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Review

# Structure, function and regulation of ammonium transporters in plants

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## Abstract

Ammonium is an important source of nitrogen for plants. It is taken up by plant cells via ammonium transporters in the plasma membrane and distributed to intracellular compartments such as chloroplasts, mitochondria and vacuoles probably via different transporters in each case. Ammonium is generally not used for long-distance transport of nitrogen within the plant. Instead, most of the ammonium transported into plant cells is assimilated locally via glutamine synthetases in the cytoplasm and plastids. Ammonium is also produced by plant cells during normal metabolism, and ammonium transporters enable it to be moved from intracellular sites of production to sites of consumption. Ammonium can be generated de novo from molecular nitrogen ($N_2$) by nitrogen-fixing bacteria in some plant cells, such as rhizobia in legume root nodule cells, and at least one ammonium transporter is implicated in the transfer of ammonium from the bacteria to the plant cytoplasm. Plant physiologists have described many of these ammonium transport processes over the last few decades. However, the genes and proteins that underlie these processes have been isolated and studied only recently. In this review, we consider in detail the molecular structure, function and regulation of plant ammonium transporters. We also attempt to reconcile recent discoveries at the molecular level with our knowledge of ammonium transport at the whole plant level.

*Keywords:* Ammonium transporter; Structure; Function; Regulation; Plant

## 1. Introduction

Ammonium[1] and nitrate are believed to be the principal sources of nitrogen for plant growth in agricultural and most natural environments. They are required in greater amounts than any other mineral nutrient. When the two compounds are provided to plants at similar concentrations, ammonium is generally taken up more rapidly than nitrate [1–3]. The preference for ammonium over nitrate is explained, at least in part, by the extra energy the plant must expend in reducing nitrate to ammonium before it can be incorporated into organic compounds [4]. However, other imperatives such as the need to maintain pH homeostasis during nitrogen assimilation [5] result in the simultaneous utilisation of ammonium and nitrate under normal conditions. Thus,

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Abbreviations: PMF, proton motive force; HATS, high-affinity ammonium transport system; LATS, low-affinity ammonium transport system; GS, glutamine synthetase; GDH, glutamate dehydrogenase; PBM, peribacteroid membrane; NTR, nitrogen regulation

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[1] In this review, ammonium is used to denote both $NH_3$ and $NH_4^+$. Chemical symbols are used only when specificity is required.

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nutrient solutions that have been optimised for plant growth typically contain millimolar concentrations of both ions.

In nature, ammonium and nitrate are rarely available in equal amounts and their concentrations in the soil can vary over several orders of magnitude, from micromolar to hundreds of millimolar [6]. Cells have evolved a repertoire of transporters that enable them to efficiently import ammonium and nitrate over a wide range of concentrations. Molecular data indicate that several solutions to the problems of N acquisition were found early during evolutionary history, well before the emergence of eukaryotic cells, and passed on genetically to the higher plants of today. Thus, there are structural and functional parallels between ammonium transporters, and between nitrate transporters in plants, animals and bacteria. There may also be regulatory parallels, although plants have almost certainly evolved novel mechanisms of regulation in order to integrate transport of these compounds with their complex and unique multicellular metabolism. This review focuses on the structure, function and regulation of ammonium transporters in plants. The complementary subject of nitrate transporters in plants is taken up by Forde (this issue).

Several previous reviews have covered various aspects of ammonium transport in plants [7–11]. In general, past reviews have followed the historical development in our understanding of ammonium transport which has progressed from the agronomic and physiological levels, through the biochemical and biophysical levels, and finally to the molecular level. To provide a different perspective of past work, and perhaps encourage new directions in future work, this review begins by considering some of the fundamental chemical and physical characteristics of ammonium, as well as some relevant biophysical and biochemical aspects of plant cells. From this perspective, we review recent advances in our understanding of the molecular structure and function of ammonium transporters, both in plants and other organisms. Next, we consider what is known about the regulation of ammonium transporters in plants and what we might learn from other organisms. We then attempt to 'close the circle' and relate current data on the molecular biology of ammonium transporters to the physiology of ammonium transport in plants: in so doing it will be clear that there are major gaps in our understanding of ammonium transport at the molecular–physiological interface. Such gaps are likely to close quickly, and in the final section we consider some of the future prospects for research on ammonium transport in plants.

## 2. Physical and chemical properties of ammonium

The physical and chemical properties of ammonium determine the nature of its interactions with transporters (carriers or channels) that are 'specific' for ammonium as well as those that transport other, possibly related ions and molecules. The compound $NH_3$ is a weak base, with a p$K_a$ of 9.25. Thus, at the neutral pH typical of the plant cell cytosol, approximately 99% of ammonium is present as the cation $NH_4^+$. By definition, a decrease of one pH unit is accompanied by a tenfold increase in the ratio of $NH_4^+$: $NH_3$. Ammonium adopts sp$^3$ molecular orbital hybridisation which minimises the interactions between bonding pairs, and in the case of $NH_3$ between bonding pairs and the lone pair of electrons. This configuration puts the protons at the corners of a tetrahedron (Fig. 1). Water adopts the same molecular orbital hybridisation, although it has one more lone pair and one less bonding pair of electrons (Fig. 1). The neutral species $NH_3$ and $H_2O$ have similar sizes and molecular dipole moments (1.47D and 1.85D, respectively). Both are able to form hydrogen bonds with other molecules. The cation $NH_4^+$ has a similar ionic radius to $K^+$ but is larger than $H_3O^+$, $Li^+$ and $Na^+$, and smaller than $Rb^+$ and $Cs^+$ (Table 1). The hydration shells surrounding $NH_4^+$ and $K^+$ are also similar in size (Table 1). The sim-

Table 1

Comparison of bare and hydrated ion radii for $NH_4^+$ and some other cations (from [12])

| Ion | Bare ion (nm) | Hydrated ion (nm) |
|---|---|---|
| $H_3O^+$ | 0.115 | 0.28 |
| $Li^+$ | 0.094 | 0.382 |
| $Na^+$ | 0.117 | 0.358 |
| **$NH_4^+$** | **0.148** | **0.331** |
| $K^+$ | 0.149 | 0.331 |
| $Rb^+$ | 0.163 | 0.329 |
| $Cs^+$ | 0.186 | 0.329 |

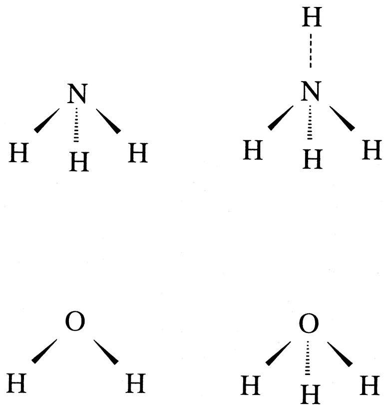

Fig. 1. Molecular structures of ammonium and water.

ilarities between $NH_4^+$ and $K^+$, and between $NH_3$ and $H_2O$, as they relate to ammonium transport, will be discussed further below (Sections 6.1 and 6.2). Finally, the neutral molecule, $NH_3$ dissolves much more readily in organic solvents than its ionic counterpart, $NH_4^+$. Consequently, the permeability of $NH_3$ across lipid bilayers is three orders of magnitude greater than that of $NH_4^+$ [13]. Whilst diffusion of $NH_3$ across the lipid portion of membranes is believed to be of biological significance, diffusion of $NH_4^+$ is not.

