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Root nitrogen acquisition and assimilation

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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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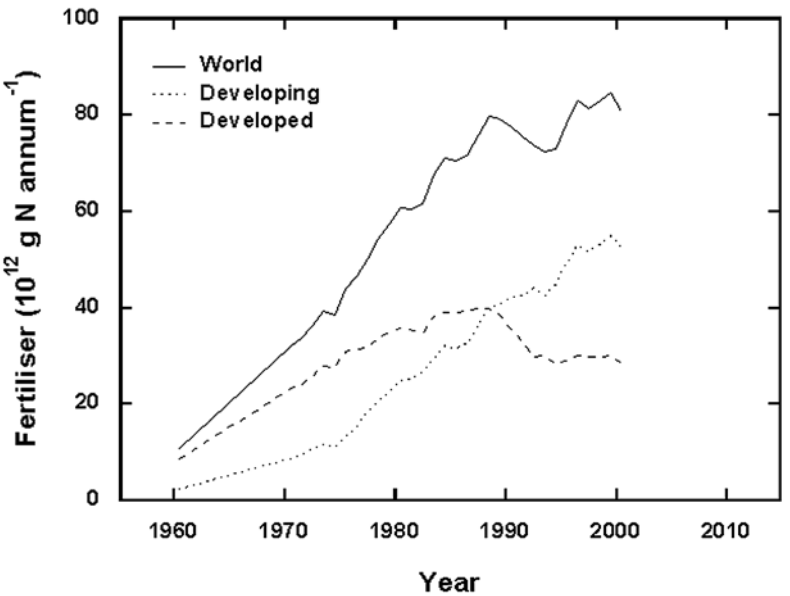

Figure 1. The consumption of N-containing fertilisers between 1961/62 and 2000/2001. The developing world was calculated from the data available for Central and South America, Africa (except South Africa), Near East (except Israel), South Asia, East Asia (except Japan), Socialist Asia and Oceania. Nitrogen-containing 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

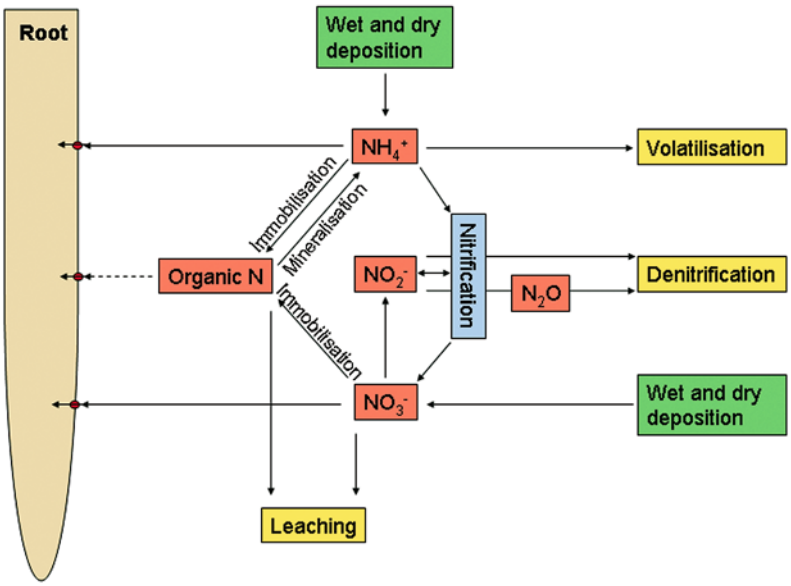

Figure 2. The main pools (boxes) and fluxes between pools (arrows) of N in terrestrial ecosystems, excluding both animals and inputs via N_2 fixation.

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.

