana*, should be developed to identify mutants disturbed in oxygen sensing, assuming that these mutants are not lethal. Lethal mutations are not completely unexpected, since low-oxygen conditions seem to be an integral part of plant growth and development, even when this takes place under normoxic conditions. More information about second messengers in low-oxygen sensing has become available during the past few years. Important signals in this respect are Ca^{2+}, pH and H_2O_2.

Interestingly, it is now recognised that responses to low oxygen can take place even before cytochrome oxidase activity becomes limited (reviewed by Geigenberger, 2003). Respiration may be inhibited already at oxygen concentrations of 8–12%, when fermentation and the 'Pasteur effect' are not yet apparent. This down-regulation of respiratory activity, followed by decreased ATP levels, can therefore not be controlled by the status of the electron transport chain, as this is not affected by these relatively high oxygen concentrations.

Following the perception of the flooding signal, several signal-transduction mechanisms will be activated that control the acclimation mechanisms. A better understanding of the signal-transduction chains that lead to root acclimation during soil flooding would increase our possibilities to manipulate the extent of acclimation, and thereby obtain tools to study the benefits and costs of acclimations. Some comparable examples are already known from flooding physiology, for instance with respect to the capacity of shoot elongation in rice in response to total submergence (Setter and Laureles, 1996). Blocking the elongation response with paclobutrazol, an inhibitor of gibberellin biosynthesis, increased survival under water considerably, probably since allocation of carbohydrates to the elongating shoot occurs at the expense of the carbohydrate reserves needed for (partially anaerobic) respiration. In another study, inhibition of ethylene perception in transgenic tobacco led to a decrease in adventitious root formation upon simulated soil flooding, which in turn decreased the biomass gain of the plants under these conditions (McDonald and Visser, 2003).

It is unfortunate that the most widely used genetic and molecular-biological plant model, *Arabidopsis thaliana*, has such a low resistance to flooding stress. Oxygen-sensing mechanisms may be present in this species, possibly allowing for screens of sensing mutants; however, the plant is largely unsuitable for studying the responses of plants to soil flooding, except for some acclimations that are surprisingly well preserved even in this flood-intolerant species (e.g., submergence-induced hyponasty of the leaves (Cox, 2004) and hypoxia-induced fermentative enzymes (Dolferus et al., 1997)). Rice may turn out to be the best alternative model species, as most acclimation responses (i.e. fermentation, aerenchyma formation, adventitious root development) are displayed by this species. A great advantage is the known sequence of the rice genome (Yu et al., 2002), which increases the speed and possibilities of molecular genetics considerably. Further development of heterologous microarray analyses, such as recently accomplished for adventitious root formation in *Pinus* (Brinker et al., 2004), may also help to circumvent the lack of model species. Another approach to pinpoint the genes or chromosomal regions that control flood tolerance is the use of crosses between waterlogging-tolerant and less tolerant species, cultivars or ecotypes. In this way a group of hybrids may be developed that encompass a wide variety in root traits such as aerenchyma content, radial oxygen loss or adventitious root formation (McDonald et al., 2001b; Colmer 2003). If genetic markers are made available for these species, QTL analysis may reveal the area on the genome where genes crucial for the expression of these traits are located. In conclusion, many advances are to be expected from the genetic and genomics field of research, which will increase our in-depth knowledge on regulatory and developmental processes in plants, including acclimation to flooding.

Acknowledgements

The work was supported by a PIONIER grant (800.84.470) of the Dutch Science Foundation to L.A.C.J. Voesenek. We thank Danny Tholen for commenting on the draft of this manuscript, Willem Engelaar and Gerard Bögemann for the cross-sections of the roots shown in Figures 1A and B, and Ankie Ammerlaan and Tim Colmer for the longitudinal section of the *Rumex palustris* adventitious root shown in Figure 1C.

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Plant and Soil (2005) 274:215–226
DOI 10.007/s11104-004-7328-9

Root defense responses to fungal pathogens: A molecular perspective

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Received 20 October 2003. Accepted in revised form 11 December 2003

Key words: disease resistance, ethylene, ISR, jasmonate, necrotrophic fungi, rhizobacteria

Abstract

This review will focus on the molecular and genetic mechanisms underlying defense responses of roots to fungal pathogens. Soil-borne pathogens, including *Phytophthora, Pythium, Fusarium,* and *Bipolaris,* represent major sources of biotic stress in the rhizosphere and roots of plants. Molecular recognition and signaling leading to effective resistance has been demonstrated to occur between host and *Phytophthora,* or *Pythium*. The hypersensitive response and apoptotic cell death, two oxidative processes that limit biotrophic pathogens, generally act to exacerbate disease symptoms induced by necrotrophic organisms. Although pathogenesis-related proteins can be expressed in roots during pathogen challenge, salicylic acid has not been implicated in root-mediated interactions. Jasmonic acid and ethylene have been found to mediate parallel as well as synergistic pathways that confer partial tolerance to necrotrophic pathogens, as well as induced systemic resistance to root and foliar pathogens. Genomics approaches are revealing new networks of defense-signaling pathways, and have the potential of elucidating those pathways that are important in root-defense responses.

Abbreviations: CEVI – constitutive expression of VSPI (vegetative storage protein 1); COI1 – coronatine-insensitive 1; E – ethylene; EDS – enhanced disease susceptibility; EIN – ethylene-insensitive; ERF1 – ethylene response factor 1; ETR – ethylene resistant; *fad* – fatty acid desaturation; *Ggt* – *Gaeumannomyces graminis* var. *tritici*; HR – hypersensitive response; LRR – leucine-rich repeat protein motif; JA – jasmonic acid/jasmonate; NBS – nucleotide binding site protein motif; PR – pathogenesis-related; ROS – reactive oxygen species; SA – salicylic acid/salicylate; TLP thaumatin-like protein; PR-5

Introduction to root pathogens and the infection process

Like the above-ground organs, roots can be attacked by a number of pathogenic and parasitic organisms. These include, in order of importance, fungi, nematodes, bacteria, viruses, and parasitic higher plants. Monetary losses due to soil-borne pathogens of vegetables, fruits, and field crops have been estimated at US$4 billion annually in the US (Lumsden et al., 1995). Compared to infection by foliar pathogens, there are many important differences in the ecology, epidemiology, life cycles, pathogenesis, and infection caused by root pathogens. Within the last few years, there have been major advances in the understanding of host–pathogen interactions, mostly involving foliar pathogens. Less well understood are the interactions and mechanisms of resistance to necrotrophic root pathogens; these do not have the high degree of host specificity that characterize

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most biotrophic foliar and root pathogens. *Arabidiopsis thaliana* has become a model host plant, but few root pathogens have been used in this system.

The most significant pathogens of the roots of crop plants are either fungi or filamentous bacteria of the genus *Streptomyces* (Loria et al., 2003). There are a few pathogenic soil-borne bacteria, such as *Ralstonia solanacerarum,* which causes a wilt, and the well-studied *Agrobacterium tumefaciens,* which causes crown gall by genetic transformation of the plant (Gelvin, 2000; Schell, 2000). However, pathogenic bacteria (with the exception of *Bacillus),* are short-lived in the soil, and susceptible to desiccation and to the high degree of rhizosphere competition (Loria et al., 2003). The only viruses known to infect roots, such as nepoviruses and tobraviruses; are introduced by nematodes, or by zoosporic fungi such *Olpidium* which transmits lettuce big vein (Lot et al., 2002) and *Polymyxa,* the vectors of soil-borne wheat mosaic virus and barley yellow mosaic virus (Driskel et al., 2002).

This review will focus on root responses to soil-borne pathogenic fungi, which make up the bulk of microbes attacking the roots. Most root pathogens are necrotrophic, that is, they kill host tissue with toxins, peptide elicitors, or enzymes that trigger host cell lysis and death, thereby providing conditions favorable to pathogen growth. Classic examples are the Oomycete *Pythium* and the Basidiomycete *Rhizoctonia*. Although some species of these genera can infect above-ground parts under wet, humid conditions, they primarily attack roots and emerging shoots. Both genera show a preference for young, juvenile tissue as compared to older woody tissues with secondary wall thickenings, and both can attack germinating seeds in the soil, causing pre-or post-emergence damping-off or seedling rot. They also can attack young root tips and feeder roots, since the newest tissue is formed at the root tip. These pathogens can directly penetrate the root epidermis, and thick-walled resistant survival structures have evolved that are capable of surviving environmental extremes in a dormant state in the absence of a susceptible host. Of the fungi that parasitize root systems, surprisingly few are biotrophic, that is, require a living plant to parasitize and obtain nutrients. Some, such as *Phytophthora sojae,* are hemibiotrophic and form haustoria or feeding structures in plant cells. Another characteristic of most root necrotrophic pathogens is their wide host range. For example, *Pythium ultimum* has been recorded on over 100 genera of plants in the US (Farr et al., 1987). In contrast to biotrophic pathogens, the majority of root necrotrophic pathogens do not appear to have closely coevolved with a specific host, or to be distinguished by races that are virulent on specific genotypes, varieties, or cultivars of domestic plants, and avirulent on closely-related genotypes.

The following is a generalized life cycle of necrotrophic root rotting soil-borne pathogenic fungi. Such fungi can survive in the soil in a dormant, quiescent state, when environmental conditions are not suitable for growth, or when the host is not present. They must also withstand microbial degradation and lysis, parasitism and predation, constituting an important trophic level in the soil ecosystem. Therefore, in many fungi, a thick-walled resistant spore or structure has evolved to serve this survival function. These survival structures are often dark-colored or melanized, making them more resistant to microbial degradation. In the case of *Pythium,* sexual spores called oospores, or thick-walled sporangia serve this purpose (Hendrix and Campbell, 1973). *Rhizoctonia* survives as microsclerotia, structures composed of fungal plectenchyma, aggregated thick-walled cells, or as dark, swollen monilioid hyphae (Parmeter, 1970). Both structures are formed in rotting root tissue, which can also offer a degree of protection.

Once environmental conditions become favorable and a root emerges or grows in close proximity to the fungal propagule, the resistant structure will germinate to form hyphae that will grow toward the susceptible root or seed. If conditions are wet enough, *Pythium* will form zoospores, motile flagellated spores that swim in the film of water around soil particles, and contact the root. Fungi have mechanisms of chemotaxis and chemotropism, and sense root exudates such as sugars, amino acids, organic acids and fatty acids (Deacon and Donaldson, 1993; Donaldson and Deacon, 1993; Ruttledge and Nelson, 1997; Tyler, 2002). They can move or grow in response to gradients of these compounds. Electrostatic charge may also be an important sensory stimulus for swimming zoospores (Van West et al., 2002). Zoospores are often attracted to the zone behind

the root tip, root hairs, or the area where a secondary root emerges from the pericycle. Once the fungal hyphae or zoospore contacts the surface of the root, there probably is a recognition event on the part of both the fungus and plant. This also involves attachment of the fungus to the root. The zoospore will encyst, form a cell wall, and germinate to form an infection hypha. When a hypha contacts the root, it can form an appressorium, a swollen structure that attaches to the root and forms the infection hypha for penetration. *Pythium* and *Phytophthora* form these structures; *Rhizoctonia* forms multicellular infection cushions, which serve a similar purpose. In order to penetrate the host cell wall, fungal hyphae excrete cell-wall degrading enzymes such as pectinases and other pectic enzymes; hemicellulases, cellulases, and proteinases (Campion et al., 1997). These macerating enzymes result in cell death. Non-host-specific toxins may also be formed, although not much is understood about their existence or structure in root pathogens (Desilets et al., 1994). Once the pathogen gains ingress, it grows intracellularly in the cortex of the root, killing the tissue ahead of the advancing hyphae, and colonizing the root. New infections can be initiated on adjacent roots by hyphae or zoospores produced on the killed tissue.

Diseases caused by soil-borne pathogens are considered to be monocyclic. New roots on the plant can become infected from initial primary infection, but there is not much plant-to-plant spread in a single season, because of the limited distance that the inoculum travels in the soil. This is unlike polycyclic foliar diseases, which produce tremendous amounts of spore inoculum which spread from plant to plant by wind or rain in an exponential fashion in a single season. Finally, when the fungal mycelial biomass is increased and the root is killed, the fungus will produce survival structures in the root, such as oospores or microsclerotia. Other secondary pathogens and saprophytes will colonize the root, and *Pythium* and *Rhizoctonia* do not have a high level of competitive saprophytic ability against these other organisms. Thus, the strategy of the necrotrophic pathogen is to grow quickly and colonize the root ahead of secondary invaders, and then convert to survival structures.

One subset of necrotrophic pathogens, the wilt pathogens, has a more specialized life cycle with an adaptation to growth in the vascular system. These include the forma speciales of *Fusarium oxysporum* and species of *Verticillium*. The forma speciales have a limited and specific host range, and often form races. Wilt pathogens also colonize the cortex of the root, but gain access to the xylem in the zone of elongation before the vascular system is fully developed and differentiated, because the Casparian strip, suberized tissue in the endodermis surrounding the stele, presents a barrier to direct penetration of the vascular system in older parts of the roots. These fungal pathogens block the movement of water in the xylem by producing mycelia, spores, and high-molecular-weight polysaccharides in the xylem vessels, while degrading plant cell walls and releasing pectic substances and other polymers that can clog the vascular system and reduce its water-transport efficiency to the leaves.

Rhizosphere pathogens can induce defense responses in roots

Plants mount resistance to pathogens using a variety of mechanisms that can target specific or multiple pathogens. These mechanisms include the production of antimicrobial metabolites, inactivation of pathogen-derived toxins and lytic enzymes, and triggering of host-defense responses by pathogen-or host-derived elicitors. Processes that serve to rapidly limit growth of the pathogen at the site of infection are essential to disease resistance, and involve the generation of reactive oxygen species (ROS) that induce localized tissue collapse and necrosis. Also essential is a systemic resistance, mediated by host-derived salicylic acid (SA) that provides protection in the non-inoculated portions of the plant. Elicitation of general resistance mechanisms by the plant growth regulators jasmonic acid (JA) and ethylene (E) also contributes to disease resistance. Here, JA and methyl jasmonate (MeJ), a naturally occurring derivative of JA, will sometimes be collectively referred to as jasmonate.

Only a small number of defense pathways and resistance mechanisms described for leaf-pathogen interactions have been reported so far in roots (Figure 1). In response to challenge by necrotrophic fungal pathogens, roots typically exhibit the JA- and E-dependent defenses (Devoto and

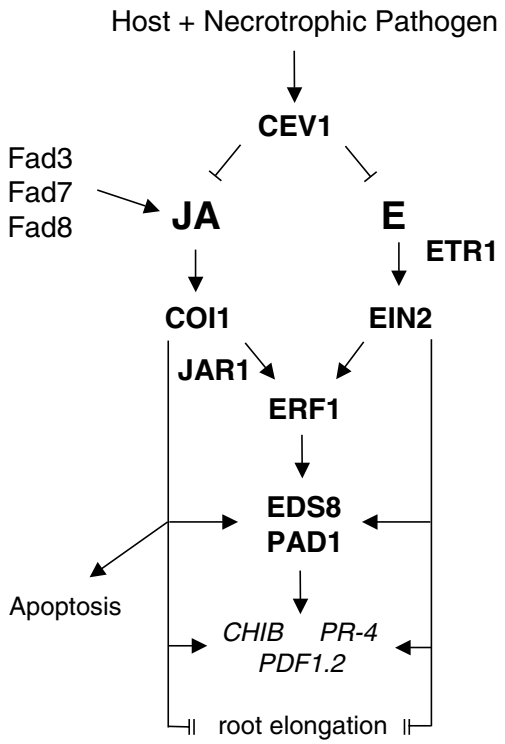

Figure 1. Proposed jasmonate (JA)-and ethylene (E)-mediated host responses to necrotrophic pathogens. Fatty acid desaturases (Fad3 Fad7 Fad8) and cellulose synthase (Cev1) are required for the wild type effects of JA and E on tolerance to such pathogens. Increased flux through either the JA or E pathway enhances pathogen tolerance, while modulating root growth and morphology.

Turner, 2003; Turner et al., 2002; Wang et al., 2002), but not genotype-specific resistance or the SA-dependent defense response. Although it is possible that roots lack effectors for triggering the SA pathway during invasion by necrotrophs, the absence of strong resistance might reflect the limited number of root–pathogen interactions that have been examined, and the difficulty in identifying genotype-specific interactions in below-ground pathosystems.

Interactions with Oomycete root pathogens are among the best studied with respect to elicitation of host defense responses by pathogen-derived factors. Many of these pathogens produce phytotoxins, enzymes that degrade host cell walls and phytoalexins, and inhibitors of pathogenesis-related proteins (Van West et al., 2003), reflecting the offensive–defensive nature of chemical signaling between plant and pathogen. *Pythium* and *Phytophthora* produce families of peptides or small proteins that can trigger a variety of host-defense responses. *Pythium oligandrum* produces an elicitor peptide, oligandrin, that stimulates host suppression of *Phytophthora parasitica* on stems of *Lycopersicon esculentum* (tomato) (Picard et al., 2000). Oligandrin induces the deposition of material at the host cell wall that blocks and contains the pathogen, and appears to disrupt hyphal wall synthesis. Picard and co-workers postulate that phytoalexins might be involved in the formation of host barriers. *Pythium aphanidermatum,* the causal agent of stalk rot and seedling damping-off in a broad range of hosts, secretes a protein that elicits programmed cell death and accumulation of the phytoalexin precursor 4-hydroxybenzoic acid (Viet et al., 2001) in the dicots *Nicotiana tabacum* (tobacco) and tomato, but not in the monocots *Zea mays* (maize), *Avena sativa* (oat), and *Tradescantia zebrina*. These findings suggest that host-dependent signal perception might determine whether the interaction will lead to susceptibility or resistance.

"Resistance" proteins associated with genotype-specific interactions, including proteins encoded by classical resistance (R) genes and the so-called pathogenesis-related (PR) proteins, are expressed in some root–pathogen interactions. The tomato *I-2* gene, conferring resistance to the wilt pathogen *Fusarium oxysporum* f.sp. *lycopersici,* encodes a member of the nucleotide-binding site/leucine-rich repeats (NBS-LRR) family of resistance proteins (Mes et al., 2000). *I-2* is expressed in lateral root primordia of young roots, and vascular regions of mature roots and foliar organs. The arrest of hyphal growth at the vascular interface is postulated to be the basis for *I-2*-mediated resistance against *F. oxysporum*.

Although the hypersensitive response (HR) has not been extensively examined in root–pathogen interactions, an interesting example of the HR in roots has been described for a soybean-*Phytophthora sojae* interaction (Kosslak et al., 1996). The investigators characterized a recessive mutation in soybean that resulted in the spontaneous formation of necrotic lesions on roots. Roots of NR (necrotic root) seedlings produced the expected HR in response to an incompatible strain of *P. sojae,* but sustained a four-fold reduction in disease incidence when inoculated with a compatible (disease-causing) strain.

Reduced incidence was temporally correlated with necrosis, and necrotic tissue was found to have high levels of two defense-related molecules, glyceollin and anionic peroxidase.

Despite the lack of strong evidence for an SA-dependent defense pathway in roots, accumulation of PR-l and PR-5, a thaumatin-like protein (TLP), has been reported in barley roots during challenge with *Bipolaris sorokiniana,* a necrotrophic root pathogen of *Triticum aestivum* (wheat) and barley (Liljeroth et al., 2001). These proteins accumulate in the root tips 6–10 days after inoculation with *B. sorokiniana,* and have also been detected in non-inoculated roots and leaves, indicating the movement of a signal that induces their expression in distal tissues. Similar patterns of induction have been observed in barley inoculated with *Blumeria graminis,* the causal agent of powdery mildew, and *Drechslera teres,* the net blotch pathogen, suggesting that the expression of PR-1 and TLP is part of a general defense response. In contrast to barley, induction of these proteins is not detected in wheat roots.

High levels of PR-l and TLP are correlated with high rates of cortical cell death (Liljeroth et al., 2001). Not surprisingly, infection by necrotrophs, including the take-all pathogen *Gaeumannomyces graminis* var. *tritici (Ggt),* is also correlated to higher rates of cortical cell death, possibly due to increased release of nutrients available to the pathogen, and a compromised ability of the host to mount a defense response. However, expression of PR-l and TLP in barley roots does not afford significant protection against necrotrophic pathogens.

General resistance mechanisms that simultaneously curtail biotrophic and necrotrophic pathogens (Heath, 2000; Thordal-Christensen, 2003) can be constitutive, thereby conferring an ongoing level of protection to the plant. For example, a PR-10-like protein is expressed in the root hairs and root epidermal cells of pea plants (Mylona et al., 1994), and is postulated to have a defense function in these tissues. Other PR and defense-related proteins have been detected in roots following pathogen infection, but do not confer significant levels of protection. Host-derived chitinase and glucanase accumulate around hyphae of *Fusarium culmorum* and *Fusarium avenaceum* that infect roots of susceptible spruce and pine (Asiegbu et al., 1999). Peroxidase activity also accumulates at root cell walls, presumably serving a role in cell-wall strengthening and free radical scavenging. The lack of effective resistance underscores the pathogen-specific nature of PR proteins, and the importance of timing of the expression of these proteins. Also, their induction might be occurring by default as a part of the wound or general stress responses, and they might have a protective role against microbes that would otherwise be pathogenic.