## 3. Biophysical characteristics of plant cells

Plants, like all multicellular organisms, exhibit specialisation or division of labour at many different levels: sub-cellular, cellular, tissue and organ. When considering ammonium transport in plants such specialisation must be taken into account. However, most of the interest in ammonium to date has been on its use as an exogenous source of N and, therefore, studies of ammonium transport have focused mainly on root cells. In this section, we consider some of the biophysical properties of a ‘typical’ plant cell which will serve as a point of reference when we consider later some of the measured aspects of ammonium transport in plants. Ammonium is present in all compartments of the cell, although its concentration varies depending on a number of factors including the concentration of ammonium in the neighbouring compartment(s), the difference in pH (ΔpH) and electrical potential (Δψ) between compartments, and the form ($NH_3$ or $NH_4^+$) in which ammonium is transported across the delimiting membrane. In compartments in which ammonium is not metabolised, such as the vacuole, the concentration of ammonium may approach its equilibrium value. In compartments in which ammonium is metabolised, such as the cytosol and plastids, the steady-state concentration of ammonium may be much lower than the predicted equilibrium value. In this review, we will consider ammonium transport into and out of several compartments of the plant cell: the cytosol, the plastid, the mitochondrion, the vacuole, and the nitrogen-fixing symbiosome of infected root nodule cells (Fig. 2).

The plant cell is bounded by the plasma membrane or plasmalemma. A proton motive force (PMF) is established across the plasma membrane, largely through the action of a P-type $H^+$-ATPase that pumps protons out of the cell into the apoplastic space [14]. The PMF consists of a chemical component ($RT/F\Delta pH$) and an electrical component (Δψ). Primary proton-translocating reactions also generate

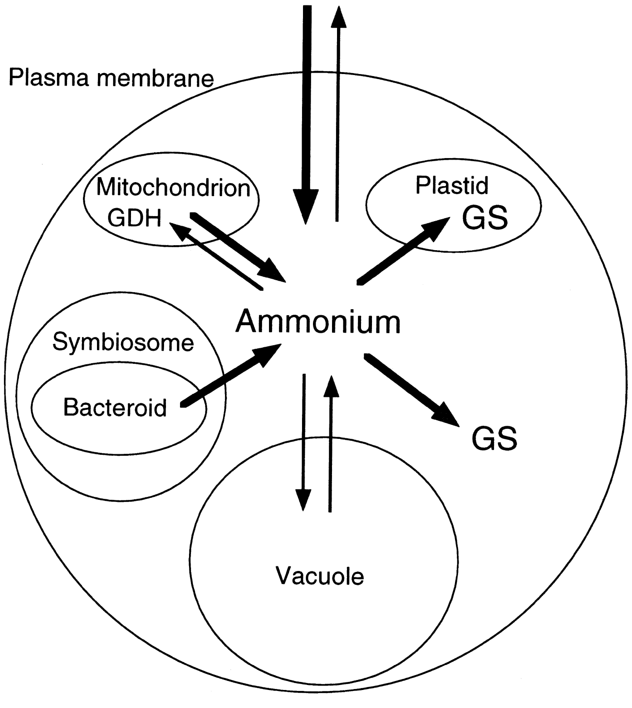

Fig. 2. Intracellular ammonium fluxes in a hypothetical plant cell. Only organelles with major roles in the ammonium economy of the cell are included. Arrows indicate directions of ammonium flux, with thicker arrows denoting predominant fluxes.

electrochemical gradients across the tonoplast (membrane surrounding the vacuole), the plastid and mitochondrial inner membranes, and the peribacteroid and bacteroid membranes of the symbiosome [6,15–17]. The range of values that have been found for $\Delta$pH and $\Delta\psi$ across some of these membranes is shown in Table 2. These values can be used to calculate the ratio of internal to external ammonium ($NH_3$+$NH_4^+$) concentrations at equilibrium for the various compartments. These ratios are dependent on the form of ammonium that is transported i.e., $NH_4^+$ or $NH_3$ (Table 3). For the hypothetical situation in which only $NH_4^+$ is transported across the membrane, the equilibrium ratio approximates the ratio of $NH_4^+$ in:out which is determined by $\Delta\psi$ alone, and can be calculated using the Nernst equation [16]. The concentration of $NH_3$ can be ignored in these calculations because physiological pH is generally less than pH 7.3 and under these conditions $NH_3$ accounts for less than 1% of the total ammonium concentration. For the alternative, hypothetical situation in which only $NH_3$ is transported across the membrane, the ammonium concentration ratio between the inside and outside of the compartment is determined by $\Delta$pH. At equilibrium, the $NH_3$ concentration is equal in both compartments but the concentration of $NH_4^+$ in each compartment is determined by the local pH. Once again, the $NH_3$ concentration is negligible compared to the $NH_4^+$ concentration, so the calculation can be simplified. Thus, for every change in one pH unit there is a corresponding tenfold change in ammonium concentration (Table 3). We will come back to these calculations in Section 8.

Table 2
Typical values of $\Delta\psi$ and $\Delta$pH for different membranes

| Organelle/membrane | $\Delta\psi$ (mV) | $\Delta$pH |
|---|---|---|
| Cell/plasma membrane | −120–−180[a] | +2[a] |
| Vacuole/tonoplast | +20–+80[a] | −2–−3[a] |
| Mitochondrion/inner membrane | −170[b] | Less than 0.5[b] |
| Plastid/inner membrane | | +0.3–+1.0[c] |

The $\Delta$pH value for the plastid inner membrane was determined for chloroplasts in the light. Values are relative to outside the particular compartment.

[a] From [6]

[b] From [15]

[c] From [16].

Table 3
Accumulation ratios for ammonium at equilibrium in different cell compartments, assuming that either $NH_4^+$ or $NH_3$ is the transported species, and that transport is passive

| Compartment | $NH_4^+$ transport | $NH_3$ transport |
|---|---|---|
| Cell cytoplasm: apoplast | 110–1100 | 0.01 |
| Vacuole: cytoplasm | 0.04–0.5 | 100–1000 |
| Mitochondrion: cytoplasm | 760 | 0.3 |
| Plastid: cytoplasm | | 0.1–0.5 |

The values of $\Delta\psi$ and $\Delta$pH for each membrane were taken from Table 2 and used to calculate ammonium accumulation ratios resulting from transport of $NH_4^+$ or $NH_3$, respectively (see text for details).

The principal fluxes of ammonium in plant cells that are considered in this review are shown in Fig. 2. Ammonium is imported from the external environment, including both the rhizosphere and atmosphere, via ammonium transporters in the plasma membrane of root cells [11] and leaf cells [18]. Ammonium entering the cell is assimilated either in the cytoplasm via glutamine synthetase, or in plastids and possibly also mitochondria, following transport into these organelles (see Section 4). Ammonium may also enter the vacuole where it is 'stored' temporarily. Cytoplasmic ammonium can leak from plant cells and this efflux can be considerable, at least under certain experimental conditions [11]. Ammonium can also be generated de novo from $N_2$ by nitrogen-fixing bacteria in some plant cells, such as rhizobia in legume root nodule cells. Most of this ammonium is transferred to the plant cytoplasm where it is assimilated by GS [19]. We will explore the molecular basis for these different fluxes in Sections 5, 6 and 8.

## 4. Biochemistry of ammonium assimilation

Ammonium assimilation in plants occurs principally, if not exclusively, via the enzyme glutamine synthetase (GS; EC 6.3.1.2). Higher plant GS is an octomeric protein. At least four different genes encode subunits of the active protein and different combinations of the subunits produce different isoenzymes of GS (reviewed in [19]). There are both cytoplasmic and plastidic forms of GS and they have high affinity for ammonium, with $K_m$ ranging from 10 to 20 μM [20]. In roots, incorporation of ammonium into glutamine appears to occur mainly via cytoplasmic GS, although the gene encoding plastidic GS is expressed, and the protein is presumably active there [19]. In leaves, the plastidic, or more specifically chloroplastic, isoenzyme is the predominant form and it appears to play an important role in recovery of ammonium derived from photorespiration [21]. Cytoplasmic GS isoenzymes are also present in shoots [19] but their role(s) there remain unclear. In nitrogen-fixing legume nodules, both cytoplasmic and plastidic forms of GS are expressed in temperate legumes but only cytoplasmic forms of GS appear to be present in tropical legumes [19]. The two intracellular locations of GS in plant cells have obvious implications for ammonium transport: we might expect to find ammonium transport activity at both the plasma membrane and the plastid inner membrane. As we shall see later, there is ample evidence for the former. Evidence for the latter, however, is lacking.