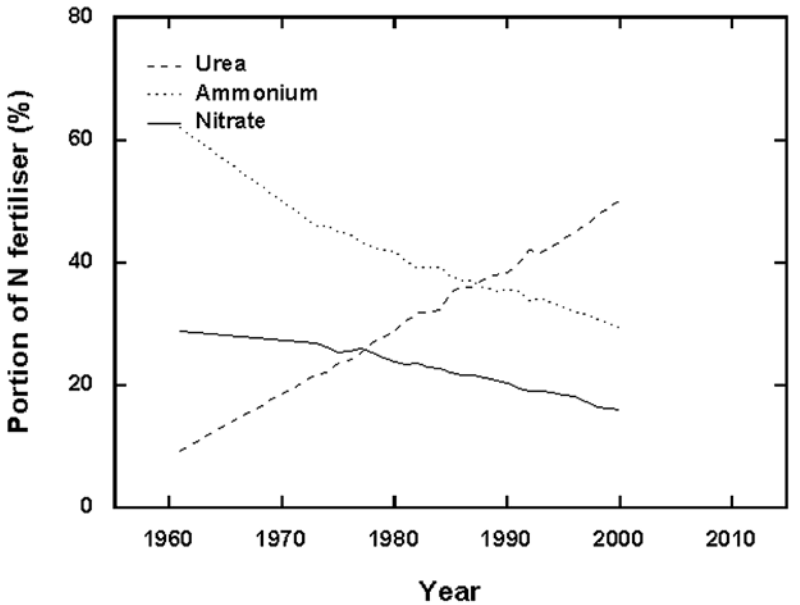

Figure 3. The proportion of total nitrogenous fertiliser applied containing urea, ammonium and nitrate or combinations of these (data plotted from that available from the International Fertiliser Industry Association, www.fertiliser.org/ifa/statistics.asp).

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

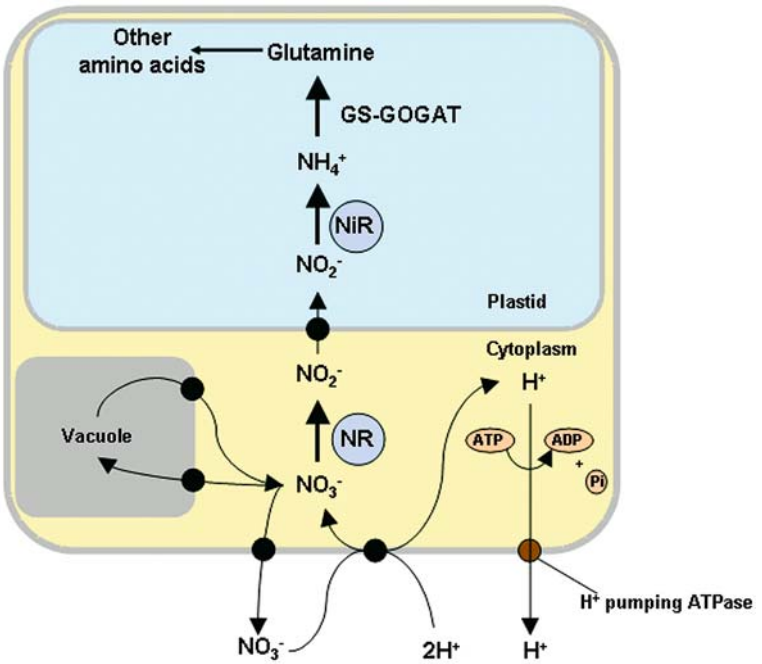

Figure 4. Schematic diagram of NO_3^- uptake and assimilation by plant cells. Key: nitrate reductase, NR; nitrite reductase, NiR; glutamine synthetase, GS; glutamate-2-oxoglutarate aminotransferase, GOGAT (redrawn from Crawford et al., 2000).