Jasmonate- and ethylene dependent signaling in root defenses

The action of JA confers a moderate degree of host tolerance to necrotrophic root pathogens, and plants that are compromised in JA biosynthesis or signaling show enhanced disease symptoms. The *Arabidopsis fad* triple mutant (*fad3-2 fad7-2 fad8*), deficient in biosynthesis of the JA precursor linolenic acid, is more susceptible to the root pathogen *Pythium mastophorum;* 90% of the *fad* plants showed disease symptoms, as compared to about 10% of wild-type plants (Vijayan et al., 1998). Roots of *fad* plants also harbor significantly more oospores of *Pythium*. Exogenously applied MeJ reduced the level of infection in *fad* roots, but did not affect the growth rate of the pathogen *in vitro*. Another class of *Arabidopsis* JA mutations, designated *jar1* (jasmonic-acid resistant), shows reduced sensitivity to jasmonate and deficient JA signaling (Staswick et al., 1998). Both the *fad* and *jar1* plants exhibit enhanced susceptibility to *Pythium irregulare*.

The *fad* triple mutant is unable to accumulate *LOX2* and *PDF1.2* mRNA following *Pythium* challenge (Vijayan et al., 1998), indicating that a functional JA pathway is required to promote the expression of these defense-related genes in roots. However, a causal link between accumulation of these transcripts and disease tolerance remains to be demonstrated.

A particularly revealing *Arabidopsis* mutant, *esa1,* displays enhanced sensitivity to necrotrophic but not biotrophic pathogens (Tierens et al., 2002). In *esa1,* induction of camalexin biosynthesis and expression of *PDF1.2* mRNA are delayed, providing indirect evidence for the involvement of JA in these responses. Functional ESA1 protein appears to be required for the action of ROS;

treatment with the ROS-generating herbicide paraquat activates *PDF1.2* mRNA accumulation in wild-type but not *esa1* plants (Tierens et al., 2002).

Using reverse genetics approaches similar to those described for the JA pathway, a number of laboratories have demonstrated that the gaseous plant growth regulator ethylene (E), mediates the localized HR response (necrosis), induction of PR proteins, general resistance to pathogenic fungi in susceptible or non-host interactions, and the wound response. The ethylene-response pathway has been reviewed in detail (Wang et al., 2002).

As for JA mutants, plants impaired in E perception show more severe disease symptoms in response to necrotrophic pathogens than do wild-type plants. Tobacco expressing a defective E signal perception component, Etr1-1, is rendered insensitive to ethylene (Knoester et al., 1998). The *etr1-1* plants show a higher incidence of stem necrosis and wilting when grown in non-pasteurized soil, and these symptoms are mimicked by inoculation of the roots with normally non-pathogenic *Pythium sylvaticum*. Symptomatic (wilted) stems of *etr1-1* tobacco harbor pathogenic strains of *Rhizopus stolonifer, Fusarium oxysporum,* and *Thielaviopsis basicola* (Geraats et al., 2002). The *etr1-1* plant is also more susceptible to necrotrophic fungi, including *B. cinerea, Cercospora nicotianae,* and the macerating bacterium *Erwinia carotovora,* but not the biotrophs *Oidium neolycopersici, Peronospora tabacina* and TMV (Geraats et al., 2003). Inoculation of *etr1-1 Arabidopsis* with the root pathogens *P. irregulare, P. jasmonium,* and *P. aphanidermatum* results in substantial wilting, necrosis and eventual plant death as compared to wild-type and mock-inoculated mutant plants (Geraats et al., 2002). *Ein2* mutants of *Arabidopsis,* defective in E signal transduction, also are more susceptible to *Pythium* (Geraats et al., 2002).

The effect of ethylene on defense responses and disease symptoms varies with the host plant. E-insensitive *Arabidopsis* and tobacco respond similarly to necrotrophic and biotrophic pathogens; however, *etr1-1 Arabidopsis* remains tolerant to one *F. oxysporum* isolate, whereas the tobacco mutant does not (Geraats et al., 2002), suggesting that the action of E can be influenced by genotype- or pathosystem-dependent factors. Soybean plants carrying mutations at two E-perception loci, Etr 1 and Etr2, display a more subtle loss of tolerance to *Rhizoctonia solani* (Hoffman et al., 1999). Furthermore, a susceptible genotype of *Arabidopsis* that carries *ein2-1* exhibits decreased symptoms when inoculated by a normally virulent strain of the soil-borne pathogen *Ralstonia solanacearum,* the causal agent of bacterial wilt (Hirsch et al., 2002).

JA and E have overlapping roles in general plant resistance, in some cases, providing a degree of pathogen-induced tolerance for otherwise susceptible hosts. It is not surprising that both of these phytohormones act synergistically in certain defense responses, such as the induction of mRNAs encoding PR-lb and thaumatin-like protein in tobacco roots (Xu et al., 1994), and ERFI (Lorenzo et al., 2003) in *Arabidopsis*. The molecular basis for the synergism is unknown, but might involve co-association of F-box proteins in the SCF-ubiquitin ligase complex, as proposed for JA and IAA (Gazzarrini and McCourt, 2003).

In addition to induction of defense genes, JA and E might be affecting defense-gene expression by modulating root development. Both metabolites inhibit root elongation, and in doing so, possibly divert metabolic resources from roots undergoing pathogen attack, while activating general resistance in those roots. The effect of E on root elongation is independent of COI1 (Ellis and Turner, 2002), suggesting that JA and E are acting on root morphology through independent, parallel pathways.

Systemic resistance induced by rhizobacteria

Interactions between roots and certain non-pathogenic soil-borne microbes, including members of the genera *Pseudomonas* and *Bacillus,* can trigger a systemic resistance to root and foliar pathogens in the host (Pieterse and van Loon, 1999). This process, known as induced systemic resistance (ISR), is distinct from foliage-mediated systemic acquired resistance by the absence of SA signaling in the root (Chen et al., 1999), the absence of local necrotic lesions on the leaves (Liu et al., 1995), and the inclusion of pathogens to which wild-type levels of tolerance in the plant are mediated by the JA and E pathways (Ton et al.,

2002b). Ecotypes of *Arabidopsis* that undergo systemic acquired resistance are not always able to exhibit ISR in response to root colonizing rhizobacteria (Ton et al., 1999), suggesting that these two types of induced resistance are not identical.

In solanaceous hosts, SA appears to have a role in leaf-localized ISR, depending upon the bacterial strain (Audenaert et al., 2002; Maurhofer et al., 1998), and ISR can be accompanied by induction of PR protein genes (Maurhofer et al., 1994; Park and Kloepper, 2000). In contrast, ISR in *Arabidopsis* is governed by the JA and E pathways, but not by SA. For instance, the protection conferred by *P. fluorescens* strain WCS417r to *P. syringae* pv. *tomato* is compromised in *jar1, etr1, ein, eds, eir1* (ethylene insensitivity in roots), and *npr1* Arabidopsis mutants (Iavicoli et al., 2003; Knoester et al., 1999; Pieterse et al., 1998; Ton et al., 2002a), indicating the requirement for JA- and E-signaling, and NPR1 in either the roots or shoots, or both. The requirement for NPR1 in ISR appears to be SA-independent; ISR is fully operable in a NahG host that has reduced levels of SA (Van Wees et al., 1997), and applied JA and 1-aminocyclopropane-l-carboxylate trigger ISR in NahG plants (Pieterse et al., 1998). Root-colonizing rhizobacteria that reduce symptoms of *P. syringae* pv. *tomato* in the *fad* triple mutant, *ein2,* and *npr1* (Ryu et al., 2003) suggest the existence of additional components or mechanisms for ISR that are distinct from the SA pathway.

Involvement of JA and E *per se* in the ISR has not been demonstrated. ISR in *eds4, eds8,* and *eds10* is not restored by applications of JA, although the E precursor l-aminocyclopropane-1-carboxylate restored ISR in *eds8* (Ton et al., 2002a), a JA pathway mutant. Neither JA nor E levels are significantly elevated in leaves of plants whose roots were treated with WCS417r, as compared to untreated plants. However, the conversion of l-aminocyclopropane-l-carboxylate to E by ACC oxidase is elevated in WCS417r-treated plants (Pieterse et al., 2000). The enhanced conversion is independent of ISR, as it occurs with variants of WCS417r that lack the ability to induce ISR, as well as in *jar1* and *npr1* hosts (Hase et al., 2003). Rhizobacteria-mediated ACC conversion is postulated to "prime" the host for stronger E-mediated defense responses upon pathogen challenge (Hase et al., 2003).

The ability to undergo ISR co-segregates with basal resistance to *P. syringae* pv. *tomato*. Classical genetics indicate that both traits are governed by a single dominant gene, called *ISR1* (Ton et al., 1999). Arabidopsis ecotypes that carry the recessive *isr1* allele exhibit JA-induced root stunting and *Atvsp* gene expression, suggesting that *ISR1* does not encode a component of the JA pathway (Ton et al., 2001). However, *isr1* plants display an attenuated E triple response and reduced expression of E-induced genes *hel* and *Pdf1.2*. Ton et al. (2001) hypothesize that the *isr1* genotype harbors reduced E sensitivity, and that ISR1 is involved in the E response pathway.

Although the molecular pathways and processes governing ISR in the leaf are being elucidated, those in the root remain obscure at present. Triggering of ISR in the root has been studied primarily in the context of biological control of root pathogens, and is proposed to involve siderophore-dependent ROS production in the host (Audenaert et al., 2002), and production of the antifungal metabolite 2,4-diacetylphloroglucinol by the biocontrol organism (Iavicoli et al., 2003). It is intriguing that both the biological control rhizobacterium and its target pathogen induce defense genes in cucumber roots (Chen et al., 2000), although this response in tomato roots is associated with specific bacterial strains (Audenaert et al., 2002). So far, studies on ISR have been limited to dicot hosts, and more information on root processes and genes that signal ISR in the below-ground portions of the plant needs to be pursued.

Future directions

Higher plants employ a number of molecular mechanisms for adaptation to abiotic and biotic stresses. As compared to most aerial portions of the plant, roots are morphologically simple organs that must nevertheless maintain nutrient and water utilization, anchorage, and other functions that support the entire plant, despite changes in soil temperature, salinity, water availability, and levels of toxic compounds. Leaves and stems exhibit diverse defense signaling pathways in response to necrotrophic pathogens;

however, root-defense responses appear to be mediated primarily by JA and E, which confer a general resistance that is relatively weak (Figure 1). The SA-mediated systemic acquired resistance that is commonly induced by foliar pathogens has not yet been demonstrated to occur in response to root pathogens and parasites, which would be expected if SA is exclusively synthesized in leaves and unidirectionally translocated through the phloem. *Arabidopsis* has been shown to display an effective foliar defense response to the biotroph *Peronospora parasitica* but in pathogen-challenged roots, the same plant fails to mount the cell death and ROS responses typical of the HR (Hermanns et al., 2003). On the other hand, roots are able to mount strong genotype-specific resistance against parasitic plants (Gowda et al., 1999) and nematodes, with evidence of an HR (Williamson, 1999), phytoalexin induction, and PR protein synthesis (Baldridge et al., 1998), and against hemibiotrophic soil-borne fungi such as *Phytophthora sojae* (Dorrance and Schmitthenner, 2000) and biotrophic fungi such as *Plasmodiophora brassicae* (James and Williams, 1980). It is possible that a limited set of genes and pathways are recruited in roots. This might be attributable to the buffering nature of soil (e.g., Loria et al., 2003), or reflect the need for a streamlined and rapid surveillance system that remains responsive to numerous stresses. Alternatively, organ specialization might necessitate limited expression of defense pathways in roots, resulting in repression of pathways that arose in the aerial organs after roots evolved from shoots and stems about 360 to 410 million years ago (Waisel et al., 2002).

It is of interest to determine whether molecular components of JA and E signaling identified in studies with foliar necrotrophic pathogens (Kunkel and Brooks, 2002) are also required for tolerance to root pathogens. Such components include ERF1 (Berrocal-Lobo et al., 2002), COI1 (Feys et al., 1994), an F-box motif-containing protein that is postulated to be a component of SCF-ubiquitin ligase complexes involved in protein degradation (Xie et al., 1998; Xu et al., 2002), and cellulose synthase CeSA3 (Ellis and Turner, 2001; Ellis et al., 2002). The causal relationship between cellulose synthesis and increased steady-state levels of JA and E remains to be determined. However, this finding, along with the enhanced transcription of JA-induced genes and atypical lignin deposition in the cell wall (Cao-Delgado et al., 2003) in *cev1,* suggests that flux through either JA or E or both are mediated by the cell wall or cell-wall-associated signals. Several other macromolecules associated with the cell wall, including cell wall proteins, transport complexes, and plasma membrane-localized kinase cascades (reviewed in Heath, 2000; Piffanelli et al., 1999), and actin (Sugimoto et al., 2000) are associated with biochemical processes involved in general resistance. These findings implicate the host cytoskeleton or a cytoskeletal element in defense responses.

Genomics approaches are helping to define whole pathways and cellular processes that underlie host resistance to foliar necrotrophic pathogens (Cheong et al., 2002; Ramonell et al., 2002; Sasaki et al., 2001; Schenk et al., 2000, 2003), and have the potential to do the same for root necrotrophic pathogens. In one comprehensive study, profiles of expressed genes were compared in 17 *Arabidopsis* mutants harboring various defects in the SA-, JA-, and E-mediated defense pathways (Glazebrook et al., 2003). By clustering differentially expressed genes according to their expression profiles, the researchers identified a group of JA-responsive genes that was only JA (COI1)-dependent, and a second group was jointly dependent upon E and SA via EIN2, EDS8, and PAD l. In this way, the expression of unique and common genes was correlated to the absence and presence of various components in the signaling pathways, and novel relationships between specific genes and signaling pathways components were identified.

Although some or many of the genes are likely to be significant in leaf–pathogen interactions, none were specifically earmarked as important for root defenses. A recent survey of wheat root ESTs expressed under several stress and non-stress conditions has revealed the presence of mRNAs involved in the HR and in the JA-, E-, and SA-signaling pathways (P. Okubara, unpublished data). However, the presence of mRNA in roots can sometimes be misleading, as in the case of those for PR-1b and thaumatin-like protein that are abundant, but not translated (Xu et al., 1994). Therefore accumulation and functional significance of these proteins in host defense need to be demonstrated.

The advent of molecular genetics and genomics is providing the means to identify genes in the pathogen that govern the infection process, pathogenicity strategies, and life cycle. Rapid and sensitive techniques such as real-time PCR will be useful for quantifying differences in the quality and timing of defense-gene expression that might indicate thresholds that delineate susceptibility and tolerance. With the exception of elicitors from *Pythium,* host recognition of necrotrophic pathogens has not been documented.

Despite extensive efforts by breeders, natural sources of resistance to many root pathogens remain elusive. So far, germplasm of the small-grain cereals, as well as close and distant relatives, does not confer adequate protection against most root necrotrophic pathogens. Presently, growers have limited options to control these diseases, as few fungicides are economical or effective against soil-borne pathogens.

Promising results in the area of enhanced resistance have recently been reported for foliar necrotrophic pathogens. Dickman et al. (2001) obtained tolerance to S. *sclerotiorum* and other pathogens of tomato by expressing genes that inhibit apoptotic host cell death. Although fungal growth was not directly inhibited, the pathogens failed to invade and kill the host tissue in the transgenic plants. Apoptosis, a mitochondrion-regulated process that occurs during normal root development (Gilchrist, 1998; Liljeroth and Bryngelsson, 2001), occurs during pathogen challenge (Lam et al., 2001), and in response to JA, E, and wounding. Apoptosis and the HR have been described in plants undergoing infection by root pathogens; *Sclerotinia sclerotiorum,* a broad host range necrotrophic root pathogen, induced nucleosomal laddering in infected tobacco leaves (Dickman et al., 2001). However, the role of this process in root defenses remains to be determined. By enhancing the flux through defense-signaling pathways or enhancing production of antimicrobial metabolites, such as saponins (Papadopoulou et al., 1999; Wilkes et al., 1999), and isothiocyanates (Tierens et al., 2001), a higher level of tolerance to necrotrophic root pathogens in adapted crop varieties might also be obtained.

The absence of recognition during the early stages of infection and/or the lack of an effective defense response suggests that plants have not yet developed or do not have resistance mechanisms against necrotrophs. Perhaps our understanding of older, more evolved pathosystems can provide leads to combat emerging diseases. Meanwhile, resistance has the potential to be a dominant component of the management of root pathogens, as it is with foliar pathogens.

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Plant and Soil (2005) 274:227–236
DOI 10.1007/s11104-004-0159-x

Soil microorganisms: An important determinant of allelopathic activity

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Received 14 January 2004; Accepted in revised form 5 March 2004

Key words: allelopathy, degradation, phenolic compounds, soil microorganisms

Abstract

Current evidence illustrates the significance of soil microbes in influencing the bioavailability of allelochemicals. This review discusses (i) the significance of soil microorganisms in influencing allelopathic expression, (ii) different ways of avoiding microbial degradation of putative allelochemicals, and (iii) the need of incorporating experiments on microbial modification of allelochemicals in laboratory bioassays for allelopathy. Several climatic and edaphic factors affect the soil microflora; therefore, allelopathy should be assessed in a range of soil types. Allelopathy can be better understood in terms of soil microbial ecology, and appropriate methodologies are needed to evaluate the roles of soil microorganisms in chemically-mediated interactions between plants.

Introduction

Allelopathy is the suppression of growth of a neighboring plant by another species through the release of chemical compounds into the environment (Lambers et al., 1998). For allelopathy to be an ecologically relevant mechanism in influencing growth of plants in field situations, allelochemicals must accumulate and persist at phytotoxic levels and come in contact with the target plant (Choesin and Boerner, 1991). However, after entering soil, processes like retention (sorption), transport and transformation determine persistence and fate of allelochemicals (Cheng, 1995). The type of allelochemical, microflora and substrate conditions each play an important role in determining the persistence of allelochemicals in soils. After isolation and identification of allelochemicals, it becomes necessary to study their behaviour in soil. Mere presence of chemicals in the donor plant and its phytotoxic activities in artificial medium (e.g., agar) does not demonstrate its allelopathic activity in natural situations. Allelopathy methodology has been criticised due to neglect of soil (Romeo, 2000). Soil microorganisms consume organic molecules and therefore chemicals may not accumulate to phytotoxic levels and donor plant may not exhibit any allelopathic activity in natural situations.

Microbial degradation of a particular allelochemical depends upon the specific microflora. For example, phloroglucinol is a simple phenolic compound with allelopathic activity and also a degradation product of several flavonoids (Rice, 1984). The inhibition of crop growth by this compound is magnified when farmers irrigate their field with water contaminated with phloroglucinol. Inderjit and Dakshini (1996a) reported phloroglucinol in water from a tube well installed in a field infested with the perennial weed, *Pluchea lanceolata*. The bacterium *Rhodococcus* sp. has potential to utilise phloroglucinol as a sole carbon source (Levy and Carmeli, 1995). This specificity might explain why allelopathic effects vary in soil from different locations (Inderjit et al., 1996). Another important aspect is the significance of a particular species of a bacterium genus in terms of degrading a compound. For example, amongst

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different species *of Cephalosporium* (*C. furcatum, C. khandalense, C. nordinii* and *C. roseum*), C. *furcatum* is most capable of degrading ferulic acid, a potent allelochemical (Rice, 1984). Although the significance of soil microorganisms in influencing allelopathy in field situations is undoubted, their role is not adequately addressed in bioassays for allelopathy.

Allelochemicals are released into the environment by the plant through foliar leachate, root exudation, residue decomposition, leaf litter or volatilisation (Inderjit and Nilsen, 2003). Allelochemicals influence various physiological processes in different plant species (Inderjit and Duke, 2003). Discussion on the state of knowledge on actual sites of action and biochemical basis of toxicity of allelochemicals is beyond the scope of this review. After their entry into environment, persistence, availability and biological activities of allelochemicals are influenced by microorganisms (Inderjit, 2001). Microbial degradation/transformation of allelochemicals in soil determines the expression of allelopathy in many situations. The aim of this review is to discuss (i) the role of soil microorganisms in influencing allelopathic activities, (ii) different ways of avoiding microbial degradation of putative allelochemicals, and (iii) adequate methodology to appreciate the role of microbial ecology in allelopathy.

Microorganisms influence allelopathic activities

Soil microorganisms are important in modifying allelopathic activity. In many situations degraded products play a key role in allelopathic growth inhibition. There are several reports on the allelopathic significance of products of microbial metabolism (Blum, 1998; Blum and Shafer, 1988; Inderjit, 1996). For example, microbial degradation of benzoxazinones is important in rye (*Secale cereale*) allelopathy (Nair et al., 1990). *Actinetobacter calcoaceticus*, a gram-negative bacterium, was isolated from field soil in Michigan, and reported to convert 2(3H)-benzoxazolinone (BOA) to 2,2′-oxo-l,l′-azobenzene (AZOB) (Chase et al., 1991). Compared to BOA, AZOB was reported to be more inhibitory to garden cress (*Lepidium sativum*) and barnyardgrass (*Echinochloa crus-galli*) (Nair et al., 1990). In soil, DIMBOA (7-methoxy-2, 4-dihydroxy-l,4(2H)-benzoxazin-3-one) and DIM2BOA (7,8-dimethoxy-2,4-dihydroxy-1,4(2H)-benzoxazin-3-one) released from roots of wheat (*Triticum aestivum*) and corn (*Zea mays*), respectively, were catabolised by microorganisms to 2-amino-7-methoxy-3H-phenoxazin-3-one and 2-amino-4,6,7-trimethoxy-3H-phenoxazin-3-one, respectively (Kumar et al., 1993). Gagliardo and Chilton (1992) reported the transformation of rye allelochemical, BOA into 2-amino-3H-phenoxazin-3-one in nonsterile soil. These authors observed 50% inhibition in the radicle growth of barynardgrass *(Echinochloa crus-galli)* by 0.7 and 0.1 mM of BOA and aminophenoxazinone, respectively. This illustrates the higher phytotoxicity of degraded byproduct aminophenoxazinone than its precursor BOA.