The $K_m$ of an enzyme for its substrate(s) often reflects the concentration of substrate normally available to the enzyme. If this is true for the two forms of GS, the implications for ammonium concentrations in the corresponding plant cell compartments under natural conditions are quite interesting. We will come back to this in Section 8.

It has been suggested that a second enzyme, glutamate dehydrogenase (GDH; EC 1.4.1.2) that is present in mitochondria may also play a role in ammonium assimilation in plants, particularly during photorespiration in leaf cells [22]. The enzyme GDH has a much lower affinity for ammonium ($K_m$ 10–80 mM; [20]) than GS, but concentrations of ammonium in leaf cell mitochondria may be as high as 5 mM [23] which is sufficient for assimilation of ammonium into glutamate. Indeed, incorporation of [$^{15}$N]ammonium into glutamate by isolated mitochondria has been demonstrated [22]. Although such observations are consistent with a role for GDH in assimilation of ammonium derived from photorespiration, it is generally accepted that mitochondria do not perform such a role. Instead, it is believed that most of the ammonium generated in mitochondria during photorespiration is transported to chloroplasts where it is assimilated via GS [21]. Strong support for this conclusion comes from studies with photorespiratory mutants that lack plastidic GS and show the classic symptoms of N-starvation [24].

## 5. Molecular structure and function of ammonium transporters

### 5.1. The AMT1 transporter family

The first ammonium transporters to be isolated from any organism were two related high-affinity $NH_4^+$ transporters from yeast (MEP1; [25]) and *Arabidopsis* (AMT1; [26]). Both were isolated by functional complementation of a yeast mutant defective in ammonium transport [27]. Related proteins have since been found in bacteria [28–32], yeast [33,34], animals (e.g., the *Caenorhabditis elegans* genome contains four AMT1 homologues), and several species of plants, including *Arabidopsis* [35], rice [36] and tomato [37]. These transporters clearly form a family, designated TC #2.49 [38] and all that have been characterised so far are specific for ammonium/methylammonium. A comparison of the predicted amino acid sequences of all AMT1-related plant proteins presently available in public databases is shown in Fig. 3. Current plant transporter gene/protein nomenclature includes the initials of the plant genus and species in addition to the gene/protein family and gene/protein number: thus the original *Arabidopsis thaliana* AMT1 has been renamed AtAMT1;1 and the second member isolated from this family in *Arabidopsis* was called AtAMT1;2 (accession no. AF110771). All but one of the proteins included in Fig. 3 are greater than 70% identical to AtAMT1;1 at the amino acid level. The exception to this is an *Arabidopsis* protein predicted from genomic sequence (accession no. AC003028). This protein is less than 25% identical to the other plant ammonium transporters and is, in fact, more closely related to some bacterial ammonium transporters (Fig. 4). A BLAST search [39] indicated that the nearest neighbours of this protein, which we have called AtAMT2;1, are ammonium transporters from *Aquifex aeolicus*, *Methanobacterium thermoautotrophicum*, *Bacillus subtilis* and *Escherichia coli*. As can be seen from Fig. 4, there has been considerable divergence between AtAMT2;1 and these proteins. We have shown that the gene encoding AtAMT2;1 is tran-

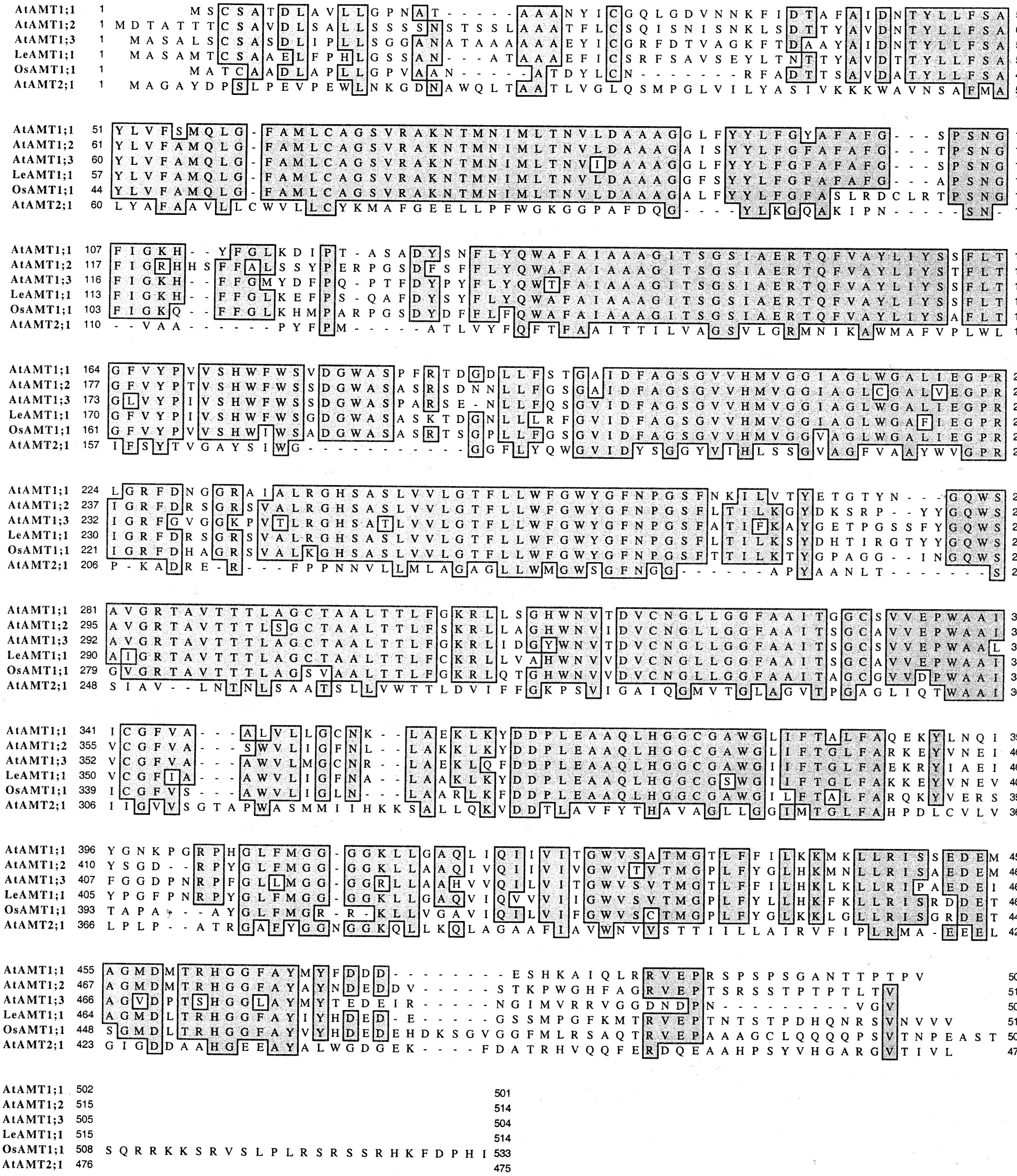

Fig. 3. Alignment of deduced amino acid sequences of plant ammonium transporters using Clustal W. Identical residues are boxed and shaded. Accession numbers are given in the legend to Fig. 4.