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-

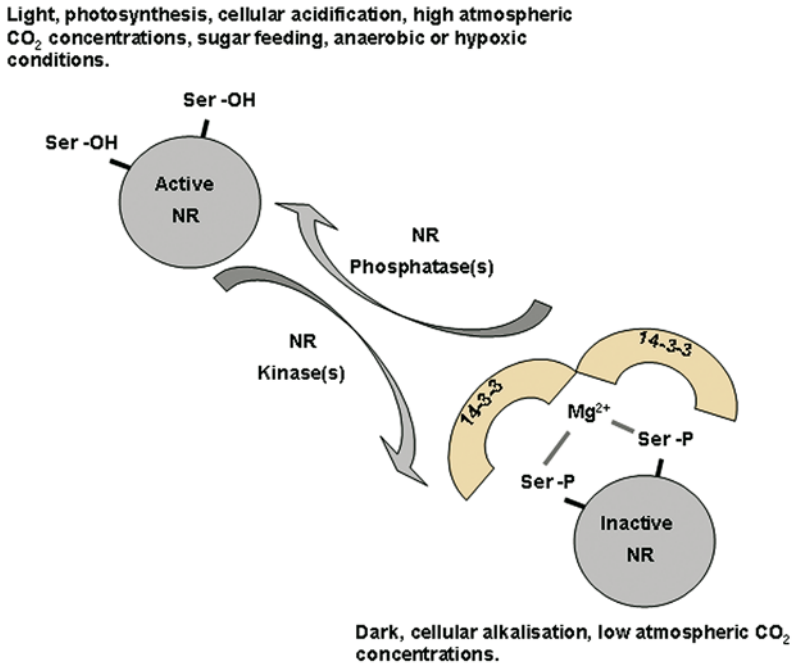

Figure 5. Schematic diagram of the environmental post-transcriptional regulation of nitrate reductase. For simplification the phosphorylation/dephosphorylation and the 14-3-3 binding steps have been shown together, but they actually occur as two separate steps. Key: nitrate reductase, NR; dephosphorylated 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

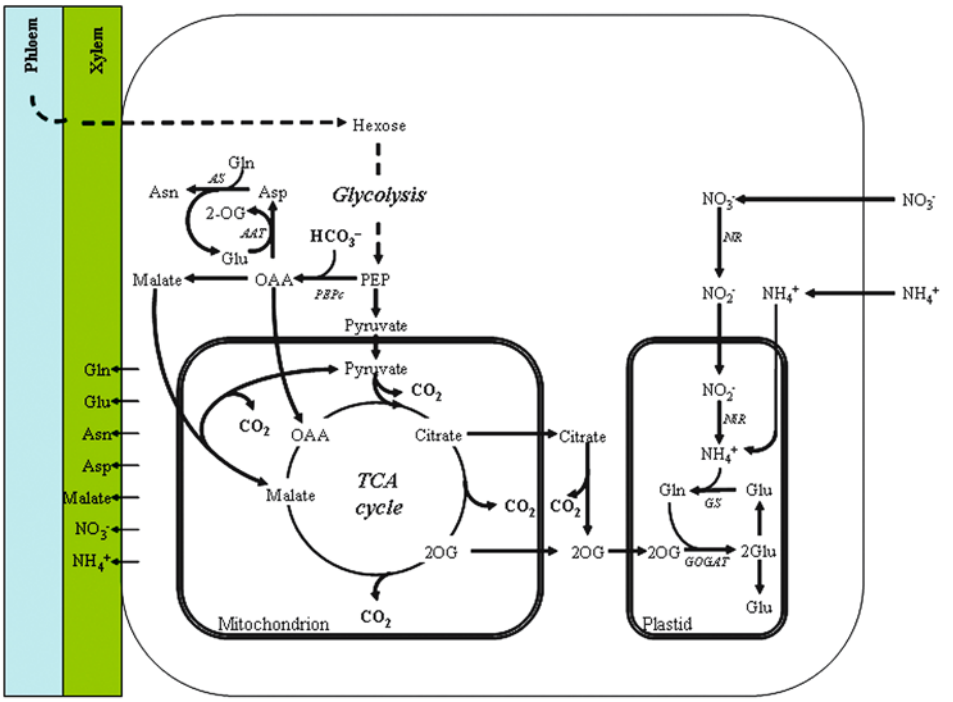

Figure 6. Simplified scheme of some C and N interactions in plant roots highlighting the role of anaplerotic C provision to the TCA cycle in plant roots. C enters the TCA cycle through pyruvate and through oxaloacetate (OAA) or malate, which may be derived from carboxylation of PEP. OAA may also be transaminated 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.

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