Black walnut (*Juglans nigra*) releases a quinone, juglone (5 hydroxy-1, 4-napthoquinone) (Willis, 2000). Juglone has been documented as a potent allelopathic compound (Jose, 2002; Ponder and Tadros, 1985; Rietveld, 1983; Willis, 2000). In Germany, Rettenmaier et al. (1983) isolated the bacterium *Pseudomonas putida*, which can catabolise juglone, from soils beneath walnut trees. The steps involved in juglone degradation are: Juglone $\rightarrow$ 3-hydroxy-juglone $\rightarrow$ 2,3-dihydroxybenzoate $\rightarrow$ 2-hydroxy-muconicacid semialdehyde (Rettenmaier et al., 1983). Schmidt (1988) reported the rapid degradation of juglone in the presence of the soil bacterium, *Pseudomonas putida* J1 in soils in the USA. *Pseudomonas* J1 can metabolise juglone as its sole carbon source. Soil microorganisms generally utilise phenolic compounds as carbon sources (Inderjit, 1996). Because of rapid degradation of juglone by the bacterium, Schmidt (1988, 1990) argued that the chances of juglone accumulating at phytotoxic levels in a field situation would be slight. Williamson and Weidenhamer (1990), however, did not agree with this conclusion, and suggested that phytotoxicity is a function of static (i.e. existing concentration in soil solution) and dynamic availability (i.e. renewal rate) of allelochemicals. Even if the bacterium catabolises juglone, the products may also be toxic. Furthermore, *Pseudomonas* J1 may not be present in all soils, and may not be evenly distributed (Williamson and Weidenhamer, 1990). If this bacterium is not present in the soil, juglone can accumulate at

phytotoxic levels. Jose and Gillespie (1998) reported a significant amount (fall, 1.03 μg/g soil; spring, 1.16 μg/g soil; summer, 1.17 μg/g soil) of juglone under a 10-year old plantation of black walnut. It was reported that juglone concentration in soil decreases with an increase in distance from the tree row. These authors investigated the effect of 3 different concentrations of juglone (10^{-6}, 10^{-5} and 10^{-4} M) on growth of corn (*Zea mays*) and soybean (*Glycine max*). When grown in solution treated with 10^{-4} M, significant inhibition in relative growth rate (RGR) of shoot (corn, 53%; soybean, 69%) and root (corn, 87%; soybean, 99%) was observed. These growth studies, however, were carried out using hydroponic solutions.

Accumulation of juglone at phytotoxic levels is largely determined by the presence of the bacterium *P. putida* in the environment. It is important to study (i) whether *P. putida* is present in soils under walnut trees, and (ii) soil factors influencing the population of *P. putida*. There is a need to carry out further studies with juglone on natural soils. Other possible factors, e.g., shading, resource competition, soil texture should also be taken into account while explaining walnut interference with growth of neighboring plants. Knowledge on soil microbial ecology from various locations may help in our understanding of persistence, fate and phytotoxicity of allelochemicals in the natural environment.

In many situations, a compound released by plants may be innocuous, whilst its microbial degradation product(s) can be toxic (Inderjit et al., 1999a). An example from Florida, USA illustrates the role of microbial ecology in structuring community composition and subsequently regulating fire cycle. Florida scrub community comprises of sandhill and sand pine communities (Fischer et al., 1994). Sand pine community is dominated by trees such as sand pine (*Pinus clausa*) and oaks (*Quercus chapmanii, Q. myrifolia* and *Q. gerinata*), and perennial shrubs e.g., wild rosemary (*Ceratiola ericoides*), *Calamintha ashei, Chrysoma pauciflosculosa* and *Polygonella myriophylla* (Weidenhamer, 1996). *Schizachyrium scoparium* is a fire fuel grass of sand pine scrub. Sandhill community is dominated by longleaf pine (*Pinus palustris*), slash pine (*P. elliotii*) and oaks (*Q. laevis, Q. geminata*), and has dense growth of native grasses and herbs. While surface fire generally occurs every three to eight years in the sandhill community, it occurs at intervals of 20 to 50 years in the fire-sensitive sand pine community. Longer fire cycles in the sand pine scrub community could be due to terpenoids released by the shrubs such as wild rosemary, which inhibits the growth of fire fuel grass, *S. scoparium* (Richardson and Williamson, 1988). Wild rosemary produces an inactive dihydrochalcone, ceratiolin. Due to abiotic factors (light, heat, and acidic soil conditions), ceratiolin undergoes transformation to produce the toxic compound hydrocinnamic acid (Tanrisever et al., 1987), which further undergoes microbial degradation to acetophenone (Fischer et al., 1994). At 200 ppm, hydrocinnamic acid inhibited root (87%) and shoot (83%) biomass of *S. scoparium* (Williamson et al., 1992).

Seasonal variation in the microbial population may influence availability of allelochemicals. Recently, Schmidt and Lipson (2003) reported the increase in microbial populations in autumn and winter following litter input in the snow of tundra soils. The winter microbial population increases to a maximum level in late winter, followed by a decline during snowmelt. This sequence likely results in an increase in available nitrogen, which can be used by summer microbial community. Phenolic compounds were released from leaf litter in autumn, and act as carbon source for microorganisms that develop during autumn and winter. Phenolic compounds were consumed by microorganisms, and therefore cannot play any role in plant growth inhibition during spring. While studying changes in soil microbial community structure in an alpine dry meadow, Lipson et al. (2002) found that microscopic counts of total bacteria (expressed as 10^9 cell/g soil) in summer (21.6 ± 1.72) were higher than those observed in winter (6.2 ± 0.34). However, fungal counts (expressed as meter/g soil) were higher in winter (192.8 ± 16.2) than in summer (126.8 ± 34.3). Seasonal variation in microflora may explain some of the seasonal variation in the allelopathic activities.

Ways to overcoming microbial degradation

Schmidt and Ley (1999) discussed ways of overcoming soil microbial breakdown of allelochemicals. One possibility is the mass release of

allelochemicals into the soil, so that some amount of allelochemicals remain available to the target plant even after partial microbial breakdown. However, there is always a possibility that inactivation can occur by sorption to both inorganic and organic particles in soil, which results, for example, slow rates of release of chemicals (Dalton, 1999). Perennial plants with allelopathic activity have greater chances of maintaining phytotoxic levels of allelochemicals in spite of microbial degradation. The evergreen habit ensures the continuous and periodic replenishment of allelochemicals. Perennial weeds such as *Pluchea lanceolata* (Inderjit, 1998; Inderjit and Dakshini, 1994a, b, 1996b), *Imperata cylindrica* (Inderjit and Dakshini, 1991), *Artemisia vulgaris* (Inderjit and Foy, 1999), *Cyperus rotundus* (Tang et al., 1995) and *Verbesina encelioides* (Inderjit et al., 1999b) can maintain phytotoxic levels of allelochemicals owing to their evergreen nature. Another way of overcoming microbial degradation is the production of compounds resistant to microbial degradation. Quercitrin, for example, is a flavonoid produced by *Pluchea lanceolata* and is resistant to fungal degradation (Rice, 1984, p 354). Inderjit and Dakshini (1995a) reported that both quercitrin and quercetin were detected from *Pluchea lanceolata*, however, only quercitrin was detected from *Pluchea*-infested cultivated soils. This is could be due to the fact that quercitrin is resistant to microbial degradation and persists in soil for longer duration compared to other easily degradable flavonoids such as quercetin (Inderjit and Dakshini, 1995a; Rice, 1984). The third strategy for overcoming microbial degradation is to release allelochemicals in close proximity of target plant (Schmidt and Ley, 1999). This reduces the distance between the point of release of allelochemicals and action site. The contact of chemicals to the roots of the target plant is more important than their concentration and availability in soil (Blum et al., 1999).

Another way of avoiding microbial degradation of allelochemicals could be preferential use of a different carbon source by microorganisms (Dalton, 1999; Inderjit 1996; Schmidt and Ley, 1999). In certain situations, allelochemicals released from the donor plant may not have allelopathic activities, but may provide carbon source to soil microorganisms which can result in microbially released toxins. For example, Kaminsky (1981) reported that growth inhibition of annuals growing in the vicinity of *Adenostoma fasciculatum* was due to the increase in the population of soil microorganisms in the rhizosphere soil. It was concluded that phenolic compounds released from *A. fasciculatum* were responsible for a shift in microbial population. Schmidt et al. (2000) hypothesised that the population of phenolic-utilising microorganisms would be higher in soils with plants producing phenolic compounds when compared with those containing plants that do not produce phenolic compounds. These authors studied the effect of *Salix brachycarpa* on salicylate-utilising microorganisms in alpine soil. Soils beneath *S. brachycarpa* had higher levels of salicylate-utilising microorganisms when compared with nearby *Kobresia myosyroides*-dominated meadows (Schmidt et al., 2000). This example illustrates the relationship between soil microorganisms and allelochemicals leached from, or exuded by, plants.

Blum and coworkers (1999) worked extensively on the utilisation of phenolic acids by microorganisms. Blum et al. (2000) reported an inverse relationship between phenolic acid-utilising (PAU) bacteria in the rhizosphere of cucumber (*Cucumis sativus*) seedlings, and absolute rate of leaf expansion for plants in a soil to which a mixture of phenolic acids were applied (Figure 1). The reduction in the magnitude of growth inhibition by phenolic acid mixture (equimolar mixture of *p*-coumaric, ferulic, *p*-hydroxybenzoic and vanillic acids) was attributed to the induced and/or selected rhizosphere PAU bacteria. An increase of 500% in rhizosphere bacteria after the addition of 0.6 μmol g^{-1} soil mixture of phenolic acids resulted in 5% decrease in the inhibition of absolute rates of leaf expansion of cucumber (Blum et al., 2000). Soil may have inhibitors (e.g., phenolic acids, methionine), promoters (e.g., nitrate) and neutral substances (e.g., glucose) towards plant growth. While studying the role of noninhibitory concentrations of neutral substances (glucose) and promoters (nitrate) on the modification of allelopathic activities of *p*-coumaric acid on seedling biomass of morning-glory (*Ipomoea hederacea*), Blum et al. (1993) found that methionine and nitrate influenced the amount of *p*-coumaric acid required to bring 10% inhibition of morning-glory biomass. In the presence of methionine (3.36 μg methionine/g soil), only 3.75 μg/g soil of *p*-coumaric

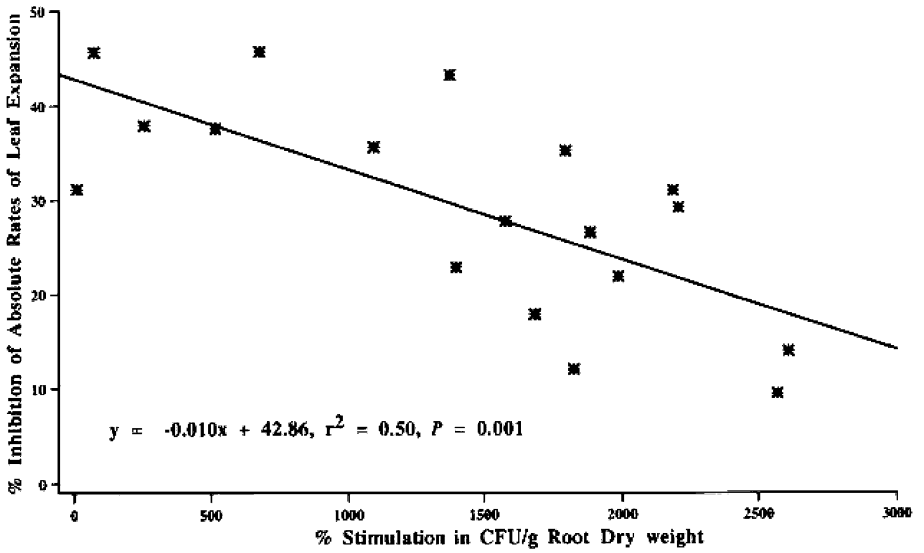

Figure 1. Relationship between percent stimulation of the colony-forming units (CFU) per gram root dry weight of phenolic acid-utilising rhizosphere bacteria and percent inhibition of absolute rates of leaf expansion for cucumber seedlings treated with 0.6 μmol g^{-1} soil of an equimolar mixture of *p*-coumaric, ferullic, *p*-hydroxybenzoic and vanillic acids. Source; Blum et al. (2000). Reproduced with permission from Kluwer Academic Publishers.

acid was required to cause 10% inhibition compared to 7.5 μg/g in the absence of methionine. However, in the presence of higher amounts of nitrate (3.5 vs 14 μg/g), the *p*-coumaric acid concentration required to cause 10% inhibition in the biomass of morning-glory was increased (7.5 vs 39.51 μg/g).

Pue et al. (1995) reported the differential utilisation of organic molecules by soil microorganisms. Glucose enhanced the inhibitory activity of *p*-coumaric acid on morning-glory seedling biomass, i.e. increasing concentrations of glucose lowered the amount of *p*-coumaric acid required for a particular level of inhibition (Pue et al., 1995). Microorganisms probably use glucose as preferential source of carbon, which makes more *p*-coumarie acid available to morning-glory. Kaur et al. (2002) advocated against correlating biological activity of isolated allelochemical(s) to that of foliar leachate or root exudates, because of the presence of neutral substances, promoters, and inhibitors in root exudates or foliar leachates.

It is important to discuss how systems can be manipulated, e.g., the increase in the solution pH of the rhizosphere can eliminate the phytotoxic effects of phenolic acids. This relationship suggests that, when economically feasible, liming can be used to minimise allelopathic effects of phenolic acids released from plants and their debris (U. Blum, pers. comm.). Dalton (1989) hypothesised that reversible sorption of phenolic acids onto soil particles may provide some protection against microbial degradation. Blum (1998) tested the validity of the hypothesis given by Dalton (1989). He found that reversibly bound phenolic acids were not protected against microbial degradation, and could not help in building phytotoxic levels of phenolic acids. Reversibly bound phenolic acids were readily used by soil microorganisms, but at slower rates compared to free phenolic acids.

Methodological impasses

One of the shortcomings of many bioassays for allelopathy is the absence of soil or employing artificial growth medium (e.g., sand, agar or vermiculite) (Foy, 1999; Inderjit and Dakshini, 1995b) which widens the gap between laboratory studies and field observations. Natural soil has an important component, i.e., microorganisms that can limit allelopathic expression once allelochemicals are released into soil. In terrestrial systems, allelochemicals (particularly nonvolatiles) encounter millions of soil microorganisms

Table 1. Studies of the degradation of allelochemicals by fungal and/or bacterial species

Compound	Fungal/bacterial species	Reference
p-hydroxybenzaldehyde	*Haplographium* sp., *Hormodandrum* sp., *Penicillium* sp., *Spicaria* sp.	Henderson (1956)
Vanillin	*Pseudomonas, Cellulomonas, Achromobactor*	Kunc (1971)
Gallate	*Pseudomonas putida*	Tack et al. (1972)
Ferulic acid	*Rhodotorula rubra, Cephalosporium furcatum Trichoderma* sp., *Mucor* sp., *Mortierella ramannians*	Turner and Rice (1975)
Rutin	*Pullularia fermentans*	Hattori and Naguchi (1959)
Rutin, quercetin	*Aspergillus flavus, A. niger*	Westiake et al. (1959)
Phlorizin	*Venturia inaequalis*	Holowczak et al. (1960)
Gallotannin	*Achromobacter* sp.	Lewis and Starkey (1969)
Condensed tannin	*Penicillium adametzi*	Grant (1976)
Juglone	*Pseudomonas putida*	Rettenmaier et al. (1983)
Cinnamic, *p*-coumaric and ferulic acids	*Streptomyces setonii*	Sutherland et al. (1983)
2,3-benzoxazolinone	*Acinetobacter calcoaceticus*	Chase et al. (1991)
Hydrocinnamic acid	*Klebsiella oxytoca, Erwinia uredovosa*	Hashidoko et al. (1993)
Phloroglucinol	*Rhodococcus* sp. BPG-8	Armstrong and Patel (1983)
1,8-cineole	*Aspergillus niger*	Nishimura et al. (1982)
(−)-cis-carveol	*Streptomyces bottropensis*	Nishimura et al. (1983)
(±)-limonenes	*Aspergillus cellulose*	Noma et al. (1992)
Cedrol	*Cephalosporium aphidicola, Aspergillus niger, Beauveria sulfjurescens*	Hanson and Nasir (1993), Gand et al. (1995)

(bacteria and fungi), which influence the availability of allelochemicals (Inderjit, 2001). A discussion on the significance of soils in bioassays for allelopathy is beyond the scope of this review.

In many situations, phytotoxicity of a compound is studied after its addition to soil (Inderjit, 2001; Inderjit and Nilsen, 2003). The observed growth inhibition after the addition of an allelochemical may not be due to the added compound, but could be due to the joint action of a mixture of compounds added, and its degradation products. For example, ferulic acid undergoes microbial degradation to yield vanillic acid and protocatechuic acid (Turner and Rice, 1975). Any growth inhibition could be a result of a mixture of these phenolic acids, and is not necessarily a result of ferulic acid alone. Rice (1984, p. 354) discussed the degradation of several flavonoids, e.g., rutin and quercetin (Table 1). The products of microbial degradation of flavonoids are protocatechuic acid, phloroglucinol carboxylic acid and phloroglucinol carboxylic acid-protocatechuic acid ester. Once flavonoids and other phenolic compounds are released into the environment, they likely undergo microbial degradation (Table 1). Products of microbial degradation are therefore important in determining phytotoxicity. Many times, however, degradation components are not studied, and the growth response is attributed to the compound added in the soil. Romeo and Weidenhamer (1998) suggested that using sterilised soil in laboratory experiments may help in assessing the role of microorganisms. Abiotic and biotic soil factors influence the qualitative and quantitative availability of various microbially degraded chemicals, and also growth of the soil microflora population and soil chemistry (Dalton, 1999; Huang et al., 1999; Inderjit and Dakshini, 1999; Inderjit and Keating, 1999; Inderjit and Weiner, 2001). The following points need attention while studying phytotoxicity of a compound in the environment.

(1) What is the mode of release of allelochemicals, and how frequently can they be replenished?

Table 2. Fungal flora of *Pluchea lanceolata*-infested soils from four locations (S1, S2, S3 and S4) in northern India Fungal species such as *Alternaria alternata, Aspergillus terreus, Drechslera tetramera* and *Penicillium javanicum* were recorded in all four sites. Reproduced with permission from National Research Council, Canada. Source: Inderjit et al. (1996)

Fungal species	S1	S2	S3	S4
Aspergillus niger	+	–	+	+
Aspergillus flavus	+	–	+	+
Aspergillus fumigatus	–	–	–	+
Cladosporium cladosporides	+	–	–	–
Curvularia pallescens	–	–	+	+
Fusarium moniliforme	–	+	–	–
Penicillium chrysogenum	–	–	+	–
Stachybotrys atra	–	+	–	–
White sterile mycelium	–	+	+	+

Note: +indicates species detected; –indicates species not detected.

(2) Does phytotoxicity of allelochemicals vary in sterilised and nonsterilised soil?
(3) What is the microbial flora of the substratum?

Phenolic compounds act as carbon source for soil microorganisms, and may cause a shift in microbial ecology (Blum and Shafer, 1988; Blum et al., 1993; Schmidt and Ley, 1999). Soil microorganisms may influence plant growth by competing with seedlings for nutrients, causing nutrient immobilisation and/or preferentially retain nutrients (Schmidt and Ley, 1999; Stark and Hart, 1997). It is therefore important to study the involvement of soil microorganisms in influencing availability of allelochemicals and thus resulting in plant growth inhibition. Sterilised soil, in addition to nonsterilised soil, can be used in laboratory bioassays to make a comparative assessment of phytotoxicity (Inderjit and Foy, 1999; Inderjit et al., 2004; Romeo and Weidenhamer, 1998). Careful attention should be given to measurement of microbial parameters. For example, microbial biomass and microbial activity may not respond in the same direction and in the same degree to the substrate amendment (Wardle and Ghani, 1995; Wardle and Nilsson, 1997). Compared with microbial biomass, microbial activity responded more to the substrate amendment (Anderson and Domsch, 1985). Michelsen et al. (1995) suggested that enhanced nutrient immobilisation was due to enhanced microbial activity. However, Wardle and Nilsson (1997) argued that enhanced nutrient immobilisation could also be due to enhanced microbial biomass. Therefore, it is important to generate data on microbial biomass and activity in allelopathy bioassay.

Next to degrading allelochemicals, microorganisms can also outcompete plants for nutrients, preferentially retain nutrients such as nitrate and can immobilise nutrients (Inderjit and Weiner, 2001; Schmidt and Ley, 1999; Singh et al., 1989; Stark and Hart, 1997). These factors may operate simultaneously with any role in allelopathy, and affect community structure. Because of the complexity of natural conditions (Weidenhamer, 1996; Inderjit and Del Moral, 1997), studying allelochemical phytotoxicity in relation to microbial ecology might be a difficult task.