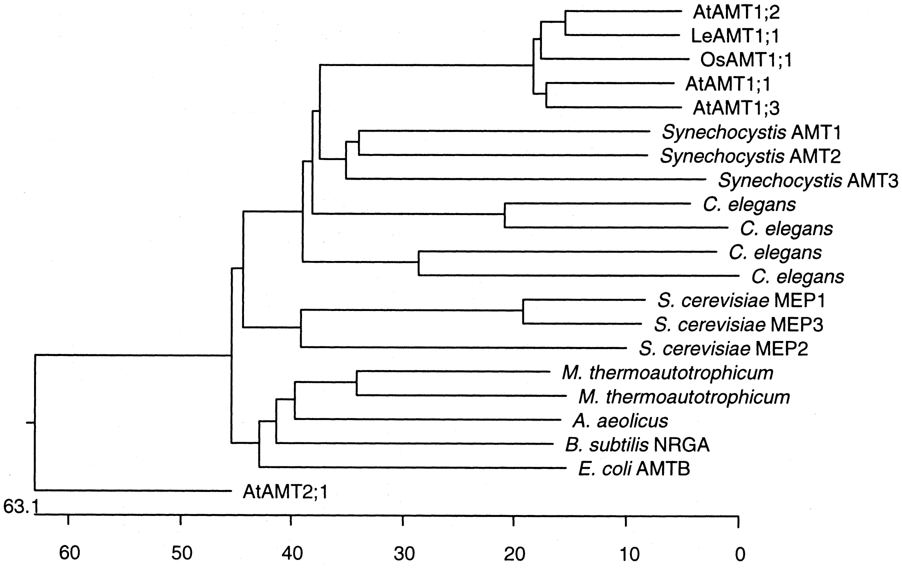

Fig. 4. A phylogenetic tree for all eukaryotic and selected prokaryotic members of the AMT protein family, generated using Clustal W. The scale indicates percent divergence. The Genbank identifier numbers for the sequences used are as follows: AtAMT1;1 (1703292); AtAMT1;2 (4324714); AtAMT1;3 (4218128); AtAMT2;1 (3335376); LeAMT1;1 (3023281); OsAMT1;1 (2160782); *Synechocystis* amt1, amt2, amt3 (1723099, 3024960, 1001374); *Caenorhabditis elegans* (1703293, 3023294, 3023293, 1255842);*Saccharomyces cerevisiae* MEP1, MEP2, MEP3 (730015, 1302091, 2131174); *E. coli* amtB (1103924); *Bacillus subtilis* nrgA (1075875); *Methanobacterium thermoautotrophicum* (3334420, 3334419); *Aquifex aeolicus* (2982857).

scribed in *Arabidopsis* [100], and given the substantial divergence between the predicted protein and the AMT1 family of proteins, we propose that it be classified in a distinct family, hence the designation AtAMT2;1. Further discussion of AMT2;1 will be left for Section 5.2.

A proposed transmembrane topology for AtAMT1;1 is shown in Fig. 5. Ten transmembrane helices are predicted, with the N- and C-termini extracytoplasmic. Such a topology is unusual for secondary transporters which usually have the N- and C-termini on the cytoplasmic side of the membrane. However, there are several examples of other secondary transporters which have been shown to have an extracellular N-terminus [40,41]. The topology shown for AtAMT1;1 is also largely consistent with the positive inside rule [42] and the charge difference rule [43]. A consideration of the AMT family indicates that the ancestral member may have had a classical 'six plus six' topology consisting of intracellular N- and C-termini with twelve transmembrane helices, grouped in two pairs of six helices separated by a large extracellular loop. Several bacterial members of the AMT family including the *E. coli* amtB and *Synechocystis* amt1 and amt2 have hydropathy plots consistent with a 'six plus six' topology. Many eukaryotic ammonium transporters, however, appear to have lost the N-terminal helix and in some cases also either helix 8 or helix 9. Interestingly, this is also true of *Synechocystis* amt3, suggesting that the loss of these helices is an ancient event. In *Synechocystis*, amt1 has a major role in ammonium uptake whereas the activities of amt2 and amt3 are very low [31]. Whether the different topologies are directly related to different functions, intracellular localisation and/or regulation remains to be determined.

In some plant ammonium transporters the N-terminal hydrophobic region appears to have evolved into a localisation signal. AtAMT1;1 has a weakly predicted signal sequence with the cleavage site between residues 20 and 21 [44] which would place the mature N-terminus outside the cell as shown in Fig. 5. OsAMT1;1 also has a predicted signal sequence. AtAMT1;2 has a predicted chloroplast transit peptide [45], making it a candidate for a plastid ammonium transporter. Other plant ammonium transporters including AtAMT2;1 and LeAMT1;1 entirely lack the N-terminal hydrophobic region/signal sequence. Multiple alignments indicate that the N-termini of these transporters must also be extracytoplasmic. These transporters may therefore be inserted into the membrane via a mechanism distinct from the Sec-dependent protein translocation pathway [46]. Clearly there is a need to determine experimentally the topology and location of members of the AMT family.

Fig. 5 also shows the position of four highly conserved, charged amino acids which are found in transmembrane helices. The energy cost involved in burying charged amino acid residues within the hydrophobic region of the lipid bilayer suggests that they are likely to have a functional role in ammonium transport. The most highly conserved region of the AMT family is helix 5 and the loop between helices 5 and 6 (the ammonium transporter signature sequence). Two charged amino acid residues, H206 and R220, are found in helix 5 and would be located on the same face of the helix. This region is therefore highly likely to be functionally important and experiments to test the role of these residues are currently under way. D83 and H372 are also conserved among most of the plant members of the AMT family. In the lactose permease from *E. coli*, extensive mutagenesis studies have identified only six amino acid residues which are essential for proton-coupled lactose transport and a molecular mechanism has been proposed [47,48]. Whether the AMT family operates in a similar manner will be interesting to determine, given its different topology as well as the fact that ammonium transport most likely occurs via a uniport mechanism in response to $\Delta\psi$ (see below).

The biochemical properties of *Arabidopsis* AtAMT1;1 have been studied in yeast [26]. Yeast cells expressing AtAMT1;1 were able to take up the ammonium analogue [$^{14}$C]methylammonium in an energy-dependent manner. The $K_m$ for methylammonium was 65 μM and uptake was competitively inhibited by ammonium with a $K_i$ of 5–10 μM. The transporter was found to be relatively specific for (methyl)ammonium: $K^+$, $Rb^+$ and $Cs^+$ had little effect on methylamine uptake. Methylammonium uptake was optimal at pH 7: rates of uptake were approximately 50% lower at pH 5 and pH 9. Uptake was retarded by inhibitors of the yeast plasma membrane $H^+$-ATPase as well as different protonophores that collapse the PMF across the yeast plasma membrane. Cells expressing AtAMT1;1 accumulated methylammonium several hundred fold. However, without precise measurements of the volume of the yeast cells or proof that methylamine was not converted to methylglutamine (or some other compound) as occurs in some bacteria [31,32], the precise magnitude of methylamine accumulation in these experiments remains unknown. This, together with the lack of data on the $\Delta\psi$ across the yeast cell plasma membrane in these experiments, makes it impossible to say whether methylammonium transport by AtAMT1;1 was active (i.e., exceeded the maximum accumulation of methylammonium predicted by the Nernst equation) or passive. Given typical values for $\Delta\psi$ across the yeast plasma membrane [49] a 100-fold accumulation of (methyl)ammonium could

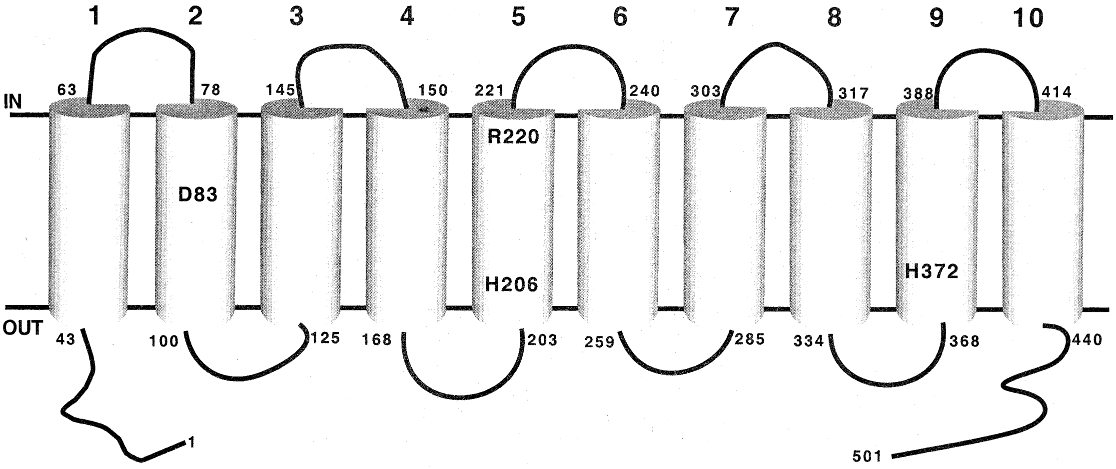

Fig. 5. A predicted transmembrane topology for AtAMT1;1. All charged amino acid residues predicted to fall within the membrane are indicated.