Inderjit et al. (1996) studied the fungal flora of *Pluchea lanceolata*-infested soils from four locations (S1, S2, S3 and S4) in northern India (26°00′–28°35′ N, 76°70′–87° 06′ E). These sites had similar level of precipitation and all the sites were cultivated in similar fashion during the past several years. The soil type was sandy-loam. The four selected sites had different levels of total phenolics (S1, 17.2 mg/100 g soil; S2, 9.5 mg/100 g soil; S3, 9.8 mg/100 g soil; S4 3.3 mg/100 g soil). While the fungus *Cladosporium cladosporides* was detected only in S1 (site with highest levels of total phenolics), white sterile mycelium was detected only from S4 (site with lowest level of total phenolics) (Table 2). Natural soil infested with donor (allelopathic) plants from different locations may have different microflora. This condition makes allelopathy difficult to investigate in field situations.

While investigating soil transformation of BOA of rye, Gagliardo and Chilton (1992) found that nonsterililised soil transformed BOA into toxic aminophen-oxazinone. The transformation of BOA, however, was not observed in the sterilised soil. Inderjit and Foy (1999) investigated the involvement of soil microorganisms in interference potential of mugwort (*Artemisia vulgaris* L.) using sterilised and nonsterilised soil. These authors reported that total phenolics and red clover (*Trifolium pratense* L.) growth in sterilised and nonsterilised soil amended with mugwort leachates indicated the potential role of soil microorganisms. For example, a significant reduction (72%) in red clover root growth was observed in

nonsterilised soil amended with mugwort leachate compared with unamended control, whereas there was no effect of the mugwort lechates in sterilised soil.

Concluding remarks

Microorganisms are known to detoxify or magnify the toxicity of allelochemicals after their entry into soil, and thus influence allelopathic activities. However, their significance in influencing allelopathic activity is largely is not investigated in bioassays for allelopathy. Incorporating studies on microbial degradation of allelochemicals in bioassay for allelopathy is needed. This evaluation is crucial in understanding the role of allelochemicals in natural situations. It is also important to determine how climatic and substratum factors influence soil microorganisms. Further, while collecting field soil, attention should be paid to differentiate rhizosphere and bulk soil. Depending upon the crop species, the rhizosphere may have a specific microflora, which can selectively degrade allelochemicals. The fate of allelochemicals can be better understood in terms of soil microbial ecology. Ecosystem components such as soil microorganisms create conditionality for allelochemicals to have, direct or indirect, effect on community structuring (Inderjit and Callaway, 2003). To appreciate the role of allelochemicals in ecosystem functioning, future allelopathy research should be designed to understand the interaction of allelochemicals with ecological processes (e.g., soil microbial ecology).

Acknowledgements

I sincerely thank Professors Udo Blum, Tim Colmer, Hans Lambers, John Romeo and Steve Schmidt for reading the manuscript prior to submission. I thank Professor Robert G. Wetzel and two anonymous referees for making constructive comments to improve the quality of the manuscript.

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Plant and Soil (2005) 274:237–250
DOI 10.1007/s11104-004-1305-1

Facilitative root interactions in intercrops

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Received 11 December 2003; Accepted in revised form 12 July 2004

Key words: exudation, facilitation, intercrop, isotope methodology, mycorrhiza, root system

Abstract

Facilitation takes place when plants ameliorate the environment of their neighbours, and increase their growth and survival. Facilitation occurs in natural ecosystems as well as in agroecosystems. We discuss examples of facilitative root interactions in intercropped agroecosystems; including nitrogen transfer between legumes and non-leguminous plants, exploitation of the soil via mycorrhizal fungi and soil-plant processes which alter the mobilisation of plant growth resources such as through exudation of amino acids, extra-cellular enzymes, acidification, competition-induced modification of root architecture, exudation of growth stimulating substances, and biofumigation. Facilitative root interactions are most likely to be of importance in nutrient poor soils and in low-input agroecosystems due to critical interspecific competition for plant growth factors. However, studies from more intensified cropping systems using chemical and mechanical inputs also show that facilitative interactions definitely can be of significance. It is concluded that a better understanding of the mechanisms behind facilitative interactions may allow us to benefit more from these phenomena in agriculture and environmental management.

Introduction

Intercropping is based on the management of plant interactions to maximize growth and productivity (Ofori and Stern, 1987; Trenbath, 1976; Vandermeer, 1989; Willey, 1979a,b). However, there have been few published studies on below-ground interspecific interactions between intercropped species (Zhang and Li, 2003). A fundamental aspect of intercropping is to avoid unfavourable intra- or interspecific competition possibly including interspecific facilitation (Callaway, 1995), where plants increase the growth and survival of their neighbours (Lambers et al., 1998).

Intercropping is defined as the growing of two or more species simultaneously on the same area of land (Trenbath, 1976; Willey, 1979a). The crops are not necessarily sown at the same time, and harvest times may also differ. However, the crops must co-occur for a significant period of their growth (Ofori and Stern, 1987).

Intercropping is an old and widespread practice in the low-input cropping systems of the tropics (Willey, 1979a), and was common in developed countries before the 'fossilisation' of agriculture (Cassman, 1999; Matson et al., 1997). The shift from mainly labour intensive systems to cropping optimised through the use of fertilisers and other chemical inputs during the 20th century constituted a significant transformation in agriculture around the world (Crews and Peoples, 2004). However, growing interest in intercropping systems in developed countries (Ofori and Stern, 1987) has been initiated by the increasing awareness of environmental damage arising from the use of non-renewable artificial fertilisers and pesticides (Fujita et al., 1992).

Connolly et al. (2001) found that 80% of published intercrop research was conducted in

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Africa and Asia. With the most common crops mentioned first, the top ten list for most frequently used intercrop crops was: maize (*Zea mays*), cowpea (*Vigna unguiculata*), groundnut (*Arachis hypgaea*), wheat (*Triticum aestivum*), millet (*Pennisetum glaucum*), clover cul-tivars (*Trifolium spp.*), beans (*Phaseolus vulgaris*), pigeonpea (*Cajanus cajari*), other beans (*Vicia faba*), barley (*Hordeum vulgare*) and pea (*Pisum sativum*). Many other species are potential intercrop components, each suiting different purposes and cropping conditions (Willey, *1979a)*.

Plants may ameliorate harsh environmental conditions or increase the availability of resources for other species (Lambers et al., 1998). Transfer of symbiotically fixed nitrogen (N) from intercropped legumes to intercropped non-legumes (Jensen, 1996a), release of phosphorus (P) from organic compounds through the work of extracellular enzymes (Dakora, 2002), or dissolution of inorganic P fixed in soil due to lowering of pH by N_2-fixing legumes (Yan et al., 1996) are examples of potential direct facilitative interactions. Facilitation may also act indirectly, by eliminating potential competitors through e.g. isothiocyanates from cruciferous plants (Kirkegaard et al., 1999) or by stimulating beneficial soil microbes (Wamberg et al., 2003) such as arbuscular mycorrhizal (AM) fungi (Francis and Read, 1994). Other more indirect facilitative interactions can also occur (Zhang and Li, 2003). Hauggaard-Nielsen (200la) showed how pea grown in intercrop with barley decrease its yield but because the barley component had greater competitive ability towards weeds soil inorganic N was consequently used for barley grain production instead of weed biomass as in the pea sole crop. The prevalence of damage caused by pest and disease organisms is often observed to be lower in intercrops than sole crops. This may result from indirect facilitation, possibly caused by a lower concentration of host plants and more barriers in the access to hosts, furthermore, changes in the environment of the intercrop, favoring natural enemies of the attacker may hold some of the explanation (Altieri, 1999; Trenbath, 1993).

Yields of intercrops may exceed the yield sum of the component species grown alone (Willey, 1979a), as a result of better use of available growth resources (Hauggaard-Nielsen et al., 2001b; Natarajan and Willey, 1980b; Vandermeer, 1989). Other benefits of intercrops include greater stability of yields compared to the respective sole crops (Willey, 1979b), resilience to perturbations (Trenbath, 1999) and reduced negative impact of arable crops on the environment (Jensen, 1996b) through reduced N-leaching (Hauggaard-Nielsen et al., 2003). When including legumes in an intercrop system, the symbiotic N_2 fixation and residue incorporation also contribute to ameliorating soil fertility (Jensen, 1996c). Furthermore, implementation of intercrops in agroecosystems has been shown to increase diversity of microbes, flora and fauna, which often have a positive impact on crop productivity (Vandermeer, 1995). However, in real life, it usually is a balance between biological and economical advantages, which decide the farming and cropping systems used. Some advantages and disadvantages of intercropping compared to sole cropping are listed in Table 1.

A common and generally accepted method of measuring intercrop advantage is Land Equivalent Ratio (LER) (De Wit and Van den Bergh, 1965). This relative unit express the percentage of land required to produce the same yield in sole crops as in the intercrop, and can be used for all forms of intercropping, where crop components do not differ substantially in duration of growth (Ofori and Stern, 1987). A LER value above one indicates a benefit of intercropping. As an example $LER = 1.3$ indicates a 30 percent advantage, meaning that 30% more land is needed to produce the same yield via sole cropping. Thus, the complementary facilitation dominates over the competitive interference (Zhang and Li, 2003) indicating that facilitative, root and/or shoot, interactions may be operative (Vandermeer, 1989).

Unless specific reference is made, we restrict our discussion to intercrops with only two species. When the term 'sole crop' is used, it refers to a component crop grown alone at optimum plant density and spacing (Willey, 1979a). We also limit our discussion of facilitation to interactions between currently growing intercrop components and do not discuss long term facilitative effects of species on soil characteristics.

Our purpose is to discuss potential. facilitative root interactions in intercrops ($LER > 1$) and to address the research that may lead to greater benefits of facilitation in agriculture and environmental management.

Table 1. A list of advantages and disadvantages when intercropping, defined as the growth of two crops simultaneously on the same area of land not necessarily sown at the same time and with possible different harvest times, but with simultaneous growth for a significant time of their growth period involving interspecific interferences in both time and space. Informations to the table are gathered from Anil et al. (1998), Ofori and Stern (1987), Trenbath (1976), Vandermeer (1989) and Willey (1979a)

Advantages	Disadvantages
Improved the utilisation of local resources for crop production.	Possible over-extraction of nutrients.
Dependence on only one crop is avoided.	Harvesting of one crop component may cause damage to the other.
Less need use of pesticides and fertilizers.	Many times very difficult to mechanize intercropping systems.
Local flora and fauna is favoured	Possible to support a proliferation of harmful flora and fauna for the crops.
Permit a gradual change in more rational external input thinking to more sustainable technologies.	Opposition from prevalent social economic and political systems.
Diversification of cultivated land.	More complex cropping systems and less understood compared to sole crops
Intercropping has biological advantages over the use of sole crops	In many actual economic systems, not considered to be economically efficient

Methods for studying root interactions

A major problem in the study of intercrops is differentiation between roots of individual plants (Martin et al., 1982). One solution is to develop a field version of the various 'pot' compartmentalisation techniques excluding below-ground interactions by nylon mesh or solid root barriers (Johansen and Jensen, 1996; Li et al., 2003; Tofinga et al., 1993). However, the soil profile is disturbed when establishing such compartments, and it may change the root system architecture significantly. Facilitative interactions are conditioned by the environment (Callaway, 1995) and therefore we only discuss methods applicable to field studies.

Investigation of root distribution

Recovery of roots from the soil by excavation, sampling into trench sides, or by means of auger boring and soil coring gives information about total root mass, diameter, length and nutrient concentration (Mackie-Dawson and Atkinson, 1991). Roots from some species may be distinguished from other roots by their morphology, while this is impossible for others. Colouring techniques have been developed for some species, allowing quantitative fractionation of roots from different species after washing (Ward et al., 1978). Shoot-applied radioisotopes may be used in combination with autoradiography of soil slices or washed roots. However, most physical. procedures likely underestimate the amount of roots present (Khan et al., 2002a). Methods where roots are observed directly through, e.g., an angled micro-rhizotron tube (DeRuijter et al., 1996; Hansson and Andren, 1987; Kirkham et al., 1998), profile wall or observation windows can be used for determination of root distribution, diameter, longevity, periodicity and length (Mackie-Dawson and Atkinson, 1991), but with no differentiation of individual roots, unless they are clearly morphologically different or have e.g. root nodules.

Soil injection of radiotracers like ^{32}P, ^{131}iodine (I) and ^{86}rubidium (Rb) is another way of gaining qualitative information on whether living, functional roots of a specific plant are present at the time and at the specific location of the injection, but not on the root density or biomass (Abbott and Fraley, 1991). A convenient way of placing radioactive substances in soil for studies of root development is to use frozen solution encapsulated in gelatine dropped in predrilled holes in the soil (Jacobs et al., 1970) or into tubes that have been installed into the soil to fixed depths (Hauggaard-Nielsen et al., 200lb). Thus, accurate amounts of radioactive tracers can be precisely placed at specific soil depths without any contamination hazards of the soil layers around the deposition due to the encapsulation (Figure 1). The shoot radioactivity *in situ* or in foliage is a qualitative indication of

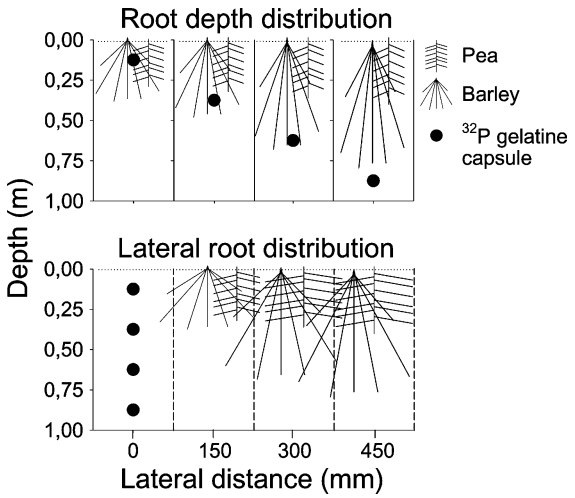

Figure 1. Schematic illustrations of the ^{32}P placements in the field when estimating root depth in four succeeding soil depths like 0–25 cm, 25–50 cm, 50–75 cm and 75–100 cm and lateral root growth. The lateral root growth illustration is exemplified using the 0–25 cm soil depth. Lateral root growth in the other layers can be estimated identically. Adapted from Hauggaard-Nielsen et al. (2001b).

functional roots present within the labelled soil volume.

A commonly used isotope is ^{32}P whose 14-day half-life permits measurements for several months. It is fixed to the soil when placed, and has the advantage of being a plant nutrient. Intercropped species are assumed to be able to exploit the same soil volumes containing ^{32}P even though, e.g., differences in absorption capacity between species due to dissimilar root surface area might be critical for such an immobile nutrient as P (Hauggaard-Nielsen et al., 2001b). This method has obvious advantages superior to excavation and soil coring techniques, because it is (a) faster, (b) more sensitive to finer roots, (c) non-destructive to plants, (d) able to differentiate between active roots and (e) able to differentiate roots of individual. plants in competitive field situations (Hauggaard-Nielsen et al., 2001b; Jacobs et al., 1970).

Non-radiotracers, e.g., strontium (Sr) and lithium (Li), have the advantages of being cheap and simple to use, and their use is not complicated by the difficulties caused by decay rates or other environment impacts. Placement of non-radioactive tracers in the field, and plant tissue sampling should be conducted as explained for the radioactive tracer method. Criteria for tracer suitability are that it should be: (i) present at low concentrations in soil, (ii) taken up freely by the roots and transported to leaf tissues, (iii) non-toxic and (iv) easy to determine quantitatively (Martin et al., 1982).

Exploring root interactions

The transfer of nutrients like carbon (C), N and P from one plant species to another requires the use of isotope labelling techniques. Labelling techniques can be divided into three approaches: (i) atmospheric labelling with $^{15}N_2$ for N_2 fixing plants, $^{15}NH_3$, $^{13}CO_2$ or $^{14}CO_2$ in gas-tight enclosures, (ii) ^{15}N stem labelling (Mayer et al., 2003) and shoot labelling of leaflets or petioles (Khan et al., 2002b), (iii) split-root labelling technique with, e.g., $^{15}NO_3$ and $^{32}PO_4$ (Ikram et al., 1994; Jensen, 1996d). In general, soil ^{15}N dilution technique is not applicable for determinations of short term N transfer in intercrops under field conditions (Ledgard et al., 1985), while the direct labelling of leaflets or petioles have a sufficiently good time resolution (Hogh-Jensen and Schjoerring, 2000).

Hyphae of AM fungi may inter-link plants of different species, and transfer nutrients between individuals of a plant community (Yao et al., 2003). Furthermore, external hyphae are well positioned to absorb the nutrients mineralised from e.g., decaying roots of one plant, and mediate the transport to a growing neighbouring plant (Johansen and Jensen, 1996). The role of AM is usually studied using a combination of isotopic tracers and compartmentalisation techniques (Jakobsen, 1994; Johansen et al., 1992; Ikram et al., 1994). These methods are based on a three-compartment system approach with a root-free hyphal compartment separated from two rooting compartments at either side by means of a fine mesh allowing passage of hyphae but not of roots (Figure 2).

Reviewing the most feasible methods to study root facilitation we realised that some were introduced 20–30 years ago, but are nevertheless still appropriate to use. One disadvantage though, is that some of these methodologies are often expensive and quite laborious. We expect that through increased attention in the scientific

community towards root facilitation as a fundamental process in the dynamics of plant communities (Callaway, 1995), methods integrating the newest technologies available for field and laboratory studies will develop. However, the excellent work of Khan et al. (2003a) provides an overview of existing methods using ^{15}N isotope techniques, mass balance and physical recovery of roots (Table 2) to quantify legume effects on soil N pools and on the N economies of crops following legumes underlining strengths and weaknesses of different methodologies crucial to remember when new techniques are developed.

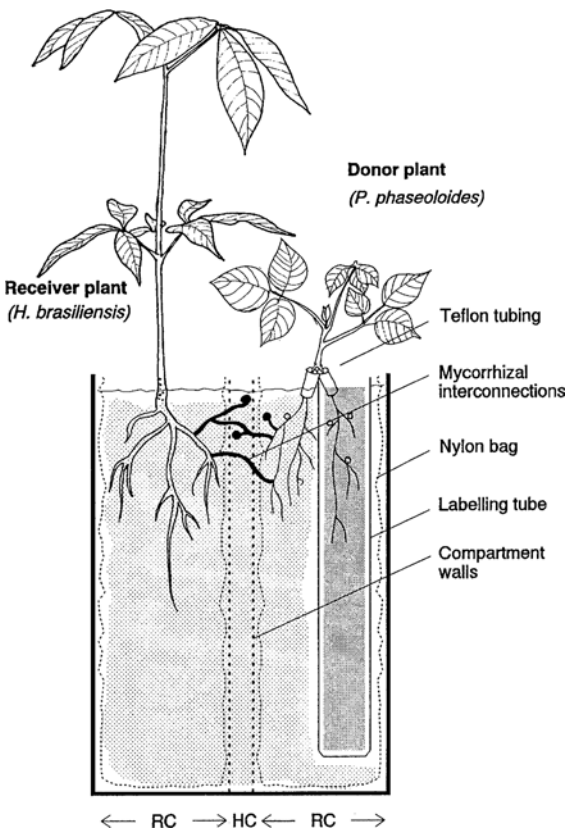

Figure 2. Illustration of experimental unit for nutrient transfer studies where bean plants (*Pueria phaseoloides*) are the donor and rubber trees (*Hevea brasiliensis*) the receiver when grown together in PVC containers where the root compartments (RC) were separated with a 3-cm root-free soil layer termed the hyphal compartment (HC). The three compartments were defined between perforated plates and the root compartment lined with bags of nylon mesh allowing passage of hyphae but not roots. ^{32}P, ^{15}N or other tracers can be applied to the labelling tube. Adapted from Ikram et al. (1994).

Facilitative interactions between intercrop components

Root distribution

When an intercrop with two species is established, early interference may involve competitive root effects. Two weeks after emergence pea plant shoot dry matter production was depressed by 15–20% when intercropped with barley (Bellostas et al., 2004). Greater root distribution of intercropped barley plants indicated the importance of early below-ground interference in this study, such as competition for space, which can be regarded as a soil resource, and limited nutrient resources (McConnaughay and Bazzaz, 1992). Growth characteristics that allow rapid establishment of barley plants are likely the determining factors leading to the often observed dominance exerted by this species when intercropped (Jensen, 1996c).

Early below-ground competitive interference may shift to facilitative effects later in the growing season. If interspecific competition forces one of the species to grow to a greater depth, this species may be able to use more nutrient sources from deeper soil layers, and be less prone to water stress later in the growing season. Barley root systems exploit more soil layers when intercropped with pea compared to sole cropped (Figure 3); thus in combination they may exploit a larger soil volume (Hauggaard-Nielsen et al., 2001b). Such root growth pattern may be explained by the 'avoid area' hypothesis put forward by Willey (1979a), pea creating areas that are more nutrient depleted than the surrounding soil, forcing barley downwards. However, such avoidance may occur among plants belonging to

Table 2. Estimates of below-ground N in roots as a percentage of total plant N for faba-bean (Vicia faba), chickpea (*Cicer arietinum*), mungbean (*Vigna radiata*), pigeonpea (*Cajanus cajan*) and wheat (*Triticum aestivum*) using various ^{15}N-based and non-isotopic methods. From Khan et al. (2002a)

Species	Physical recovery	^{15}N shoot labelling	^{15}N balance	Soil ^{15}N dilution	Mass N balance
Faba-bean	13	39	33	11	30
Chickpea	11	53	43	52	52
Mungbean	4	20	15	39	37
Pigeonpea	15	47	57	32	37

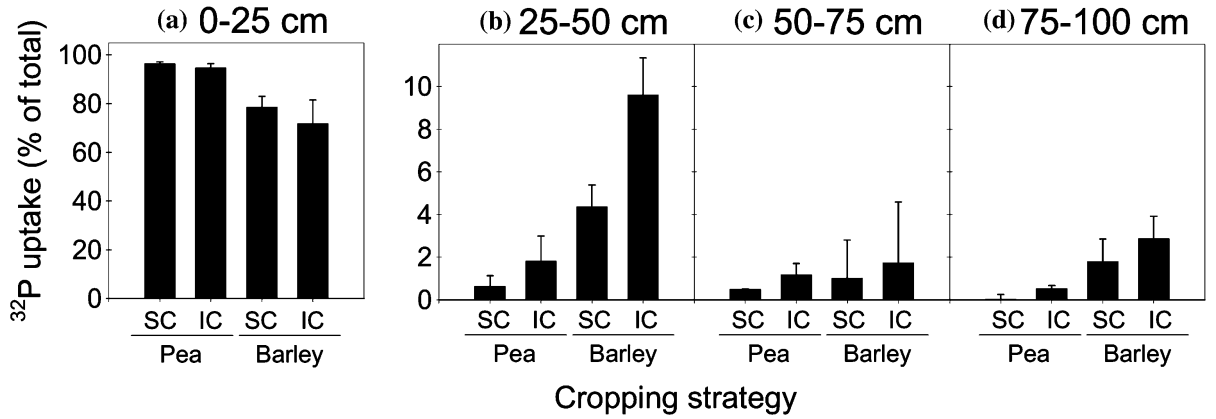

Figure 3. Radioactive phosphorus (^{32}P) uptake from four soil depths as percentage of total ^{32}P activity in harvested biomass 81 days after emergence. SC = sole cropping and 1C = intercropping. Values are means ($n = 4$; ± SE). Modified from Hauggaard-Nielsen et al. (2001b).

the same or differing species so it might not be caused by interspecific interactions. Spatial distribution of roots in a sorghum-pigeon pea intercrop showed how roots of the two crops intermingled freely (Natarajan and Willey, 1980a).

More efficient water and nutrient uptake could increase productivity in intercrops under unfavourable environmental conditions (Fujita et al., 1992), such as in many subtropical and tropical regions. Likewise, under temperate conditions it is important to capture water to achieve suitable yields, but also to limit leaching in the wet autumn and winter months (Hauggaard-Nielsen et al., 2003). The intercrop is able to explore a larger total soil volume if the component crops differ in rooting pattern (Hauggaard-Nielsen et al., 2001b). However, field experiments conducted under temperate climatic conditions indicate that grain legumes and cereals use equal amounts of water measured as volumetric soil water content in the soil profile (Hauggaard-Nielsen et al., 2001a,b). Competition for water under such climatic conditions may not be important in determining intercrop efficiency (Ofori and Stern, 1987).

Nutrient transfer between plants of different species

Rhizodeposition is a major source of N, C and other nutrients for the soil microbiota (Walker et al., 2003) affecting the density and activity of microorganisms in the rhizosphere, and the turnover and availability of plant nutrients (Hogh-Jensen and Schjoerring, 2001).

During growth N is deposited in the rhizosphere as a result of the continuous turnover of roots and nodules (Walker et al., 2003). It is deposited as ammonium, amino acids and sloughed-off cells, and can be reabsorbed by the legume itself or taken up by a neighbouring plant. Using a direct ^{15}N-labelling technique in a glasshouse experiment Giller et al. (1991) estimate that up to 15% of the N in N_2-fixing beans could be transferred to intercropped maize. Using a direct split-root ^{15}N-labelling technique (Figure 2) in pea-barley intercrop pot experiments, Jensen (1996a) supported this by demonstrating that the amount of pea-derived N in barley increased markedly with time. This was probably due to the accelerated turnover of root and nodule material with a transfer of 19% of the N in barley derived from pea after 70 days. However, in the field N transfers from pea to barley was not found (Figure 4) (Hauggaard-Nielsen et al., 200la; Jensen, 1996c). Other field studies with annual pea-barley and pea-mustard (*Sinapis alba* L.) intercrops also failed to demonstrate significant N transfer from the legume to the non-legume (Izaurralde et al., 1992; Waterer et al., 1994).

Considerable variation is often found in the ^{15}N-enrichment measurements derived from field studies, possibly because the transfer of N from legumes to non-legumes in annual intercrops only causes a slight dilution of the soil and fertiliser N in the non-legume. Laboratory studies

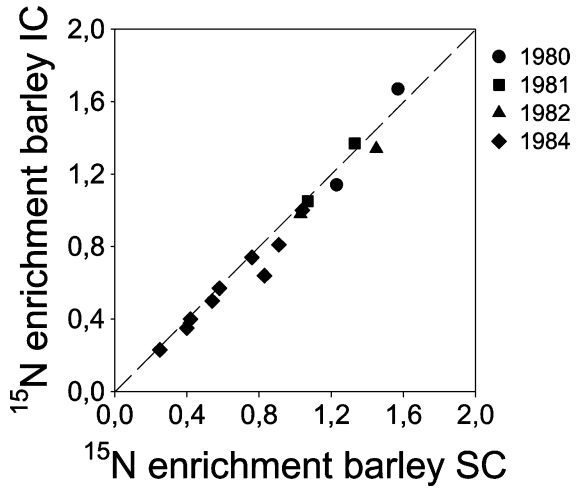

Figure 4. ^{15}N enrichment (atom % ^{15}N excess) of barley (*Hordeum vulgare* L.) grown as sole crop (SC) and in a pea (*Pisum sativum* L.) -barley intercrop (IC) with variable harvest numbers in the four years of experimentation. Values are means (n = 4), Modified from Jensen (1996c).

have shown that N transfer occurs (Giller et al., 1991; Jensen, 1996a), but the lack of evidence from the field presumably shows that an indirect methodology may be unable to detect N transfer (Jensen, 1997). The indirect ^{15}N-isotope dilution method only gives an estimate of apparent transfer, since the rooting depth and pattern of N uptake for the non-legume can differ between sole and intercrop situations (Hauggaard-Nielsen et al., 2001b; Jensen, 1996c) differing in the exploitation of the soil profile. The significance of legume N rhizode-posits for the N nutrition of an intercropped plant mainly depends on (i) the effect of rhizodeposits on the mineralisation-immobilisation turnover of N in soil, (ii) the competition between the legume and the associated plant for plant-available soil N, (iii) the capacity and time course for N uptake in associated plants, and (iv) the availability of other N sources (Jensen, 1997). Through differences in rates of rhizodeposition and litter production crop species affect soil nutrient cycling and thereby plant growth conditions to varying degree (Van der Krift et al., 2001).

In perennials such as clover and grass pastures, N transfer between species is quantitatively greater than in annual intercrops and crucial for N cycling (Hogh-Jensen and Schjoerring, 2000; Jorgensen et al., 1999). However, it is difficult to distinguish whether 'indirect' transfer (Trannin et al., 2000) through mi-crobial decomposition from previous years causes this, or 'direct' transfer (Van Kessel et al., 1985) from N fixed in the same growth period.

In poor soils with only limited soil mineral N availability; N transfer might play a dynamic and quantitative important role. Direct ^{15}N-leaf-labelling techniques used to quantify the amounts of fixed N inputs to the soil from legumes (Evans et al., 1989) are developed to enhance our understanding of leguminous below-ground N contribution and processes (Table 2). Hogh-Jensen and Schjoerring (2000) estimated N transfer from clover to grass based on both the soil ^{15}N-dilution technique and a direct leaf-feeding ^{15}N technique. Summarised over the first production year, N transfer from white clover to ryegrass estimated by the direct technique (4.5 g m^{-2}) was more than double of that estimated by the indirect technique (1.3 g m^{-2}). However, to our knowledge the direct ^{15}N leaf-labelling technique has only been used in a few cases to validate the N transfers in field-grown annual intercrops (e.g., Ledgard et al., 1985).

Mycorrhiza-mediated facilitation

Common mycorrhizal networks (CMN) can interconnect individual species or cultivars when intercropped by extending AM mycelia from one plants roots to another plant roots (He et al., 2003). However, there is controversy about whether transfer takes place through such CMNs or indirectly through the soil. A direct transfer of N from soybean (*Glycine max* L.) to maize mediated through AM has been shown (Martin et al., 1982; Van Kessel et al., 1985). Nevertheless, AM hyphae appear not to be of quantitative significance in the transfer of N or P between living plants (Ikram et al., 1994; Johansen and Jensen, 1996) Below-ground N transfer from a legume to a non-legume rather occurs via decomposing roots, and is therefore an indirect transfer (Trannin et al., 2000). When the root system of the donor plant is decomposing, AM hyphae may enhance the nutrient transfer from the dead tissue to a living plant (Johansen and Jensen, 1996). Thus, in an intercrop with crops of differing growth durations, one might assume that the

amount of P, N and potentially other nutrients originating from AM hyphae increases with time because of accelerating turnover of hyphae and P-rich root materials, as discussed for pea-derived N in barley in a study by Jensen (1996a). Furthermore, He et al. (2003) review not only one-way transfer of leguminous N to a non N_2-fixing mycorrhizal plant but also a few studies showing transfer from non N_2-fixing mycorrhizal plants to N_2-fixing mycorrhizal plants as a kind of bidirectional transfer approach. A bi-directional transfer of nutrients by AM warrants further investigation (He et al., 2003) including development of experimental designs and tracers to provide nutrient movement informations.

Martensson et al. (1998) assessed the genetic variability related to N uptake by screening five varieties of chicory (*Cichorium intybus* L.), four of peas and three of red clover in combination with eight isolates of AM. In pea-chicory intercrops the percentage of N in the chicory derived from transfer ranged between 3% and 50% between the varieties. Equivalently, red clover-chicory intercrops showed that 20 to 34% of N in the chicory roots came from transfer of legume N, with variation between fungal isolates used. It is suggested that more research effort is put towards strategic selection of suitable cultivars and AM partners for investigation of possible N transfer increase in intercropping systems.

The role of mycorrhizal fungi in the P nutrition of crop plants has been widely studied (Schweiger and Jakobsen, 1999; Schweiger et al., 2001). Nevertheless, it is widely accepted that AM fungi colonise many plant species with significant functions in the ecosystem due to a wide range of interactions with other microorganisms in soil (Francis and Read, 1994). Despite the effect of root exudation (Walker et al., 2003), including the N transfer effect (Martensson et al., 1998), AM colonisation may also influences the role of bacteria in promoting AM formation and of soil animals (e.g., collembola) in grazing the external mycelium (Wamberg et al., 2003). Grazing of mycelium, and thereby mineralisation of P, N and other nutrients, obviously affects potential transfer in intercrops, but it may also modify the interactions of plant pathogens, such as root-inhabiting nematodes and fungi (Brussaard et al., 2001).

Altering substrate characteristics

Plants can affect microbial activity and the decomposition rates of soil nutrient pools either through exudative growth induced changes in the soil, or via the composition of the incorporated plant residues, while microbial activity and related soil processes in turn regulate nutrient availability for plant growth (Kumar and Goh, 2000). Release of root exudates can solubilise and/or mobilise nutrients from the mineral or organic fractions in soil. In a faba bean-maize intercrop pot experiment, Li et al. (1999) show a significant positive yield effect on maize significantly increasing its P uptake with root intermingling compared to other pots with nylon mesh separation and solid root barriers. Faba bean may influence both N and P acquisition in the intercrop. In a chickpea (*Cicer arietinum* L.)-wheat intercrop study, Zhang and Li (2003) conclude that even though chickpea can mobilise P in the root zone a competitive wheat cultivar acquires more P from the root zone than chickpea resulting in P depletion in the chickpea rhizosphere. Another example from temperate climatic conditions is pea-barley intercropping, where barley roots penetrate rapidly down the soil profile (Hauggaard-Nielsen et al., 2001b) and start soil N uptake before pea roots appear (Jensen, 1996c). Such growing conditions may stress pea and increase exudation (Rengel, 2002). Thus, root exudation may change the distribution of growth factors influencing exploitation of the soil volume and the general utilisation of environmental resources by the intercrop in both time and space.

Legumes excrete more amino acids than non-legumes (Walker et al., 2003) and this can have implications for the plant uptake of soil nutrients. Nodulated soybean can take up more soil N than non-nodulated and fertilised soybean (Jensen and Sorensen, 1988) maybe due to variation in the release of exudates of nodulated and non-nodulated soybean plants. Thus, if the C:N ratio of exudates in the nodulated soybean was lower than that of the non-nodulated soybean less soil N would likely immobilise and more mineral N would be available for uptake (Jensen and Hauggaard-Nielsen, 2003) by itself or neighbouring species.

If the roots of the two intercropped species are sufficiently close, extra-cellular enzymes, such as phosphatases from one species may facilitative

nutrient availability to the associated species. Phosphatases can catalyse the release of phosphate from organic P sources (Tarafdar and Jungk, 1987). Extra-cellular phosphatases occur in many plant species; the rate of their exudation is increased with decreasing P availability (Tarafdar and Jungk, 1987). Thus, interspecific competition for P may enhance the exudation of phosphatases from roots of the intercropped species. Recently, it was demonstrated that chickpea might facilitate uptake of P by intercropped wheat, from an organic P source (Li et al., 2003). However, overlap of rhizospheres (depletion zone around roots) of the species is required for facilitation of immobile nutrients (like P) to occur, which is not always the case (Fusseder et al., 1988).

Exudation of carboxylates like malonate, citrate and malate has also been shown to play a role in mobilising nutrients when plants grow on poor soils. Chickpea and lupin have been shown to exude carboxylates, thereby increasing the availability of P to the plant (Veneklaas et al., 2002). Other soil chemical factors, aluminium (Al) and iron (Fe) or formation of stable complexes with cations such as Ca are modified by a range of root exudated carboxylates (Lambers et al., 2002). However, as concluded by Veneklaas et al. (2002) patterns of variation in root exudation need to be understood to a higher degree before it is possible to use such trait to enhance crop nutrient availability and uptake.

Increasing the availability of a resource

The ability to form 'proteoid' or 'cluster' roots of longitudinal. rows of hairy rootlets emerging on lateral roots is common in the non-mycorrhizal proteaceous species known for a larger absorptive surface than normal roots and a very efficient absorption of moisture and nutrients (Lambers et al., 2002). However, the nutritional. significance of these roots is largely inferred from information on lupine (*Lupinus albus*) (Lambers et al., 2002). Exudates from cluster roots of lupine have been shown to recover considerable amounts of P and Fe from a limited soil volume (Lambers et al., 2000; Shen et al., 2003) possibly making otherwise unavailable nutrients available to intercropped species improving the adaptation to nutrient-deficient soils.

In general, if legumes fix N_2 as their main N source more cations are taken up than anions, because uncharged N_2 enters the roots, causing roots to excrete protons in order to maintain internal. pH (Jensen, 1997). Using a pot experiment Yan et al. (1996) showed how the soil is acidified during cultivation of field beans (*Vtcia Saba*) because of net proton release. Thus, symbiotic N_2 fixation and ammonium assimilation cause soil acidification, whereas plants that assimilate nitrate raise the soil pH. Using a nitrogen-fixing alfalfa crop as an example, Israel and Jackson (1978) calculated, that the yields of 101 ha^{-1} of green matter acidifies the soil to such an extent that 600 kg CaCOs ha^{-1} $year^{-1}$ would need to be applied to maintain constant soil pH. The acidifying effect of leguminous N_2 fixation may be beneficial to the other crop in the intercrop by solubilizing nutrients, like P, potassium (K), calcium (Ca) and magnesium (Mg), which are otherwise fixed in unavailable forms. Legumes might be able to 'mine' a nutrient source that is not available to the non-legume, implying the operation of facilitation (Vandermeer, 1989).

Exudation of growth regulating substances

Natural plant growth regulators may be involved in root facilitation interactions. Since a plant growth regulator is any compound that has the ability to alter the amount of dry matter production and the development of the plant it can either promote or retard growth. Plant hormones as growth promoters are well known, and e.g. Toro et al. (2003) enhanced plant growth in cabbage (*Brassica oleracea*) (Toro et al., 2003) with the new group of plant hormones; Jas-monates. The jasmonates are known for their effects on plant response to biotic and abiotic stresses possibly influencing companion crops if intercropped Ries et al. (1977) found that triacontanol from Lucerne (*Medicago sativa*) hay can stimulate the growth and yield of plants of several other species. Equivalently, Søgaard and Doll (1992) reported a positive effect (from 20 to 50% increase in dry matter production) of corn cockle plants (*Agrostemma githago*) on wheat plant. As they used a design with a fixed number of wheat plants and an increasing number of

corn cockle plants it was possible to show that corn cockle may release compounds that stimulate a better use of growth factors by wheat compared with sole cropped wheat. However, it was not possible to demonstrate the same effect in the field (Doll et al., 1995). The exudation of such growth regulating substances may contribute significantly to the competitive relationship among intercropped species including weeds and thereby influence the final intercrop composition. This should be further investigated.

Biofumigation

Of all the factors that may be involved in facilitative interactions, the one most cited is the reduction in pest attack in intercrops (Vandermeer, 1989). When some pests or diseases attack a sole cropped species, it is often found that the same species intercropped with other plant species is less damaged (Trenbath, 1993). 'Biofumigation' is the term for suppression of soil-born pests and pathogens by cruciferous plants for grain production or green manure (Kirkegaard et al., 1999). The effects of the cruciferous plants are caused by isothiocyanates (TTC), compounds originated from the hydrolysis of glucosinolates (GSL) in soil, which are often released after mechanical. damage to the root tissues (Choesin and Boerner, 1991).

Despite recent published work on intercrops including oilseed rape (*Brassica napus* L.) (Andersen et al., 2004) we are not aware of specific biofiimigation studies including intercrops. However, Rumberger and Marschner (2003) concluded that oilseed rape affects soil microbial community structure during growth, thereby suggesting that facilitative interactions may occur in intercrops including cruciferous plants. The complex rate of GSL hydrolysis and ITC release in an intercrop will be influenced by the soil-plant-atmosphere system as a whole (Figure 5a) involving local climatic (e.g., water stress), edaphic (e.g., nutrients) or biotic (pest and diseases) factors. Furthermore, the competitive interactions between the individual crops in an intercrop brought about by a shared requirement for a resource in limited supply (Begon et al., 1990) may alter GSL production dependent on the species or cultivars. Sarwar and Kirkegaard (1998) included eight entries from five *Brassica* species grown in the field and in pots in the glasshouse to show interactions between species and environments with total shoot and root GSL concentration changing with environment. New ways of using this trait from the cruciferous plants in the development of intercropping systems seems promising, but needs more research.

Facilitative root interactions in practice significance and perspectives

If the niches of crops in an intercrop differ in any way that affects the capture of resources, then a mixture should have higher ecological resource use efficiency than a sole crop (Vandenneer, 1989). Thus, in cropping systems limited in plant growth resources increased competitive and facilitative interactions using intercropping are most likely to be of significance. In a recent review on competitive and facilitative interactions in Chinese intercropping systems (not considered as low input systems) Zhang and Li (2003) conclude that facilitative interactions definitely can be of significance, observing improved iron nutrition in peanut intercropped with maize, increased P uptake in maize when intercropped with faba bean and chickpea facilitation of P uptake by associated wheat from phytate-P. Despite crop management through chemical and mechanical inputs in more intensified cropping systems interspecific regulations such as facilitative root interactions may play a role. However, the importance or significance of root facilitation very much depends on local cropping conditions and the aim of the intercropping system ranging from e.g. enhancements in micronutrient accumulation to improve quality or avoid malnutrition to reductions of nutrient losses from cropping systems.