be achieved by passive uniport of $CH_3NH_3^+/NH_4^+$ in response to $\Delta\psi$. The $\Delta\psi$-dependence of (methyl)ammonium transport and the lack of exponential increase in transport rate above pH 7 (the concentration of the neutral form increases approximately exponentially between pH 7 and 9) indicate that $NH_4^+$, not $NH_3$, is the form of ammonium transported by AtAMT1;1. The fact that decreases in external pH below pH 7, i.e., increases in external $H^+$ concentrations, did not increase (methyl)ammonium uptake indicates that the mechanism of $NH_4^+$ transport by AtAMT1;1 probably does not involve cotransport of $H^+$. Thus, AtAMT1;1 is likely to be a uniporter for $NH_4^+$. A similar conclusion was reached in a more rigorous study of the *amt* gene product from *Corynebacterium glutamicum. C. glutamicum* was unable to metabolise methylammonium and it was found that the level of accumulation of methylammonium in response to a defined $\Delta\psi$ was consistent with uniport of $CH_3NH_3^+$ [28]. The fact that the methylammonium concentration never exceeded the equilibrium value predicted from the Nernst equation indicates that uniport of $NH_4^+$ by Amt is a passive process. A quite different mechanism has been proposed for ammonium transport by the AmtB homologue in *E. coli* [32]. Although their experiments did not directly assess the nature of the transported species, it was hypothesised that AmtB is a passive $NH_3$ transporter in *E. coli*. We believe it is more likely that AmtB, like its homologues in other organisms, is an $NH_4^+$ uniporter.

Recently, the kinetic properties of *Arabidopsis* AtAMT1;1, AtAMT1;2 and AtAMT1;3 were compared in yeast [35]. The $K_m$ for methylammonium of these transporters was estimated to be 8 μM, 24 μM and 11 μM, respectively. The $K_i$ for ammonium inhibition of methylammonium uptake was determined to be less than 0.5 μM, 25 μM and 40 μM, respectively. The values for AtAMT1;1 were significantly lower than those reported earlier [26]. Interestingly, the affinity of these transporters for ammonium ranges over two orders of magnitude. Despite these differences, it is likely that all AMT1 homologues share a similar transport mechanism, as discussed above. Multiple AMT1 family members with different but complementary affinities in any one organism may allow the organism to react appropriately to a wide range of ammonium concentrations in the environment. This appears to be the case in yeast [33]. In multicellular organisms such as higher plants, multiple forms of AMT1 probably allow greater regulatory flexibility and organelle-, cell-, tissue- or organ-specialisation, in addition to enabling cells to take up $NH_4^+$ over a wide range of concentrations [35]. Aspects of AMT1 regulation are discussed in Section 7 below.

### 5.2. The AMT2 transporter family

As mentioned above, there has been considerable divergence between AtAMT2;1 and all other ammonium transporters isolated so far. The closest relatives of AtAMT2;1 are putative ammonium transporters from thermophilic bacteria of the genera *Aquifex* and *Methanobacterium* (see Fig. 4), none of which have been functionally characterised. This raises the question of whether AtAMT2;1 is, in fact, an ammonium transporter. We have isolated a full-length AtAMT2;1 cDNA from *Arabidopsis* roots and have functionally expressed it in a yeast ammonium transport mutant defective in all three MEP genes [100], confirming that it is a bona fide ammonium transporter. A comparison between hydropathy plots for AtAMT2;1 and members of the plant AMT1 family suggests that AtAMT2;1 has a topology similar to that shown for AtAMT1;1 in Fig. 5. Three of the four charged amino acid residues within the membrane identified in Fig. 5 (H206, R220 and H372) are also present in AtAMT2;1. It is therefore likely that the tertiary structure and molecular mechanism of AtAMT2;1 are similar to other plant AMT proteins, despite the considerable difference in primary structure.

### 5.3. SAT1: a novel ammonium transporter in plants?

Although most plants rely on an external source of mineral or organic nitrogen for growth, some plants are able to grow in the complete absence of such nitrogen in the soil. Legumes, and some non-legumes, are able to establish symbioses with nitrogen ($N_2$)-fixing soil bacteria that enable them to colonise N-depleted environments. Many of these interactions, such as those between legumes and bacteria of the family *Rhizobiaceae* (rhizobia), are intracellular symbioses in which the bacteria live within the cytoplasm of infected root nodule cells, albeit surrounded by a host-derived membrane. The plant membrane that encloses the nitrogen-fixing rhizobia (bacteroids) in infected cells is called the peribacteroid membrane (PBM). The novel ‘organelle’ defined by the PBM and the enclosed bacteroids is called the symbiosome. Ammonium, the product of bacteroid nitrogen fixation, is transported to the plant cytoplasm via the bacteroid and peribacteroid membranes. In return, the plant provides the bacteroids with a varied menu of metabolites, including dicarboxylic acids that serve as a source of energy and carbon for metabolism [17]. Transport of ammonium to the plant cytoplasm is believed to involve $NH_3$ transport across the bacteroid membranes [50] and $NH_4^+$ transport across the PBM [17,51]. Although rhizobia possess an ammonium transporter homologous to ammonium transporters from other organisms, it is down-regulated genetically during symbiosis [52]. This is probably a prerequisite for a successful symbiosis, not only because it prohibits bacteroids from competing for the ammonia that is lost to the peribacteroid space (the space between the bacteroid outer membrane and the PBM) by diffusion of $NH_3$, but also because it avoids futile cycling of ammonium and associated uncoupling of the bacteroid inner membrane in the face of meagre ammonium assimilation by the bacteroid [17]. In contrast to the bacteroid inner membrane, the PBM has a channel for $NH_4^+$ that may represent the principle conduit for ammonium transport to the cytoplasm [51]. Once in the cytoplasm, ammonium is assimilated into glutamine via GS, as discussed above.

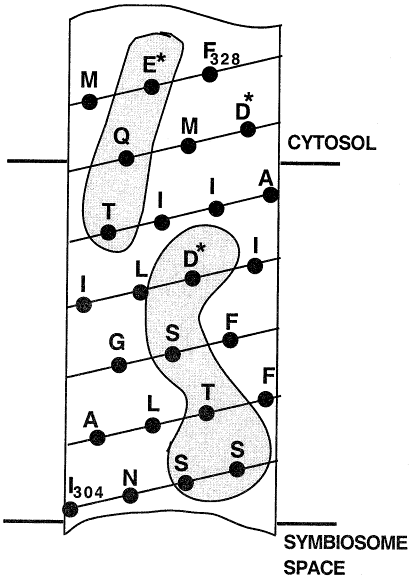

Fig. 6. Helical plot of the putative transmembrane helix of GmSAT1. Hydrophilic patches are indicated by shading and negatively charged amino acid residues are indicated by asterisks.

In an attempt to isolate a cDNA encoding the PBM ammonium channel, Kaiser et al. [53] employed the same yeast mutant that had been used successfully to isolate AtAMT1;1 [26]. The yeast mutant is defective in two (MEP1 and MEP2) of three endogenous ammonium transporters. The mutant grows very poorly on 1 mM ammonium compared to the wild type. A novel cDNA, GmSAT1, that complemented the growth defect of the double mutant, was isolated from a soybean root nodule cDNA library. The biophysical properties of GmSAT1 in yeast were similar to those of the native PBM ammonium channel. Surprisingly, the GmSAT1 protein is predicted to have only a single transmembrane domain. However, Western blot analysis showed that GmSAT1 was located on the peribacteroid membrane from nodules, and the plasma membrane from transformed yeast [53]. Immunolocalisation experiments confirmed the location of GmSAT1 on the PBM in infected soybean nodule cells (Kaiser et al., unpublished). The predicted transmembrane domain of GmSAT1 contains a number of hydrophilic amino acid residues clustered on one helical face (Fig. 6). These residues mostly contain hydroxyl groups which have been found to have an important role in lining the pore in other cation channels [54]. If several GmSAT1 proteins interacted to form a homo oligomer, the polar faces could line an aqueous pore. A second hydrophilic region extends from the membrane on the cytosolic side of the membrane. There is also a cluster of three negatively charged amino acid residues at the surface of the membrane and these may have a role in cation selectivity. There are a number of other examples of ion channels formed by proteins with a single transmembrane helix, including viral ion channels [55] and peptide toxins [56].

GmSAT1 does not share extended sequence similarity to any proteins of known function. However, a

BLAST search [39] indicated that GmSAT1 does contain a basic helix–loop–helix motif that also occurs in some transcription factors. The functional significance of this domain in GmSAT1 is unknown. The location of the protein on the PBM [53] appears inconsistent with a role in transcriptional regulation. Perhaps the helix–loop–helix domain is required for dimerisation/oligomerisation of GmSAT1 subunits and constitution of a functional transporter, whilst the positively charged basic amino acids facilitate binding to membrane phospholipids. Current work in our group and others should help to clarify the biochemical and physiological roles of this interesting protein.

## 6. Other transporters for ammonium?

### 6.1. $K^+$ Channels

In Section 2 we pointed out the similarities in the ionic radii of $NH_4^+$ and $K^+$. Likewise, the size of the hydration shell surrounding $NH_4^+$ is similar to that of $K^+$. It might, therefore, be expected that some channels and transporters distinguish only poorly between $K^+$ and $NH_4^+$. In fact, most $K^+$ channels do allow significant levels of $NH_4^+$ to permeate. The first $K^+$ transporters to be cloned from plants were two related channels from *Arabidopsis*, KAT1 and AKT1 [57,58]. Like their animal counterparts, both are also permeable to $NH_4^+$ [59,60]. More recently, unrelated high-affinity $K^+$ transporters have also been cloned from wheat and barley [61,62]. The high-affinity systems are better able to discriminate and exclude $NH_4^+$ (compare [59,61]). The corollary to this also seems to be true; namely that high-affinity $NH_4^+$ transporters, as exemplified by AtAMT1;1, do not transport $K^+$ [26]. Thus, methylammonium transport by AtAMT1;1 was inhibited strongly by $NH_4^+$ but not by $K^+$. The selectivity filter of animal $K^+$ channels has been extensively studied and the data are consistent with the movement of dehydrated ions through a narrow pore (discussed in [63]). Both $K^+$ and $NH_4^+$ are able to pass through the narrowest region of the pore but larger ions are excluded. The molecular basis for the discrimination between $K^+$ and $NH_4^+$ by high-affinity transporters is at present unknown.

### 6.2. $H_2O$ Channels

Water channels are, as the name suggests, permeable to water. Some water channels are also permeable to small solutes such as glycerol and urea (see [64]). The purpose of this section, which consists almost entirely of speculation, is twofold. First, we hope to fuel the debate about other substrates for water channels, which in our opinion is lacking amongst plant scientists. Second, we wish to explore the possible implications for $NH_3$ transport of the physical and chemical similarities between $NH_3$ and $H_2O$ noted in Section 2. Well before the discovery of water channels, much thought and discussion had gone into the question of how water gets across membranes. Some believed that it moves relatively freely through the lipid portion of the membrane; others argued that some kind of pore, probably proteinaceous is needed. Some see the discovery of water channels as vindication for the latter view. Be that as it may, the same considerations are of relevance to $NH_3$ transport.

Physiological studies indicate that transport of $NH_3$ across membranes does occur and that it can become biologically significant at high ammonium concentrations or high pH. Diffusion of $NH_3$ across the membrane of yeast or bacterial cells is sufficient to support optimal growth rates on low ammonium concentrations at neutral to alkaline pH [32]. In this study, it was shown that at pH 7, an *E. coli* mutant lacking amtB grew as well as the wild type, even at ammonium concentrations as low as 200 μM. However, at pH 5, the amtB mutant had a marked growth defect at ammonium concentrations lower than 1 mM. Similar results were obtained for yeast although the loss of the three MEP genes had a slightly more severe effect [32].

The permeability of some water channels to urea as well as water makes it highly likely that $NH_3$ would also be permeant. The apical membrane of mouse renal tubule cells has been shown to have extremely low permeability to both $NH_3$ and water [65]. One could speculate that this may be due to the absence or low activity of water channels which are permeable to both molecules. Clearly, further work needs to be done to clarify these issues. Much of the work on water channels has been done in oocytes where expression of these proteins leads to cell swelling and breakage in hypertonic solutions (see review by Kjellbom, this issue). It should be possible to determine if such cells are also able to equilibrate $NH_3$ more rapidly than untransformed cells, perhaps using [$^{14}$C]methylammonium or [$^{13}$N]ammonium to monitor uptake. Alternatively, it might be useful to monitor rates of cell swelling or changes in intracellular pH following addition of ammonium.

Water channels represent some of the most abundant proteins in plant cell membranes, including the plasma membrane and tonoplast. If they are able to transport $NH_3$, then they may represent an important route for ammonium entry (and exit) into the cell and other compartments. We think they warrant a closer look.

## 7. Regulation of ammonium transporters in plants

### 7.1. Genetic regulation

Until very recently little was known about the regulation of *AMT* genes in plants. The first study of *AMT1* found that *AtAMT1;1* was transcribed in all major organs of *Arabidopsis*, although transcript levels were highest in roots [26]. In contrast, *LeAMT1;1* in tomato exhibited root-specific expression [37]. In fact, *LeAMT1;1* appeared to be restricted largely to root hairs where it was expressed under all nitrogen conditions tested. Recently, it was shown that *AtAMT1;1* transcript levels in roots increase rapidly during nitrogen deprivation [35,66], and decrease rapidly in response to high nitrogen supply (5 mM $NH_4NO_3$ [66]). This decrease in *AtAMT1;1* mRNA was blocked by methionine sulfoximine which inhibits the conversion of ammonium to glutamine [66]. Several other lines of evidence supported the conclusion that glutamine, or an unidentified product of its metabolism, but not ammonium was responsible for the down-regulation of *AtAMT1;1* expression in *Arabidopsis* [66]. Regulation of other *AtAMT1* family members has also been studied recently [35]. Whilst *AtAMT1;1* was expressed in all major organs, *AtAMT1;2* and *AtAMT1;3* transcripts were found in roots only. Interestingly, all three *AtAMT1* family members exhibited diurnal variation in transcript levels in roots, with *AtAMT1;3* showing the most dramatic changes. Unlike *AtAMT1;1*, transcript levels of the other two family members did not increase significantly following nitrogen deprivation.