Root facilitative interactions within intercropped species integrate a wide-range of ecological services, each influenced by local climate and growth conditions as well as choice of species. The aim of this review is not to point to specific mechanisms or to give quantitative estimations of effects, but more to exemplify potential possibilities of including mechanisms of competition and facilitation in the development of future agricultural systems and environmental

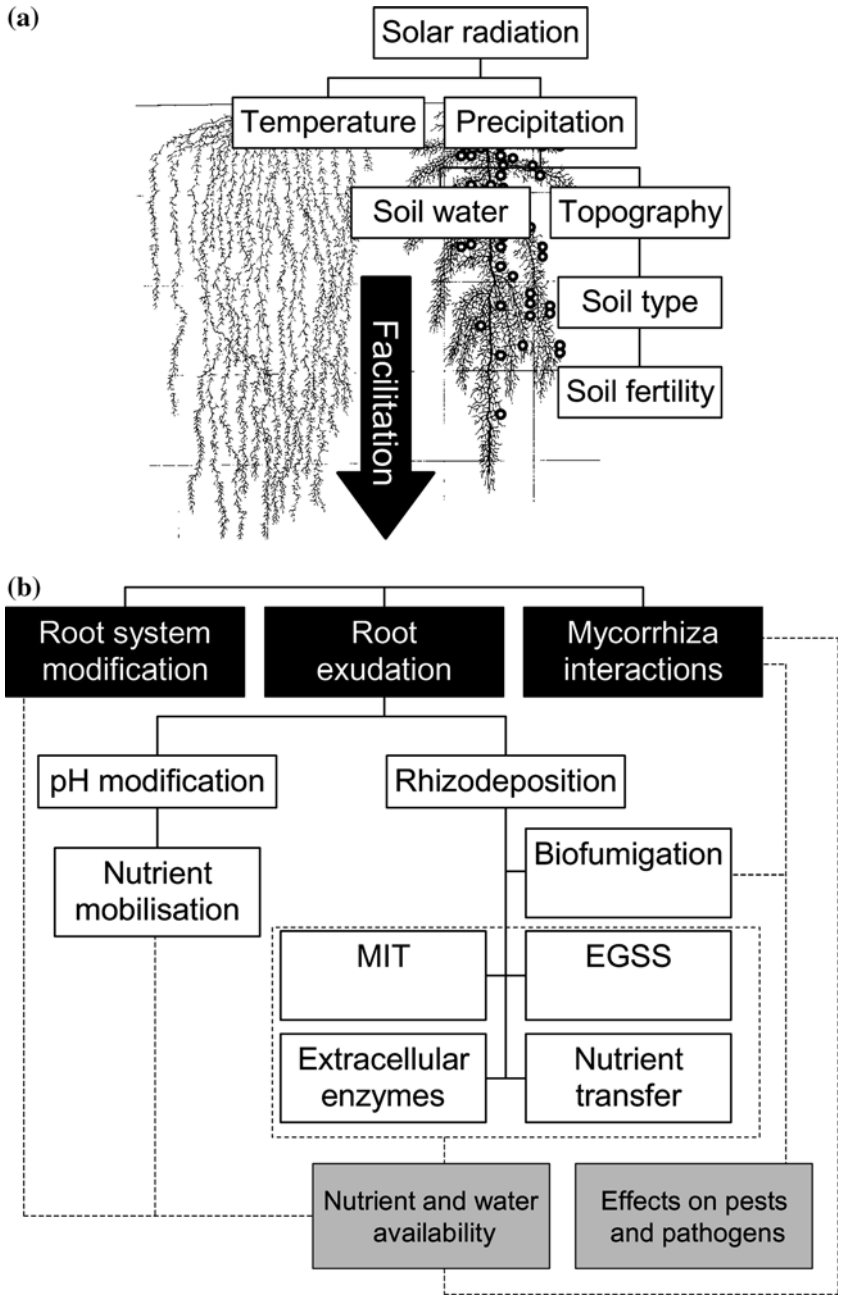

Figure 5. Conceptual model that identifies the major parts and processes in the soil-plant system involved in the general crop growth (a) and the potential facilitative interactions amongst intercropped species (b) discussed in the text. MIT, mineralisation-immobilisation turnover; EGSS, exudation of growth stimulating substances. Solid lines indicate physiological processes involved in facilitative interactions, and dotted lines the effect on plant regulating growth parameters like nutrients, water, pest and pathogens influencing the combined crop growth in an intercrop.

management. Root facilitation is regarded as a fundamental process in the dynamics of plant communities (Figure 5) and is important to include among other agronomic factors affecting the efficiencies of various intercropping systems.

Acknowledgements

The Research Centre for Organic Farming (DARCOF) funded this study.

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Plant and Soil (2005) 274:251–270
DOI 10.1007/s11104-004-0966-0

Root-to-shoot signalling: Assessing the roles of 'up' in the up and down world of long-distance signalling *in planta*

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Received 24 October 2003. Accepted in revised form 23 January 2004

Key words: abscisic acid, aminocyclopropane carboxylic acid, cytokinins, partial rootzone drying, stomatal conductance, xylem sap

Abstract

An important mediator of shoot physiological processes can be the supply of signal molecules (other than water and nutrients) from the root system. Root-to-shoot signalling is often considered to be important in regulating shoot growth and water use when soil conditions change without any demonstrable change in shoot water or nutrient status. Changes in xylem sap composition are often thought to be synonymous with changes in root-to-shoot signalling, even though there is considerable re-cycling of compounds between xylem and phloem. Techniques used to collect xylem sap are reviewed. Elucidating the roles of putative root signal molecules *in planta* has usually taken priority over identifying the sources of signal molecules in xylem sap. The roles of several signal molecules are considered. This choice is selective, and the failure of known signals to account for observed physiological changes in some systems has lead to the conclusions that other novel signals can be important. The efficacy of a given signal molecule can depend on the shoot water and nutrient status, as demonstrated by variation in stomatal responses to abscisic acid. If such variation is widespread in crop species, this may have implications for the increasing intentional use of root-to-shoot signals to modify crop water use and shoot architecture. Research into root-to-shoot signalling may become increasingly reductionist, in trying to evaluate the contribution of root signals *versus* local processes to observed physiological changes. However, future challenges are to successfully integrate this basic research into improved crop production systems.

Abbreviations: ABA – abscisic acid; ABA-GE – ABA glucose ester; ACC – aminocyclopropane-1-carboxylic acid; AOA – aminooxyacetic acid; AVG – L-α -(2-aminoethoxyvinyl)glycine; CK – cytokinin; DHZ – dihydrozeatin; DHZ-9G – dihydrozeatin-9-glucoside; DHZ-OG – dihydrozeatin-O-glucoside; DHZR – dihydrozeatin riboside; DHZR-OG – dihydrozeatin riboside-O-glucoside; DI – deficit irrigation; GA – gibberellin; g_s – stomatal conductance; IAA – indole-acetic acid; iP – isopentenyladenine; *ipt* – isopentenyl transferase; PRD – partial rootzone drying; WT – wild-type; Z – zeatin; Z-OG – zeatin-O-glucoside; ZR – zeatin riboside.

Introduction

Traditionally, the root system has been perceived as providing essential resources (water and nutrients) to the shoot and in many circumstances reductions in shoot growth can be explained by the inability of the root system to adequately supply these resources. By adjusting the rate of nitrogen (N) supply to the roots, it has been possible to precisely control shoot N concentration and plant relative growth rate (Ingestad and

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Lund, 1979). When water is withheld from the roots, leaf water status can decline in parallel with leaf gas exchange and leaf growth (Kramer, 1974). However, shoot physiology cannot always be explained by the availability of resources in the shoot.

When plants are grown in large soil volumes with adequate nutrition, and the soil moisture is slowly depleted, stomatal conductance and leaf growth can decline without detectable changes in leaf water status (Saab and Sharp, 1989; Zhang and Davies, 1990a). Another way of preventing changes in leaf water status following soil drying or osmotic stress has been to grow plants in specialised pressure chambers with pneumatic pressure applied to the roots. This technique maintains xylem sap continuously on the verge of bleeding from a cut leaf, so that the plants remain fully turgid (Passioura and Munns, 1984), yet pressurised water-stressed or salt-stressed plants show the same reduction in leaf growth as unpressurised plants (Termaat et al., 1985; Passioura, 1988). One explanation of such results is that the roots sense a change in soil water status, and transmit chemical signals to the shoots to regulate shoot physiology.

There is also evidence that long-distance signalling can regulate shoot gene expression following changes in nutrient supply to the root system, independently of changes in nutrient delivery to the shoot. Re-supply of N to N-starved maize (*Zea mays*) plants increased delivery of both N and cytokinins to the shoot and promoted shoot expression of a gene (*ZmCip1*) encoding a cytokinin-inducible protein (Takei et al., 2002). Further experiments that simulated changes in the xylem delivery of compounds to detached N-starved leaves showed that supplying millimolar concentrations of inorganic N sources (nitrate and ammonium ions) via the transpiration stream did not promote *ZmCip1* expression, while supply of nanomolar concentrations of various cytokinins promoted *ZmCip1* expression.

Research into root-to-shoot signalling has proliferated in recent years. It is therefore timely to review techniques to investigate root-to-shoot signalling and some prospective signal molecules and their putative roles *in planta*. Root-to-shoot signalling incorporates changes in both essential resources (water and nutrients) and other chemical signals. Indeed, the effectiveness of various chemical signals in eliciting a physiological response can depend on the availability of other root-sourced resources. Although such variability can give rise to uncertainty in the quantitative impact of a given change in root-to-shoot signalling, recently considerable attention has been given to manipulating root-to-shoot signalling in commercial systems. Such developments are likely to stimulate research as a means of optimising production systems.

Defining, quantifying and manipulating root-to-shoot signalling

Features of root-to-shoot signals

To fulfil the criteria of a root-to-shoot signal, a compound must

- move acropetally in the plant via apoplastic (predominantly the xylem) or symplastic pathways
- influence physiological processes in a target organ (such as leaves or fruit) that is remote from the putative site of synthesis (the root)

Experiments will usually measure the concentration of a signal both in the xylem and in the target organ. The latter criterion is analogous to that used to define animal hormones, and much work on root-to-shoot signalling has concentrated on examining roles for several (classes of) compounds known as the plant hormones, which are xylem-mobile (Table 1). Since some plant hormones can be synthesised by several different tissue types, some putative target organs might also be capable of autonomous hormone production. In this case, only detailed 'hormone budgets' (quantifying steady-state hormone levels and hormone delivery to, and export from, an organ) will establish whether root-to-shoot hormone delivery is important to the hormone level of a given organ. Grafting studies with mutants or transgenics blocked in the synthesis of plant hormones can assist this aim.

Demonstration of root hormone synthesis has been achieved using isolated roots. Exudate from de-topped root systems maintained a relatively stable cytokinin activity for four days after de-topping (Kende, 1965), implying continued root

Table 1. Xylem-mobility of the classical plant hormones

Hormone Class	Xylem-mobile compounds (species - reference)
Abscisic acid	ABA, ABA-GE
Auxin	IAA
Cytokinins	DHZ-9G, ZR, iP (sunflower – Hansen and Dörffling, 2003)
	ZR, Z, iP-type CKs (pea-Beveridge et al., 1997)
	ZR, DHZR, Z-OG, DHZ-OG, DHZR-OG, nucleotides of Z, DHZ, DHZ-OG (bean - Palmer and Wong, 1985)
Ethylene	ACC
Gibberellins	$GA_{15,17-19,23,44,53}$ (apple-Motosugi etal., 1996)

Abbreviations: ABA – abscisic acid; ABA-GE – ABA glucose ester; ACC – Aminocyclopropane-1-carboxylic acid; CK – cytokinin; DHZ – dihydrozeatin; DHZ-9G – dihydrozeatin-9-glucoside; DHZ-OG – dihydrozeatin-O-glucoside; DHZR – dihydrozeatin riboside; DHZR-OG – dihydrozeatin riboside-O-glucoside; GA – gibberellin; IAA – indole-acetic acid; iP – isopentenyladenine; Z – zeatin; Z-OG – zeatin-O-glucoside; ZR – zeatin riboside.

cytokinin synthesis. Supply of radioactive [8-^{14}C] adenine, a precursor of cytokinins, to isolated pea (*Pisum sativum*) roots and quantification of radioactive cytokinins in root extracts established that roots can synthesise cytokinins (Chen et al., 1985). Dehydration of detached roots of several species (Milborrow and Robinson, 1973) and of various ages and branching orders (Simonneau et al., 1998) stimulated ABA synthesis. Root expression of hormone-biosynthesis genes provides further circumstantial evidence of root hormone biosynthesis, but sites of high gene expression may not necessarily be sites of high hormone synthesis. While the above studies indicate that roots are capable of hormone synthesis, the extent to which they actually contribute to shoot hormone levels is more difficult to assess.

Many studies have quantified xylem sap composition in response to various changes in soil conditions. Even when the concentrations of putative signals are accurately quantified, there can be no guarantee that the compounds present are indeed synthesised in the roots. Considerable re-cycling of compounds between xylem and phloem occurs, and this can be quantified in certain species that spontaneously exude phloem sap following wounding. Following collection and analysis of both xylem and phloem sap, upward and downward flows of putative signals can be modelled. This approach indicated ABA recirculation in salt-stressed lupin (*Lupinus albus*) plants (Wolf et al., 1990) and in several experiments where castor bean (*Ricinus communis*) plants were grown with different nutrient regimes (Peuke et al., 2002 and references therein).

Another approach to demonstrate that recycling of compounds from the phloem can result in a supposed root-to-shoot signal is to disrupt basipetal phloem transport by girdling the stem at the root-shoot junction (e.g., Liang et al., 1997; Vernieri et al., 2001). The importance of ABA recycling in root-to-shoot signalling of environmental stress likely varies according to the effect of the stress on root and shoot water relations (since decreased turgor stimulates ABA biosynthesis – Pierce and Raschke, 1980). When hydroponically grown bean *(Phaseolus vulgaris)* plants were chilled to 5 °C, leaf turgor declined by 0.5 MPa within 24 h while root turgor was scarcely affected. The loss of leaf turgor stimulated leaf ABA biosynthesis. Export of ABA from those leaves and subsequent recycling via the phloem was apparent, since chilled plants showed a 3-fold increase in xylem ABA concentration which was absent in chilled, stem-girdled plants (Vernieri et al., 2001). In this case, disruption of phloem transport from the shoot abolished a putative root-to-shoot signal. In contrast, when maize plants were grown in drying soil (where turgor of some root tips is likely to decrease prior to any change in leaf turgor – Sharp and Davies 1979), stem girdling only moderately decreased (25–30%) the increase in xylem ABA concentration (Liang et al., 1997).

Stem girdling will not only disrupt the basipetal flow of the compound of interest (and thus potentially disrupt recycling of that compound). Also, the flow of other compounds, which may stimulate or repress the synthesis or metabolism of plant hormones in the roots and /or their

export from the roots, will be disrupted. For example, stem girdling disrupted the basipetal flow of sucrose to the roots of *Sinapis alba* plants induced to flower by exposure to a single long day, and also decreased root cytokinin export. Exogenous supply of sucrose to the roots of girdled plants restored root cytokinin export, suggesting that root sucrose levels regulated root cytokinin export (Havelange et al., 2000). The effects of stem girdling on root-to-shoot signalling would be worth studying in a species from which phloem sap is easily collected, to gauge the relative magnitude of hormone recycling and root hormone export.

Plant hormones are also present in the rhizosphere due to microbial and/or fungal synthesis (Costacurta and Vanderleyden, 1995) and as a result of hormone efflux from root cells (Jovanovic et al., 2000), As water moves into the root system along water potential gradients, some hormone molecules will be dragged along and these can be transferred into the xylem (Freundl et al., 1998), Detection of a compound in xylem sap is often taken as evidence of root-to-shoot signalling, even when the contributions of various sources of the compound (rhizosphere, root, shoot) are not assessed. Irrespective of the source of root-to-shoot signals, it is their ability to influence signal concentration at the active site that is physiologically important.

Demonstrating physiological activity of a putative root-to-shoot signal in a target organ (e.g., leaves) often takes precedence over determining the origin of the signal supply to, or within, that organ. Preliminary evidence of a compound's activity is a correlation between the concentration (or delivery) of the compound and the rate of a physiological process. [Concentration is the number of molecules in a given volume, while delivery or flux is the concentration multiplied by the flow rate of the transpiration stream.] Correlations become more convincing when time-course studies indicate that changes in concentration (or delivery) of the compound precede a change in rate of a physiological process. When time courses show that putative cause and effect co-occur, a problem is to differentiate between the two. This is especially the case where the physiological effect is likely to modify concentration or delivery. For example, stomatal closure should passively increase the concentration of many xylem sap compounds by decreasing transpirational flux. This would give negative correlations between stomatal closure and the concentrations of many compounds, irrespective of whether any given compound is actually causing stomatal closure. For this reason, more rigorous tests of the physiological significance of a given compound have been formulated (Jackson, 1987). *Correlation* and *duplication* experiments apply synthetic compounds to intact plants or isolated plant parts, aiming to duplicate the relationships seen *in vivo*. *Deletion* and *re-instatement* experiments aim to alter the concentrations of specific compounds in xylem sap, using mutant and transgenic lines *in vivo*, or immunoaffinity columns in collected xylem sap. Another type of deletion experiment decreases the supply of root-sourced signals by excising part of a spatially divided root system.

Much work has sought to identify the role of a given hormone class in a given physiological process, using the approaches outlined above. This classical approach will be necessarily limited by a worker's appreciation of what signals are likely to be important, and the ability to measure those signals. The advent of increasingly sophisticated analytical techniques capable of simultaneously identifying multiple compounds in a metabolic pathway or pathways (termed 'metabolomics') allows examination of a given xylem sap sample for multiple compounds, irrespective of pre-conceptions of which compounds might be important. This approach will increasingly be applied where the classical approach is unable to explain certain physiological processes where root-to-shoot signals are implicated. Regardless of the degree of sophistication employed in sap analysis, it is important to collect an authentic sample of xylem sap, free of contamination from other signal sources.

Xylem sap collection

Various techniques have been used to gain a sample of xylem sap:

- root pressure from de-topped roots;
- applying a pneumatic pressure or vacuum to de-topped roots;
- applying a pneumatic pressure or vacuum to detached leaves or shoots;
- applying a pneumatic pressure to intact plants grown in specialised root pressure chambers.

An important consideration is the site of xylem sap collection, and whether root export of substances, or the delivery of those substances to a target organ in the shoot is to be quantified. Several studies have shown gains or losses in xylem solutes during long-distance transport in the stem (Schill et al., 1996; Jokhan et al., 1999).

As discussed above, the concentration of a given compound in xylem sap will depend on sap flow rate, and the concentrations of many xylem constituents increase with decreasing sap flow rate (Munns, 1985; Else et al., 1995; Schurr and Schulze, 1995). Sap collection from de-topped root systems under root pressure alone will over-estimate true concentrations in xylem sap (Else et al., 1995; Schurr and Schulze, 1995). Similarly, applying an arbitrary overpressure to detached roots may under- or over-estimate xylem sap hormone concentration. For this reason, it is necessary to collect xylem sap at several different overpressures to encompass the range of sap flow rates found *in vivo*. The laborious nature of sample collection has prompted searches for short-cuts to ensure a realistic sap flow rate. A promising approach was to apply a pressure to a de-topped root system that was equal in magnitude to shoot water potential (Liang and Zhang, 1997). However, further tests showed that the resultant sap flow rate exceeded that occurring *in vivo* due to significant hydraulic resistances in the shoot (Jokhan et al., 1999).

Another approach to collect xylem sap from root systems is to apply suction using a syringe attached to the de-topped root system (e.g., Lejeune et al., 1988; Beveridge et al., 1997). While the suction generated (< 0.10 MPa) will enhance sap flow rate relative to collection via root pressure alone, there has been no attempt to compare solute concentrations of sap collected this way with samples obtained via the application of pneumatic pressure to the roots. Attaching the syringe to the de-topped root system will be expected to release solutes from cut cells as in the case of attaching a collecting tube (Else et al., 1994), and the effect of this release on final solute concentrations will depend on the duration of sap collection.

Measurement of root system export in field-grown plants will be limited to collection from de-topped root systems under root pressure alone or via suction, with the attendant concentration of xylem sap solutes discussed above. For this reason, xylem sap samples from plants in the field will more commonly be collected from the shoot. Pressurisation of detached leaves or shoots in a Scholander-type pressure chamber is widely applied, although the sample will represent contributions of both apoplastic and symplastic sap if the volume of sap collected is greater than that present in the xylem vessels of that tissue (which can be estimated using anatomical measurements). Following measurement of leaf water potential in sunflower (*Helianthus annuus*) leaves, small sequential overpressures ($+0.02$–0.04 MPa) were applied to determine the origin of sap on the basis of sap osmolarity (Jachetta et al., 1986). As sap osmolarity declined, sap was identified as coming from different leaf compartments: petiole and midrib ($+0.02$–0.04 MPa), minor veins and cell walls ($+0.13$–0.22 MPa) and symplastically filtered sap ($> +0.22$ MPa). Commonly, overpressures of 0.2–0.5 MPa (e.g., Correia and Pereira, 1994; Ben Haj Salah and Tardieu, 1997) are applied to leaves to ensure sufficient xylem sap for analysis, and such samples represent a mixing of apoplastic and symplastic sap. Despite this mixing, some compounds maintain a stable concentration at various overpressures, as in the case of ABA collected from maize leaves (Dodd et al., 1996). Possible phloem contamination of a xylem sap sample can be checked (and corrected for) by measuring sample sugar concentrations, as xylem sap of most species is sugar-free (Correia and Pereira, 1994). Regression analysis plotting the concentration of the compound of interest against sugar concentration allows determination of the compound's concentration when sugar concentration equals zero. Although a valuable test, this approach has not yet been extensively used.

Xylem sap can also be collected from dicotyledons (especially woody species) after the cessation of internode elongation by applying suction to detached shoots. Small lengths of stem are successively cut from the shoot tip to release the xylem contents (Bennett et al., 1927). There can be pronounced gradients in solute concentrations along the stem. Sap collected with this technique showed similar changes in amino acid and ureide composition as did sap collected from de-topped root systems under root pressure alone, even

though solute concentrations were much higher in the latter (Herridge, 1984). While this method is useful to gauge the relative abundance of different compounds, it is uncertain whether the solute concentrations detected are similar to those actually occurring in the transpiration stream.

Growth of plants in a root pressure chamber (Passioura and Munns, 1984) allows collection from an intact leaf with minimal wounding of the plant during xylem sap collection, allowing xylem sap to be repeatedly collected from the same plant. A small piece of the target leaf is removed to allow a balancing pressure (the pressure at which the xylem is on the verge of bleeding) to be manually set. After setting the balancing pressure, another, larger portion of leaf can be removed to induce xylem flow (Figure 1), or the pressure can be further raised above balancing pressure. The rate of xylem flow will increase in proportion to the leaf area removed (as hydraulic resistance is less), and will affect the concentration of xylem sap constituents. During collection of a single xylem sap sample, the concentration of xylem sap constituents in sequential samples can be affected by flow rate due to dilution effects (Figure 1). Although root pressure chambers are not commonly available, this technique allows xylem sap to be collected in a target leaf at flow rates similar to *in vivo* leaf transpiration rates.