There appear to be parallels between nitrogen regulation of *AtAMT1;1* in *Arabidopsis* and ammonium transport in yeast [33] and bacteria [29,30,32]: parallels that may provide insight into the molecular mechanisms of *AMT1* regulation in plants. However, the mechanisms underlying nitrogen regulation in yeast and bacteria are not the same. In bacteria, transcription of genes involved in N-scavenging, including ammonium transporters, is regulated by the global nitrogen regulation system (NTR). The NTR system consists of at least four proteins: a uridylyltransferase/uridylyl-removing enzyme (Utase/UR), encoded by the *glnD* gene; the PII protein encoded by *glnB*; and a two-component regulatory system consisting of the protein (histidine) kinase NtrB and the response regulator NtrC [67]. During N-deprivation, NtrC is first phosphorylated by NtrB and is then capable of activating transcription of genes involved in nitrogen acquisition and utilisation, including the ammonium transporter AmtB in *E. coli* [32], *Azospirillum brasilense* [29] and *Azorhizobium caulinodans* [30].

In the yeast *Saccharomyces cerevisiae*, nitrogen control of *MEP* genes is mediated by two GATA family transcription factors, GLN3p and Nil1p, that are involved in the regulation of many other nitrogen-regulated genes [33]. These GATA factors activate *MEP* gene transcription under conditions where nitrogen supply is poor (e.g., growth on proline or low ammonium concentrations). Under the opposite conditions, when nitrogen supply is good (e.g., growth on glutamine, asparagine, or high ammonium concentrations) nitrogen catabolite repression reduces transcription of nitrogen regulated genes. GATA factors bind to the activating sequence 5′-GAT(A/T)A-3′ which is often represented several times in the upstream region of genes that respond to nitrogen control. Little is known about the other end of the nitrogen signalling pathway(s) in yeast, namely the sensor(s). However, a recent genetic study implicates one of the ammonium transporters, MEP2, in ammonium sensing and pseudohyphal differentiation in yeast [34]. Nitrogen assimilation and pseudohyphal differentiation were studied in mutants lacking one, two or all three MEP genes. All double mutants were able to grow on low concentrations of ammonium, indicating that any one of the three transporters was sufficient to supply ammonium for growth. However, all strains lacking MEP2 were defective in pseudophyphal differentiation regardless of whether or not MEP1 or MEP3 were also present. These results suggested that it is not internal ammonium pools that act as the signal but some property of MEP2 itself. It was hypothesised that MEP2 may interact with components of a transcriptional regulatory pathway, possibly via an as yet undiscovered intermediate.

There are tantalising hints in the literature and DNA databases that higher plants may have inherited parts of one or both of the nitrogen regulatory systems that operate in bacteria and yeast today. For instance, the promoter regions of at least two genes involved in nitrogen metabolism, the tomato *nia* gene that encodes nitrate reductase [68] and the *Arabidopsis AtAMT2;1* gene (accession no. AC003028), contain multiple 5′-GAT(A/T)A-3′ sequence motifs. Interestingly, the *nia* gene is subject to nitrogen regulation and its promoter region is able to bind to the GATA transcription factor/nitrogen regulator NIT2 from the fungus, *Neurospora crassa* [68]. In an attempt to isolate GATA transcription factors from plants, two *Arabidopsis* cDNAs were found to complement a yeast *gln3* mutant [69]. Although similar to each other, the two *Arabidopsis* clones are not homologues of *GLN3*. It is not yet known whether the *Arabidopsis* gene products bind to 5′-GAT(A/T)A-3′ sequences or if they are involved in nitrogen regulation *in planta*.

Recently, plant homologues of the bacterial PII protein described above were found in *Arabidopsis* and castor bean [70]. This raises the prospect that other NTR system homologues may also be present in plants. It is clear that homologues of bacterial two component regulatory systems do exist in plants, and these have received much attention over the past few years [71]. However, time appears to have obscured the evolutionary tracks of these sequences and no obvious orthologues of NtrB or NtrC are apparent. Nonetheless, with the sequence of the entire *Arabidopsis* genome on the horizon and powerful reverse genetics techniques being used in many labs, it is only a matter of time before possible NtrB/C orthologues are tested for a role in nitrogen regulation of ammonium transporter and other genes in *Arabidopsis*.

## 7.2. Biochemical and biophysical regulation

### 7.2.1. Passive regulation

As we have already mentioned, transport of $NH_4^+$ and $NH_3$ are influenced by $\Delta\psi$ and $\Delta$pH, respectively. Changes in $\Delta\psi$ and/or $\Delta$pH will, therefore, affect the rate and amount of ammonium transport. However, unless changes in $\Delta\psi$ and $\Delta$pH directly affect gating, or open-closed configurations of ammonium transporters, their effect on ammonium transport will be passive, and their role in regulation minor. This must be the case because changes in $\Delta\psi$ and $\Delta$pH will have global consequences for transport of many other ions and metabolites. Indeed, plants seem to maintain a fairly constant electrochemical gradient across membranes like the plasma membrane, at least over the long term, despite fluctuations in the supply of major nutrients like ammonium and nitrate. Such constancy is underpinned by exquisite regulation of primary active transporters such as the plasma membrane $H^+$-ATPase (see review by Morsomme and Boutry, this issue).

A potential mechanism for regulation of transporter activity by pH could involve the protonation state of histidine residues since their p$K_a$ is close to physiological pH. Within the membrane, however, the p$K_a$ may differ due to the local environment. Ion binding or transport could be affected by whether key histidine residues are positively charged or in the neutral form. Either of the two conserved histidine residues identified in Fig. 5, or a number of others which would be located in the phospholipid headgroup region of the membrane, could be involved in pH regulation. Histidine residues have been implicated in pH regulation of membrane proteins, for example the M2 channel of the influenza A virus [72] and the NhaA $Na^+/H^+$ antiporter of *E. coli* [73].

Biochemical events downstream of ammonium transport, such as assimilation of ammonium by glutamine synthase, will also affect ammonium transport, either passively via their effect on ammonium concentration in a particular intracellular space, or actively if assimilation and transport are physically coupled. Although there is no evidence for the latter in the case of ammonium transport, there is some evidence that other transporters may be physically coupled to enzymes that accept the transported species as substrate. For example, there is genetic evidence that sulfate transporters and assimilatory enzymes may be coupled in yeast (see [74]). Physical coupling of transporters to downstream enzymes is obviously an interesting area for future research, not least because of its significance for transporter regulation.

### 7.2.2. Active regulation

Ammonium transporters, like other transporters, are likely to be actively regulated by post-translational mechanisms because genetic mechanisms are too slow to provide the fine-tuning demanded by metabolism. Although there is no direct evidence for regulation of isolated ammonium transporters by chemical modifications, analysis of the sequences of the cloned proteins indicates potential sites for phosphorylation in members of the plant AMT family. A conserved protein kinase C site is found in the putatively intracellular loop between helices 5 and 6 in AtAMT1;2 and LeAMT1;1. Given that both of these transporters are expressed constitutively in roots [35,37], it is possible that they are regulated post-translationally by phosphorylation. AtAMT1;1 and OsAMT1;1 share a site for cAMP-dependent protein kinase in another putative intracellular loop between helices 7 and 8.

### 7.2.3. On the need to regulate ammonium transporters

Obviously, there is a need to regulate ammonium transport in plants and other organisms in order to match nitrogen supply to the metabolic demands of the cell. Regulation is also important for optimal energy-use efficiency in cells, simply because accumulation of ammonium in any intracellular compartment requires the expenditure of energy. Are there other compelling reasons to regulate ammonium transport? The answer is almost certainly yes! Ammonium can act as an uncoupler of some membranes. One possible mechanism of uncoupling involves the movement of $NH_4^+$ in one direction and $NH_3$ in the other direction across the membrane, which results in net transfer of $H^+$ and dissipation of the membrane PMF. Therefore, if efficient mechanisms for the transport of both $NH_4^+$ and $NH_3$ coexist in a membrane, then one or both of these must be regulated to avoid membrane uncoupling under normal conditions. The toxic effect of ammonium on plants may result when such regulatory safeguards are insufficient.