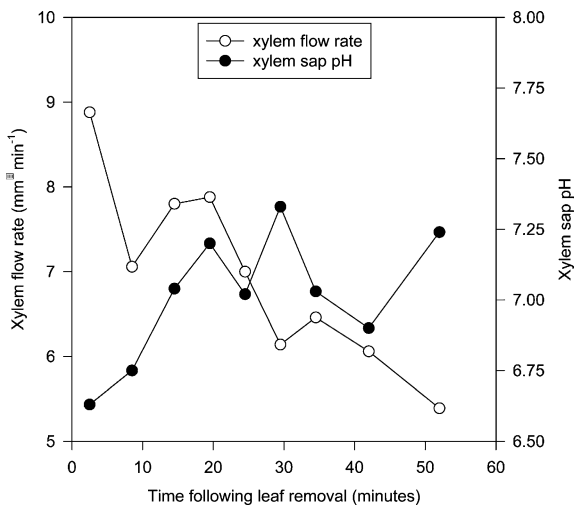

Figure 1. Changes in xylem sap flow rate (O) and pH (•) during collection of xylem sap from an expanding *Capsicum annuum* leaf using a root pressure chamber (I.C. Dodd, unpublished results). Prior to collection of sap, the leaf tip (*circa* 20% of individual leaf area) was removed to allow a balancing pressure to be set. At 0 min, half the remainder of the leaf (*circa* 5% of total plant leaf area) was removed to allow xylem flow. Sap was collected in pre-weighed vials at 5–10 min intervals.

Grafting studies

Grafting can be useful to establish how changing the genetic make-up of the root (and shoot) can affect xylem sap composition and the phenotype of root and shoot. When combined with mutant or transgenic lines that are deficient in or over-produce a putative root-to-shoot signal, reciprocal grafting theoretically allows leaf and xylem hormone composition to be manipulated independently. The re-circulation of compounds between xylem and phloem means that xylem signal concentration of reciprocally grafted mutant and wild-type (WT) plants can be intermediate between self-grafted plants (e.g., ABA concentrations in Table 2), and it is thus difficult to evaluate the specific contribution of the root. The site of the graft union will also influence potential sources of hormones in the xylem. Grafting the scion onto a stem with several nodes (e.g., Borel et al., 2001) allows contributions of the rootstock stem (and leaves if attached) to influence scion xylem composition; while epicotylar grafting (e.g., Beveridge et al., 1997) allows the cotyledons to influence scion xylem composition. To properly evaluate the contribution of a grafted root system to xylem composition, grafting at the root-shoot junction is needed.

Grafting has been used as a tool to investigate root-shoot interactions governing a range of physiological responses, such as the role of root-sourced ABA in water-stress-induced stomatal closure (Holbrook et al., 2002), the genetic control of shoot branching (Beveridge, 2000) and the role of gibberellins in stem extension (Proebsting et al., 1992). A powerful demonstration of root-to-shoot signalling in controlling developmental responses is the use of epicotyl interstock grafts. When a small piece of WT stem tissue was grafted between the rootstock and scion of either the *dad1* petunia (*Petunia hybrida*) branching mutant (Napoli, 1996), or the *rms1* pea branching mutant (Foo et al., 2001), branching of the mutant scion was inhibited. This result was interpreted as the WT tissue blocking the

Table 2. Xylem signal concentrations from reciprocally grafted wild-type (WT) and mutant (w) plants

Reference	Genes and species	Xylem signal	w/w	w/WT	WT/w	WT/WT
Holbrook et al., 2002	*sitiens* and WT tomato	[Abscisic acid] (nM)	1	7	11	42
Beveridge et al., 1997	*rms4* and WT pea	[ZR-type CKs] (nM)	1.5	2.2	19	13

movement of a root-to-shoot signal allowing lateral branching (Foo et al., 2001).

Some root signal molecules and their roles *in planta*

Abscisic acid (ABA)

Stomatal conductance (g_s)

The potency of ABA in closing stomata in epidermal strips and detached leaves (perfusion of epidermal strips with as little as 100 pM ABA can elicit substantial stomatal closure – Trejo et al., 1995) has promoted considerable research into the possibility that changes in endogenous ABA concentrations cause stomatal closure during water stress (discussed below) and other stresses (Dodd, 2003). Initial work focused on the hypothesis that leaf-sourced ABA moved towards the guard cells during periods of water stress, and only later were changes in xylem ABA concentration dynamically linked to changes in stomatal conductance (Loveys, 1984). Subsequently, xylem ABA concentration was shown to increase much earlier and to a greater extent than bulk leaf ABA concentration during a soil drying cycle, and this increase correlated with decreased g_s (Zhang and Davies, 1990a). Field and glasshouse studies showed that xylem ABA concentration and g_s are correlated (when xylem sap was collected by pressurisation, from the same leaves in which g_s was measured) in several species (*Zea mays* – Tardieu and Davies, 1992; *Helianthus annuus* – Tardieu et al., 1996; *Nicotinia plumbaginifolia* – Borel et al., 2001). In a given species, this relationship is commonly unified across different growing conditions, from leaf to leaf on individual plants, and from day to day as the plant develops (Tardieu et al., 1992). Soil application (Zhang and Davies, 1990b) or stem injection (Tardieu et al., 1996) of ABA to well-watered plants generates a similar relationship between xylem ABA concentration and g_s to that found in water-stressed plants. Removal of ABA from maize (*Zea mays*) xylem sap using an immunoaffinity column eliminated its antitranspirant activity, as assessed using a detached leaf transpiration assay (Zhang and Davies, 1991).

However, several studies indicate that variation in xylem ABA concentration alone cannot always explain the extent of water-stress-induced stomatal closure. Although the ABA concentration in xylem sap can promote stomatal closure when fed to detached leaves in some species (*Vitis vinifera* – Loveys, 1984; *Zea mays* – Zhang and Davies, 1991; *Lupinus albus* – Correia and Pereira, 1994), in other species xylem ABA concentration is apparently too low to influence stomatal conductance (*Prunus armeniaca* – Loveys et al., 1987). Similarly, the antitranspirant activity of wheat (*Triticum aestivum*) xylem sap could not be explained in terms of its ABA concentration as assessed using a detached leaf transpiration assay, and removal of ABA from this xylem sap using an immunoaffinity column did not eliminate its antitranspirant activity (Munns and King, 1988). Water-stress-induced stomatal closure of bean (*Phaseolus vulgaris*) preceded increases in xylem ABA concentration (Trejo and Davies, 1991). Reciprocal grafting of WT and ABA-deficient tomato (*Lycopersicon esculentum*) genotypes compared water-stress-induced stomatal closure and ABA concentrations of the graft combinations (Holbrook et al., 2002). Irrespective of whether WT shoots were grafted on WT or ABA-deficient roots, stomatal closure occurred in both graft combinations, despite a 4-fold difference in xylem ABA concentration. Such data suggest that other chemical regulators, in addition to ABA, can act as signals of the degree of soil drying and influence stomatal behaviour.

Supplying detached *Commelina communis* and tomato leaves with neutral or alkaline

buffers (pH ≥ 7) via the transpiration stream can partially close stomata (Wilkinson and Davies, 1997; Wilkinson et al., 1998). These buffers were believed to increase apoplastic pH, which was demonstrated to decrease sequestration of ABA by mesophyll cells. The resultant increase in apoplastic ABA concentrations ultimately closed the stomata (Wilkinson and Davies, 1997). Stomatal closure in response to increased xylem alkalinity was ABA-dependent, as leaves detached from an ABA-deficient tomato mutant (*flacca*) did not show stomatal closure when fed pH 7 buffers, and in some cases transpiration actually increased. This pH response of detached *flacca* leaves is consistent with stomatal conductance increasing when *flacca* plants dried the soil within a certain range of soil water contents (Holbrook et al., 2002). Alkalisation of xylem sap is a common response to various edaphic stresses (Wilkinson et al., 1998), and may account for observations where stomatal closure could not be readily explained by an increased xylem ABA concentration. Changes in xylem or apoplastic pH may close stomata, via a mechanism that involves ABA, prior to any *de novo* ABA synthesis.

It is important to realise that stomatal response to an increased xylem sap pH is variable. *Capsicum annuum* leaves were less responsive to a pH signal as leaves aged (Dodd et al., 2003). In contrast to experiments described above (Wilkinson et al., 1998), supplying detached tomato leaves with a pH 7 buffer did not elicit stomatal closure (Jackson et al., 2003), presumably because this buffer did not influence apoplastic pH sufficiently to increase apoplastic ABA concentration. In *Vicia faba*, a 1 unit increase in the pH of buffers fed to detached leaves had only minimal (0.1 unit) effects on apoplastic pH (Felle and Hanstein, 2002) which may not have altered apoplastic ABA accumulation. While xylem pH-induced stomatal closure is an attractive hypothesis, it should be tested using detached leaf transpiration assays in cases where it is suspected.

In some species, xylem sap pH can acidify (*Ricinus communis* – Schurr and Schulze, 1995) or show no change (*Nicotinia plumbaginifolia* – Borel et al., 2001) in response to water stress. In these species, increased xylem ABA concentration may be necessary to allow stomatal closure, or other antitranspirant compounds (as yet unidentified) may be important. Another possible explanation is that some species show high concentrations of ABA conjugates in the xylem sap (Sauter et al., 2002), which are not detected by standard immunological or physico-chemical methods for quantifying ABA. [Conjugates are detected by subjecting xylem sap to alkaline hydrolysis.] In the leaf, apoplastic glucosidases can liberate free ABA from the ABA glucose ester (ABA-GE). The antitranspirant activity of saps containing significant quantities of ABA-GE may not be explicable in terms of their ABA concentration, but sufficient ABA may accumulate at the active sites at the guard cells to initiate stomatal closure.

Leaf elongation

Although several research groups have demonstrated that leaf expansion of plants grown in drying soil can decrease even when plant water status is maintained (reviewed in Davies and Zhang, 1991), the identity of chemical signals regulating leaf expansion has proved elusive. Early experiments showed that the relationship between leaf growth inhibition and xylem ABA concentration was similar for maize plants fed ABA hydroponically in a nutrient solution and those from which water was withheld (Zhang and Davies, 1990b), indicating a possible regulatory role for ABA in controlling leaf growth of water-stressed plants. Detached maize shoots supplied with ABA via the xylem showed a similar relationship to that generated in intact plants between xylem ABA concentration and leaf growth, with 100 nM ABA inhibiting leaf elongation by *circa* 15% (Figure 2). The xylem ABA concentrations detected in field- and glasshouse-grown droughted maize plants are high enough to partially inhibit leaf growth in detached shoots, although the effectiveness of ABA as a growth inhibitor depended on prevailing evaporative demand (Figure 2; Ben Haj Salah and Tardieu, 1997). However, time-course studies have shown that leaf growth can be restricted prior to any water-stress-induced increase in xylem ABA concentration (Munns and Cramer, 1996). When xylem sap was collected from intact water-stressed wheat and barley (*Hordeum vulgare*) plants using the root pressure chamber, it contained 2–8 nM ABA and inhibited growth by 60% (compared to plants fed distilled water)

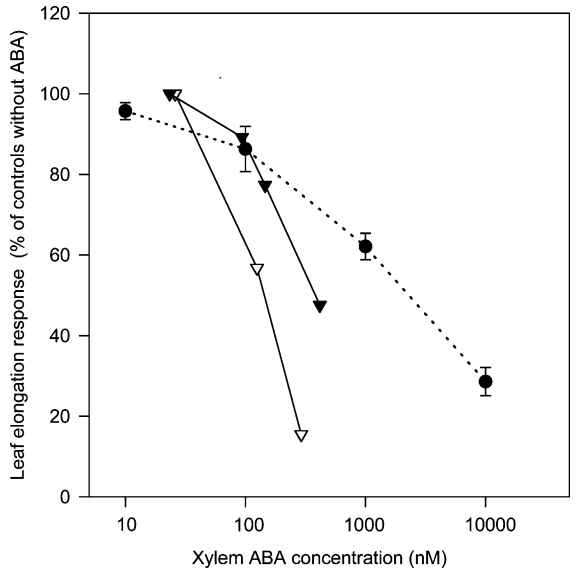

Figure 2. Relative leaf elongation response of maize leaves as a function of xylem sap ABA concentration in detached shoots (●) and intact plants grown in the greenhouse (▼,▽). For detached shoots, symbols are mean ± S.E. of n experiments for 10^{-8} M (n = 3), 10^{-7} M (n = 9), 10^{-6} M (n = 11) and 10^{-5} M (n = 8) ABA at an average temperature of 27 °C (I.C. Dodd, unpublished results). For intact plants, measurements were made during the middle of the photoperiod (▽) when air temperatures were between 30–35 °C, and the middle of the night period (▼) when air temperature was 25 °C (Re-drawn from Ben Haj Salah and Tardieu, 1997).

when fed to detached barley shoots. However, 10 μM synthetic ABA was required to elicit the same leaf growth inhibition, indicating that xylem ABA was not regulating leaf growth in these species (Munns, 1992). The evidence for and against the involvement of xylem ABA concentration in regulating leaf expansion is analogous to that for the regulation of stomatal conductance.

Water-stress-induced leaf growth inhibition can be correlated with increased xylem pH. Soil drying increased xylem pH by only 0.6 unit and almost completely inhibited barley leaf elongation (Bacon et al., 1998). When shoots detached from well-watered barley plants were fed artificial xylem saps of pH 6 and 7, the pH 7 buffer inhibited leaf growth by 37%, 54% and 71% in replicate assays (Bacon et al., 1998). It seems likely that increased xylem sap pH partially inhibited leaf growth, and that other factors (not necessarily root-to-shoot signals) were also involved. Feeding alkaline buffers to an ABA-deficient barley mutant (*Az34*) did not inhibit leaf growth unless 30 nM ABA was also present in the buffer. This pH response is directly analogous to the effect of alkaline buffers on detached leaf transpiration (Wilkinson and Davies, 1997) and interpretation of the effects of alkaline xylem pH on leaf elongation has relied on mechanisms elucidated with isolated stomata. At the more acidic apoplastic pHs found in well-watered plants, ABA is presumably partitioned into alkaline compartments in the symplast and away from ABA receptors on the guard cell apoplast (Wilkinson and Davies, 1997) and presumably also on the apoplast of expanding cells.

Initial work growing ABA-deficient mutants at low soil water status supported the contention that water-stress-induced ABA accumulation inhibits shoot growth, as shoots of the maize *vp5* mutant grew more than WT shoots when transplanted to vermiculite at −0.3 MPa (Saab et al., 1990). However, extending the experiment beyond 60 h after transplanting resulted in the WT shoots growing faster than *vp5*, indicating that the effects of ABA varied with the stage of plant development (Sharp and LeNoble, 2002). Further work with ABA-deficient mutants has reinforced the view that an important function of ABA is to restrict ethylene synthesis (Sharp and LeNoble, 2002). However, it is not yet clear whether xylem ABA concentrations (rather than bulk leaf ABA concentrations) can moderate leaf ethylene synthesis during water-stress episodes.

Aminocyclopropane carboxylic acid (ACC)

Petiole epinasty and elongation during soil flooding
Tomato responds to soil flooding by showing epinastic leaf curvature, which decreases the radiation load on the leaves. Although soil flooding can increase soil ethylene concentrations, and radioactive ethylene fed to the roots can be detected in the shoots and cause epinasty (Jackson and Campbell, 1975), attention has focused on ACC (the immediate precursor of ethylene) as a root-sourced signal. The anaerobic environment caused by soil flooding prevents the oxidation of ACC to ethylene in the roots, increasing root ACC concentrations (an 11-fold accumulation after 12 h flooding in tomato) (Bradford and Yang, 1980). Vacuum collection of sap from detopped roots showed that increased xylem ACC

delivery preceded petiole ethylene production and epinastic curvature. Application of inhibitors of ACC synthase (30 μM aminooxyacetic acid [AOA] or 10 μM L-α-(2-aminoethoxyvinyl)glycine [AVG]) to an anaerobic nutrient solution prevented the increase in xylem ACC delivery and petiole ethylene evolution and epinasty (Bradford et al., 1982). Supplying xylem ACC concentrations typically found in xylem sap of flooded tomato plants (at least 3 μM) to shoots detached from well-drained plants induced petiole ethylene evolution and epinasty (Bradford and Yang, 1980), suggesting that root-sourced ACC was responsible for shoot responses.

In the experiments described above, xylem sap was collected by applying an arbitrary vacuum suction to de-topped shoots, and the resultant sap flow rate may have been less than that occurring in flooded plants, thus over-estimating xylem ACC concentration. In subsequent experiments, root pressurisation was applied to flooded tomato plants to generate a range of sap flow rates. At flow rates that were equivalent to whole plant transpiration rate, plants that had been flooded for 24 h showed a 10-fold increase in xylem ACC concentration, and a 3-fold increase in xylem ACC delivery (Else et al., 1995). Rhizospheric ACC concentration was also measured to assess its contribution to the root-to-shoot signal. Assuming that rhizosphere ACC could move into the plant similarly to an apoplastically mobile dye that was watered into the soil, it was calculated that the doubling of soil water ACC concentration caused by soil flooding contributed only 3% to the increase in xylem ACC delivery (Else et al., 1995). Time course studies indicate that increased xylem ACC delivery co-occurred with petiole epinasty in flooded tomato, even though a stimulation of petiole ethylene evolution occurred after a lag of 12 h (Else et al., 1998). Flooded tomato plants also showed an increased (2–2.5 fold after 24 h flooding) petiole ACC oxidase activity, and transgenic plants with a decreased ACC oxidase activity showed a decreased epinastic response (English et al., 1995), indicating that shoot processes can mediate the response to a root-to-shoot signal.

Further (circumstantial) evidence that root-sourced ACC is important to the epinastic response of flooded tomato plants is provided by transformation of plants with a bacterial enzyme ACC deaminase under both constitutive (35S) and root specific (*rolD*) promoters (Grichko and Glick, 2001). Plants expressing ACC deaminase only in the roots showed a delayed epinastic response to soil flooding in comparison with plants constitutively expressing ACC deaminase and non-transformed plants, however the effects of the two transformations on root ACC concentrations and xylem ACC delivery were not determined.

Submergence of certain *Rumex* species increased petiole elongation rate (Voesenek and Blom, 1989), an adaptation that aims to shorten the duration of submergence. Application of 0.5 Pa ethylene to non-submerged plants increased petiole elongation rate (Voesenek and Blom, 1989), and application of inhibitors of ethylene synthesis or action to submerged plants decreased petiole elongation rates (Voesenek et al., 1997). Petiole elongation continued rapidly even when leaf tips emerged above the floodwater, coincident with stomatal re-opening, the resumption of transpiration and a burst of ethylene production (Voesenek et al., 2003). Three sources of ACC were identified to contribute to the burst of ethylene production following de-submergence: oxidation of ACC that had accumulated in the shoot during submergence, *de novo* shoot ACC synthesis, and root-sourced ACC that was transported in xylem sap to the shoot following the increase in transpiration that accompanied stomatal opening (Voesenek et al., 2003).

Leaf growth, abscission and water stress

Contrary to the role of ethylene in promoting shoot extension of flooded plants, in many situations ethylene is inhibitory to shoot growth (Abeles et al., 1992). There is some evidence for the involvement of ethylene in leaf growth responses to edaphic stress. Tomato plants with part of their root system in compacted soil show leaf growth inhibition and enhanced ethylene evolution (Hussain et al., 1999). [It is not yet clear whether this enhanced ethylene evolution is a response to increased soil strength *per se*, or due to a decreased oxygen availability that sometimes occurs in compacted soil (Bengough and Mullins, 1990).] Leaf growth of transgenic tomato plants, with a decreased ACC oxidase activity and decreased stress-induced ethylene production, was not inhibited by the same compaction stress (Hussain et al., 1999). Excising the roots growing

in compacted soil from WT plants restored leaf area, and prevented the increase in ethylene evolution, indicating the involvement of a root-sourced signal. ACC is a likely candidate. Salt shock (200 mM NaCl) increased (3-fold after 9 days) xylem ACC concentrations in hybrid citrange (*Citrus sinensis* × *Poncirus trifoliata*) (Gomez-Cadenas et al., 1998). Xylem ACC concentrations also decreased exponentially following re-hydration of mandarin (*Citrus reshni*) seedlings in dry soil (Tudela and Primo-Millo, 1992). These studies did not explicitly correlate the increase in xylem ACC concentration with decreased leaf expansion. Instead, the work with citrus has revealed a role for root-sourced ACC in controlling leaf abscission following water stress.

Water stress was induced by transplanting bare-rooted mandarin seedlings to dry sand for 24 h, and leaf water potential decreased to −3.0 MPa (Tudela and Primo-Millo, 1992). Plants that remained in dry sand for a further 24 h had no leaf abscission but rehydration of seedlings caused 30–50% leaf loss after a further 24 h. Root-sourced chemical signals were important to the response as re-hydration of de-rooted seedlings prevented leaf abscission. The initial water-stress induced substantial (25-fold) root ACC accumulation and xylem ACC concentration decreased following rehydration. Leaf abscission induced by re-hydration was inhibited when roots were pre-treated with 1 mM AOA (which inhibits ACC synthase) or when shoots were sprayed with 0.1 mM cobalt ions (which inhibit ACC oxidase) (Tudela and Primo-Millo, 1992). Since the de- and re-hydration treatments caused parallel changes in root and xylem ACC and ABA concentrations, the role of root ABA accumulation was addressed by pre-treatment of roots with 1 mM norflurazon, an inhibitor of ABA biosynthesis. Norflurazon pre-treated seedlings showed less leaf abscission and diminished oscillations of root and xylem ABA and ACC concentrations during the water stress/re-hydration cycle, suggesting that ABA upregulated the ethylene response at the level of ACC synthesis (Gomez-Cadenas et al., 1996). This response contrasts with that occurring in maize roots grown at low soil water potential, where decreasing ABA concentrations using a carotenoid biosynthesis inhibitor (fluridone) increased ethylene evolution (Spollen et al., 2000).

Cytokinins (CKs)

Senescence

Observations that feeding CK solutions to detached leaves can delay senescence (e.g., Badenoch-Jones et al., 1996) have suggested that root-derived CKs influence senescence *in vivo*. Correlative evidence is provided by the decline in xylem CK concentration as plants age (e.g., Nooden and Letham, 1993), ostensibly when plants are grown with adequate moisture and nutrition. Since most such developmental studies have collected xylem at flow rates much less than whole plant transpiration rate, there has been concern that xylem CK concentrations reported in pre-senescent plants are much higher than occurring *in vivo*, and that actual CK concentrations may not delay senescence if fed to detached leaves (Jackson, 1993). Irrespective of flow rate issues, the most detailed measurements of xylem and shoot CKs during development are in fruiting soybean (*Glycine max*) (Nooden and Letham, 1993). In this species, variations in actual CK delivery to the shoot may be more closely related to other developmental events (e.g., seed set, pod extension) than senescence. Measurement of CK concentrations in xylem sap collected at realistic flow rates, in a model system comprising a single vegetative axis (to avoid effects of shoot branching and reproduction on CK status) would strengthen the case that xylem CKs can regulate shoot senescence.

An alternative approach to probing the role of root-derived CKs in senescence has used transgenic plants containing the isopentenyl transferase (*ipt*) gene (which catalyses *de novo* CK biosynthesis) under the control of inducible promoters. Tetracycline induction of root CK synthesis increased the CK concentration of sap collected from the root system 10-fold, but grafting non-transformed tobacco scions on to the root system of a CK-overproducing transgenic did not influence shoot CK concentrations or prevent normal leaf senescence (Faiss et al., 1997). In a similar experiment where the *ipt* gene was placed under control of a copper-inducible promoter allowing root specific expression, shoot senescence was delayed and leaf CK concentrations increased relative to non-transformed plants (McKenzie et al., 1998). Presumably CK delivery from the root system was increased

(although this wasn't actually measured). Measurement of xylem sap collected from the leaves in both systems may reconcile these seemingly contradictory results.

Stomatal conductance (g_s)

Experiments with isolated leaves and epidermes have shown variable responses to applied CKs including promotion of transpiration and stomatal aperture (reviewed in Incoll and Jewer, 1987; Dodd, 2003). Environmental stresses that promote stomatal closure usually also decrease xylem CK concentration and delivery, thus there has been much speculation that stomatal behaviour is moderated by root-to-shoot delivery of CKs. Although there is some evidence that the changes in xylem CK concentration likely encountered *in vivo* can increase detached leaf transpiration (Badenoch-Jones et al., 1996), in many species the xylem CK concentrations required to affect transpiration are orders of magnitude greater than those found in actual xylem sap (Dodd, 2003). Rather than affecting stomatal conductance independently, CKs may antagonise ABA-induced stomatal closure, and a recent experiment measuring detached leaf transpiration shows this can occur within the concentration range of CKs and ABA found in sunflower xylem sap (Hansen and Dorffling, 2003). The availability of transgenic lines with altered CK status, when coupled with reciprocal grafting, may allow any role of root-sourced CKs to be discerned. The use of inducible promoters linked to CK biosynthetic genes may be important, as studies with pea branching mutants have indicated that the shoot may control the root export of CKs (Beveridge et al., 1997).

Shoot branching

A widely used model for the hormonal regulation of shoot branching (Sachs and Thimann, 1967) proposed that axillary bud growth was inhibited by auxin derived from the shoot apex, and promoted by root-sourced CKs. Shoot apex decapitation has been applied as an experimentally convenient means of testing this model. Although decapitation apparently increased the concentration of specific CKs in bean xylem exudate by up to 1.6-fold (Bangerth, 1994), IAA concentrations in axillary buds do not always decrease (Gocal et al., 1991). In intact plants, there is considerable doubt that the Sachs-Thimann model applies, as highlighted by studies of the *rms1-5* pea branching mutants (Beveridge, 2000; Morris et al., 2001). None of these mutants are deficient in IAA or in the basipetal transport of this hormone, contrary to the predictions of the model. Furthermore, 4 out of 5 mutants show a greatly decreased xylem CK concentration (e.g., Table 2 gives zeatin riboside concentrations of self-grafted *rms4* and WT plants). Reciprocal grafting studies with WT and *rms4* plants show that root CK export is determined by the shoot genotype, as WT/*rms4* (scion/rootstock) grafts have normal xylem CK concentrations, while *rms4*/WT grafts have greatly decreased xylem CK concentration (Table 2; Beveridge et al., 1997). Such data suggest that the shoot is controlling some or all of the following processes: root CK biosynthesis, the transfer of root-derived CKs into the xylem and the recycling of CKs between xylem and phloem.

Floral induction

Flowering of many plant species is photoperiod sensitive and in some species floral induction is accompanied by a transient increases in the CK concentration of xylem sap collected from the root system by mild suction (Lejeune et al., 1988; Machackova et al., 1996). [Paradoxically, floral induction in *Xanthium strumarium* is accompanied by a transient decrease in xylem CK concentration (Kinet et al., 1994).] Remarkably, the increased CK response is found in two species that require very different light regimes to induce flowering (Figure 3). Evidence that this transient increase in CK is necessary to promote flowering was provided by girdling experiments, which decreased root CK export *circa* 4-fold and decreased the percentage of plants flowering by half. Restoration of root CK export, by supplying 4% sucrose to the root system of girdled plants 8 h after the beginning of an inductive long day, increased the percentage of plants flowering to that achieved by non-girdled plants (Havelange et al., 2000).

Gibberellins

Gibberellins are involved in the control of leaf expansion (Ross et al., 1993) and stem extension

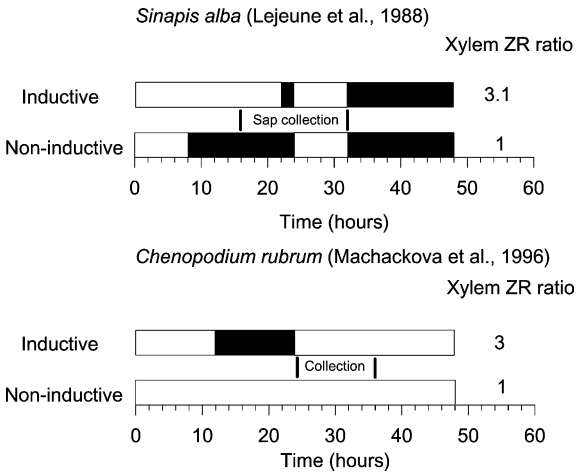

Figure 3. Photoperiodic changes causing floral induction and increased zeatin riboside (ZR) concentration of sap collected by a mild vacuum from the root system. Filled bars indicate when plants were in the dark. The timing of sap collection, and xylem ZR ratio between induced and non-induced plants during that time are indicated. All plants were grown under non-inductive conditions prior to the imposition of the indicated treatments.

(Reid et al., 1983) and much of the work establishing physiological roles for gibberellins has relied on the use of mutants. Although early bioassay work indicated that gibberellins were xylem-mobile (reviewed in Jackson, 1993) and their concentrations could be decreased by flooding (Reid et al., 1971), there are few published data where xylem gibberellin concentrations have been measured using physico-chemical techniques. The availability of gibberellin-deficient mutants in species which can be easily grafted (e.g., pea, tomato) has allowed the role of root-sourced gibberellins to be evaluated, especially their contribution to stem elongation. The ability of a WT rootstock to restore stem elongation of a gibberellin-deficient scion is dependent on the position of the lesion in the gibberellin biosynthesis pathway. WT rootstocks restored stem extension of *nana* (which is blocked in an early step of the pathway: the conversion of ent-kaurenoic acid to GA_{12} aldehyde) pea scions (Proebsting et al., 1992). In contrast, WT rootstocks did not restore stem extension of *le* (which is blocked in the conversion of inactive GA_{20} to active GA_1) pea scions (Reid et al., 1983). This suggests that root-derived GA_1 is not transported in sufficient quantity to produce a tall phenotype, and that GA_{20} or earlier metabolites in the GA synthetic pathway are transported in the xylem, and are metabolised to GA_1 in the shoot. The influence of root-derived GA_{20} on shoot growth (other than in the *nana*/WT graft combination) remains to be shown.

Nitrate

Perhaps the best example of nitrate as a signal (rather than a resource) controlling growth concerns the control of lateral root initiation (Forde and Zhang, 1998). Lateral roots can be initiated by the presence of high external nitrate concentrations even when root N status is adequate. In this case, a local signal concentration is causing a local response. It is tempting to speculate on the existence of a similar mechanism, where low xylem nitrate concentration could inhibit leaf growth independently of changes in bulk leaf N status, which would allow leaf growth to respond sensitively to any change in soil N conditions. Conversely, it has been speculated that cytokinin-inducible response regulators (Takei et al., 2002) might be involved in regulation of leaf growth of N-deprived plants (Forde, 2002). Xylem nitrate concentration is most likely to be an important root-to-shoot signal in species that reduce nitrate in the shoot (Andrews, 1986). Is there any evidence that xylem nitrate can act as a signal regulating leaf growth?

When nitrate is the N source, N deprivation decreased xylem nitrate concentration prior to any change in growth being detected (Chapin et al., 1988). This could conceivably function as a signal of reduced soil N availability. This possibility is strengthened by the observation that xylem nitrate delivery is well buffered against changes in transpiration rate (Shaner and Boyer, 1976; Else et al., 1995), thus reducing 'noise' in the signal. Certainly there is an abundant literature on the role of nitrate as a signal mediating the activity of enzymes involved in nitrogen and carbon metabolism (Stitt, 1999). The importance of xylem nitrate concentration is demonstrated by the observation that leaf nitrate reductase activity is more sensitive to xylem nitrate delivery than actual leaf nitrate concentration (Shaner and Boyer, 1976). It has been suggested that

xylem nitrate concentration can regulate leaf growth (McDonald and Davies, 1996), presumably by influencing the activity of enzymes involved in cell-wall yielding and hardening. However, reciprocal grafts of nitrate-reductase-deficient and WT pea plants, which produced a 2–3-fold difference in xylem nitrate concentration between shoot and root reducing plants, showed no difference in biomass growth across a range of solution N concentrations (Lexa and Cheeseman, 1997).

Other signals

Reviews of root-to-shoot signalling such as the present one will be selective, and attention has so far focussed on signal/process combinations that have been considered in the literature for years. However, history shows that the number of root-to-shoot signal molecules with apparent functions has increased over time, and in many systems observed changes in physiology cannot be explained in terms of existing signals. Mutational analyses in several systems have lead to the conclusions that other novel signals play a role, and several additional signal molecules such as polypeptides and mRNA are considered to be important (Jorgensen, 2002; Beveridge et al., 2003; Carrington and Ambros, 2003). Furthermore, several features of the classical plant hormones make it technically difficult to determine roles of other signals (Beveridge et al., 2003). The classical plant hormones:

- operate in complex networks involving cross-talk and feedback;
- are multifunctional and reliant on other longdistance cues or pre-set conditions to achieve specificity;
- act as 'master regulators', thus masking roles of other long-distance signals in several physiological processes.

Influence of shoot resource status on response to root signals

Much of the research on root-to-shoot signalling has sought to establish that such signals are indeed important in controlling plant response. A corollary of this is that it has been equally important to establish that shoot resource (water and nutrient) status cannot explain plant response. This 'either-or' mentality hinders the realisation that the impact of a given signal can depend on shoot resource status. An illustration of this concerns the variability in stomatal response to ABA according to leaf water and nutrient status.

In field-grown maize crops, the slope of the relationship between xylem ABA concentration and stomatal conductance varied diurnally, with the most sensitive stomatal closure occurring at lower leaf water potentials (Tardieu and Davies, 1992). [However, some species show no variation in stomatal response to xylem ABA concentration as leaf water potential decreases (Tardieu et al., 1996).] Since an increased leaf water potential increases the rate of catabolism of xylem-supplied ABA (Jia and Zhang, 1997), such a result might be explained in terms of differences in the amounts of ABA reaching the guard cells. To obviate this problem, isolated epidermes were incubated on ABA solutions of decreasing medium osmotic potential, to control apoplastic ABA concentrations around the guard cells. Stomata in epidermes incubated at lower osmotic potentials showed a greater stomatal closure at a given ABA concentration (Tardieu and Davies, 1992). This interaction may be thought of as a sensitive dynamic feedback control mechanism to ensure homeostasis of leaf water status. Any decrease in leaf water status (e.g., resulting from the sun appearing from behind a cloud) will enhance stomatal response to ABA thus decreasing transpiration and returning leaf water status to its original value.

While this variation in stomatal response is attributed to current leaf water status, there is also evidence that the water-stress history of a plant can affect current behaviour. Plants of *Commelina communis* were subjected to a slow soil-drying treatment (over 15 days), and every day isolated epidermes were incubated on ABA solutions of a constant medium osmotic potential to determine stomatal sensitivity to apoplastic ABA concentration (Peng and Weyers, 1994). In this case, sensitivity can be defined as the slope of the relationship between stomatal response and ABA concentration. Initially, water stress sensitised stomata to ABA (at the time that stomatal closure occurred in intact plants), but a

later de-sensitisation of stomata to ABA occurred when leaf relative water content began to decline and stomata had effectively closed completely. Stomatal sensitivity to ABA was thus greatest when stomatal closure effectively ensured homeostasis of leaf water status, and then declined when hydraulic influences would act to ensure continued stomatal closure.

Soil drying also decreases nutrient transport to the shoot (Gollan et al., 1992) and long-term soil drying can greatly decrease shoot N concentrations (Heckathorn et al., 1997). A series of experiments studied stomatal responses of nutrient-deprived plants to soil drying. At a given leaf water potential, nutrient deprived cotton (*Gossypium hirsutum*) plants showed greater stomatal closure than plants grown with adequate fertilisation (Radin et al., 1982; Radin, 1984). One explanation for this response was that nutrient-deprived plants showed greater stomatal closure when a given ABA concentration was supplied via the xylem to detached leaves (Figure 4).

While variation in stomatal response to ABA with differing leaf water status or nutrition has been demonstrated when either factor has been varied intentionally under closely controlled conditions, it is difficult to know how significant these responses will be to the stomatal behaviour of field-grown crops. Although some crops are managed with water and nutrients supplied 'on demand', there is increasing pressure for agriculture to improve water use efficiency and nutrient use efficiency. A recently developed irrigation technique actually aims to modify root-to-shoot signalling in the plant as a means of saving water and modifying shoot architecture. Optimising this technique to save water likely depends on greater knowledge of how the effectiveness of root-to-shoot signals varies as shoot water and nutrient status changes throughout the cropping cycle.

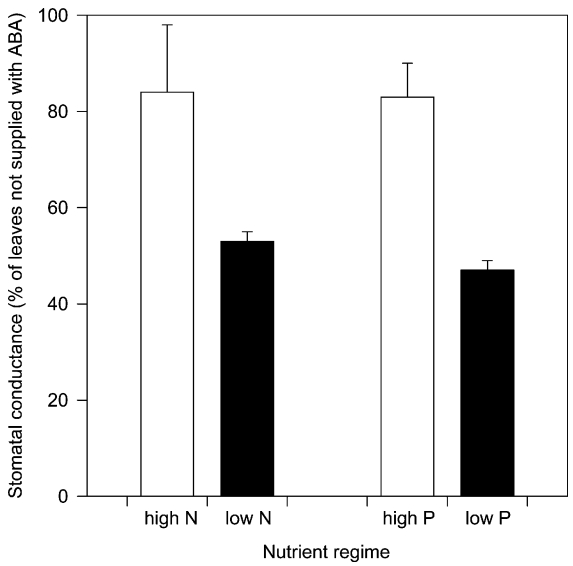

Figure 4. Relative stomatal response of cotton leaves detached from high nutrient (hollow bars) and low nutrient (filled bars) plants and supplied with 1 μM ABA via the xylem. Data are means ± SE of 3 replicate leaves and modified from Radin et al. (1982) (high and low N) and Radin (1984) (high and low P).

Exploiting root-to-shoot signals

One of the earliest demonstrations of root-to-shoot signalling of water stress, independent of shoot water status, was provided by growing maize plants with roots split between two pots of soil (Blackman and Davies, 1985). By keeping one pot well-watered and allowing soil in the other to dry, stomatal closure occurred in the absence of changes in leaf water status. Further evidence that roots were controlling shoot responses in such split-root plants was provided by an experiment showing that re-watering the roots in drying soil, or excising them, allowed growth recovery and stomatal re-opening of apple (*Malus* × *domestica*) seedlings (Gowing et al., 1990). The prospect of controlling vegetative vigour using a controlled water stress, while applying half the water of conventional irrigation, has stimulated the development of partial root drying (PRD) as a field irrigation technique. A significant outcome of PRD has been increased water use efficiency, as half the water can be applied with minimal reductions in crop yield (Dry et al., 1996; Davies et al., 2000; Kang et al., 2000; dos Santos et al., 2003).

PRD works in drip- or furrow-irrigated crops where each side of the row can be watered independently. Since only one side of the row receives water at a given irrigation, irrigation volumes are commonly halved under PRD compared to conventional irrigation where both sides of the row are irrigated (e.g., Davies et al., 2000;

dos Santos et al., 2003). It is hypothesised that roots exposed to drying soil produce chemical signals, which are transmitted to the shoots to partially close the stomata, decreasing crop water use. This stomatal response may allow the wet side of the root system to supply enough water to the shoot, thus preventing shoot water deficits (e.g., Stoll et al., 2000). Under some situations, the initial decline in stomatal conductance is not sustained (Stoll et al., 2000), presumably as roots in very dry soil contribute little to the transpiration stream. In these circumstances, the wet and dry sides of the root system are alternated to ensure that at least part of the root system is exposed to drying (but not too dry) soil, presumably to sustain the production and delivery of a root-sourced signal(s). Re-hydration of the dry part of the root system might also transiently enhance the output of root-to-shoot signals (as water flux through those roots increases) and maintain root viability.

Alternation of wet and dry sides during PRD poses two questions to an irrigation manager. Is it necessary to alternate wet and drying parts of the root system? If so, when? Irrigation trials have empirically determined the effects of fixed (the dry side is not re-watered) versus alternate partial rootzone drying (e.g., Kang et al., 2000), and the effects of different periods between alternation of wet and drying parts of the root system. However, if stomatal closure during PRD is dependent on the action of root-to-shoot signals, measurement of those signals or more likely, their effects (decreased stomatal conductance or homeostasis of leaf water potential) will be necessary to resolve the questions posed above.

Another issue is whether PRD has physiological effects that are significantly different from deficit irrigation (DI), in which a decreased amount of water is applied to the whole root system? It is conceivable that at some point(s) during the crop cycle, both treatments could expose a similar proportion of the root system to drying soil, with PRD plants showing horizontal heterogeneity of soil water within the soil profile and DI plants showing vertical heterogeneity. When the same irrigation volume was applied to both treatments, grapevines (*Vitis vinifera*) in southern Portugal grown with PRD showed a lower g_s than vines grown with DI, when measured under controlled conditions (de Souza et al., 2003). Also, PRD vines maintained a higher pre-dawn water status after veraison than those grown with DI. However, in the following year (in which total pre-season rainfall was higher) the opposite trend in pre-dawn water status was observed (dos Santos et al., 2003). In both years PRD vines showed less vegetative vigour than DI vines (dos Santos et al., 2003). In field-grown maize, physiological differences between PRD and DI treatments depended on the irrigation volume applied At higher irrigation rates there was no yield difference between PRD and DI treatments, while at lower irrigation rates, yield of PRD plants was 23% higher than DI plants (Kang et al., 2000). Further analysis of the relationship between yield and water applied (or better still, actual water use), and crop quality attributes, under different irrigation techniques in different crops will be necessary to establish the utility of the PRD technique.

Although the commercial development of PRD as a management technique started with the Australian winegrape industry (Dry et al., 2001), it is increasingly being applied to a wide range of crops in many parts of the world. Much of this work has aimed to determine the agronomic outcomes of the technique in terms of product yield and quality, and implications for crop management, rather than determining the output of root-to-shoot signals and their impact on crop water use and growth. Such an empirical approach may be necessary as much remains to be learnt about the importance of root-to-shoot signalling in different species and under different environmental conditions, such as soil type and nutrient supply. It is hoped that greater understanding of the physiological mechanisms of root-to-shoot signalling will assist in evaluating the likelihood of whether PRD will deliver favourable agronomic outcomes in a given production system.

Conclusions

Concepts of root-to-shoot communication are now entrenched in the physiological literature, and many investigators seek to explain shoot responses to changed soil conditions, or developmental events, in terms of altered root system export of signal molecules. However, root-to-shoot

signalling is only one mechanism by which the concentrations of a compound can be altered at an active site, and local processes should also be considered. Under carefully controlled conditions, this reductionist approach may allow the contribution of root-to-shoot signalling to physiological responses in a target organ (e.g., leaves) to be quantitatively evaluated, especially when coupled with grafting experiments and the use of mutant lines or transgenes under the control of root- or shoot-specific promoters. Another challenge, important from an agronomic perspective, is to understand how the root system integrates a heterogeneous soil environment into a given signal output. This issue will be important in the management of crops from which water is deliberately withheld (e.g., during PRD), and in ensuring reproducible responses in genomics or plant breeding programmes characterising root-system output in different lines. While it is likely that fundamental research will continue to discover new root-to-shoot signals and new roles for known molecules, translating this knowledge into applied outcomes is likely to stimulate future research.

Acknowledgements

I thank Christine Beveridge and Rana Munns for helpful comments on this manuscript, and DEFRA for financial support.

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