### 7.2.4. Regulation by ammonium transporters

As we pointed out above, the MEP2 protein has been implicated in the developmental switch to pseudohyphal growth in yeast, and it has been proposed as a possible sensor of ammonium in this organism [34]. Several transporters of other compounds have been similarly found to have a signalling role in yeast [75–77]. Regulation by transporters, as the first step in an assimilatory pathway, may be an efficient use of resources. However, it is not known whether plant homologues of these transporters also have a sensing role. The possibility that one of the plant ammonium transporters is an ammonium sensor is an intriguing one, and one that deserves further investigation. Complementation of the pseudohyphal growth defect in the yeast mep2 mutant with a plant homologue could open the door to such studies.

## 8. Relating molecular biology to physiology

Much of what we know about ammonium transport in plants comes from the work of plant physiologists over the last few decades. Studies of ammonium uptake kinetics in roots and leaves indicated the existence of at least two distinct mechanisms for ammonium transport into both root and leaf cells [1,78–82]. And studies of the effects of nitrogen nutrition on ammonium transport hinted at the existence of nitrogen regulation at the genetic or biochemical levels well before the first ammonium transporter genes and proteins were isolated [80,81,83–86]. Measurements of charge transfer associated with ammonium movements by electrophysiologists [78,87–91], together with estimates of intracellular ammonium concentrations by others [86,92–94] have also given us insight into the types of ammonium transporters that may be at work in plant cells. In this section, we look in some detail at the physiology of ammonium transport, and try to reconcile what we have learned about ammonium transporters at the molecular level with processes at work in living plants.

Biphasic kinetics for ammonium uptake (uptake vs. concentration) in the green alga, *Chlamydomonas reinhardtii* [95], and higher plant roots [1,78,80–82] and leaves [79] indicate that there are at least two distinct transport systems for ammonium: one high- and one low-affinity system. The high-affinity transport system (HATS) in roots is saturable, and estimates of $K_m$ for ammonium are typically below 100 μM [78,80,82]. The HATS is dependent on metabolism for its activity [10]. Uncouplers of the plasma membrane PMF inhibited the activity of the HATS substantially [10]. Changes in pH in the range 4.5–9.0 had little effect on the activity of the HATS in rice [80] which makes it unlikely that ammonium transport is coupled to $H^+$ transport in this system. Taken together, the results of uncoupler and pH experiments indicate that $NH_4^+$ is the substrate for the HATS. This has been substantiated by electrophysiological experiments [78,87–91] that show depolarisation of root membranes during ammonium uptake by the HATS.

The HATS is regulated by the nitrogen status of plants [35,66,81,83–86,96,97]. In general, conditions of nitrogen deprivation lead to increases in the activity of the HATS, while high concentrations of ammonium or products of its assimilation, such as glutamine lead to repression of HATS activity. In addition to possible regulation of the HATS at the genetic level (see below), the HATS, in rice at least, appears to be regulated at the protein level, possibly allosterically, by nitrogen availability [80]. The $V_{max}$ of the HATS in rice roots increased and its $K_m$ for ammonium decreased with decreasing availability of ammonium in the nutrient solution which coincided with decreasing concentrations of ammonium inside the cell.

Several features of the AMT1 family indicate that it is responsible for HATS activity in plants. First, the biochemical characteristics of AMT1 proteins expressed in yeast, in particular their high affinity for ammonium and their apparent $NH_4^+$ uniport mechanism, are very similar to the characteristics of the HATS in roots [26,35]. Second, *AtAMT1* transcript levels correlate with HATS activity in intact roots [35,66]. For example, changes in *AtAMT1;1* transcript levels parallel those in HATS activity following changes in nitrogen supply to plants [35,66]. Diurnal changes in *AtAMT1* mRNA levels, especially those of *AtAMT1;3*, are also accompanied by matching changes in HATS activity in roots [35]. Finally, the predicted N-terminal signal sequence of AtAMT1;1 is compatible with a location in the plasma membrane, for this protein at least. The existence and differential regulation of the AMT1 multigene family in plants implies that different family members fulfil different physiological roles. Thus, AtAMT1;1 may play the major role in $NH_4^+$ uptake into roots when nitrogen availability in the soil is low, while AtAMT1;3 may provide an important link between nitrogen assimilation and carbon metabolism in roots during the diurnal cycle [35]. In the future, immunolocalisation studies, and isolation and analysis of mutants affected in specific *AMT* genes will help to define more exactly the physiological role of this family of proteins.

The so-called low-affinity ammonium transport system (LATS) exhibits a linear increase in activity in response to increases in ammonium concentration [66,80,82]. In other words, the LATS appears to be a non-saturable system. The LATS is expressed constitutively; unlike the HATS it is insensitive to nitrogen regulation [9]. Despite the fact that it is much less sensitive to inhibition by membrane uncouplers, Wang et al. [80] have suggested that the LATS, like the HATS, transports $NH_4^+$. This is not the case with the LATS in leaves which appears to transport $NH_3$, on the basis of its response to changes in pH [79]. These results may reflect real differences in the mechanism of the LATS in roots and shoots, although we think this is unlikely. Measurements of ammonium efflux from plant roots indicate that the mechanism of efflux probably involves $NH_3$ diffusion [86,94]. If this is the case, it is probably a freely-reversible process and we speculate that efflux, like influx at high concentrations occurs via the LATS. The corollary to this would be that the LATS is indeed an $NH_3$ transport system. The isolation of plant mutants affected in the LATS would not only demonstrate the involvement of a protein in the LATS activity, but also would help to clarify the mechanism of transport.

Measurements of intracellular ammonium concentrations can be used to gain insight into the mechanisms of ammonium transport operating in different membranes (see Section 3). Such measurements are, however, difficult to make and to interpret. Two different methods, compartmental efflux analysis [86,94] and nuclear magnetic resonance spectroscopy [92,93] have been used to estimate cytoplasmic and vacuolar concentrations of ammonium. Estimates for ammonium concentration gradients across the plasma membrane (i.e., cytoplasmic ammonium: apoplastic ammonium) ranged from approximately 5 to 1800 at external concentrations of 1.5 mM and 2 μM, respectively [86,92]. Whilst accumulation ratios of several hundred could be achieved by passive influx of $NH_4^+$ in response to typical plasma membrane potentials ($\Delta\psi$: see Section 3) a value of 1800 certainly could not. Largely for this reason, Wang et al. [86] proposed the existence of an active transporter for $NH_4^+$ in root cell plasma membranes. However, it is difficult to reconcile the estimates of cytosolic ammonium concentration made by this group [86] with the high affinity of cytosolic GS for ammonium. For plants grown with 2 μM ammonium in the nutrient solution, a cytoplasmic ammonium concentration of 3.6 mM was estimated [86]. This value is approximately three orders of magnitude higher than the $K_m$ of GS for ammonium (see Section 4). Soil ammonium concentrations are probably rarely much lower than 2 μM and this is reflected in the affinity of the HATS (see above) for ammonium. If plants can really accumulate ammonium to millimolar concentrations in the cytoplasm under such conditions, one must ask why the affinity of cytoplasmic GS for ammonium remained so disparately high during evolution. We have no answer to this question and suggest, therefore, that steady state concentrations of ammonium in the cytoplasm of these plants was considerably lower than the estimated value. Such a conclusion is supported by estimates of cytoplasmic ammonium concentrations (between 3 and 10 μM for external concentrations up to 1 mM) made for maize roots using NMR spectroscopy [93].

Estimates of the ammonium concentrations in the vacuole of maize root cells indicate that they are two- to fivefold higher than in the cytoplasm [92]. Unlike the accumulation of ammonium in the cytoplasm relative to the apoplast, further accumulation of ammonium in the vacuole cannot be explained by a passive $NH_4^+$ uniport mechanism because the interior of the tonoplast is positive relative to the cytoplasm. Accumulation of $NH_4^+$ against the electrical gradient would require an active transporter. However, it is more likely that this is achieved by passive transport of $NH_3$ coupled to an acid-trap mechanism. Such a mechanism could account for a tenfold accumulation of ammonium in the vacuole if the pH of the vacuolar space were one unit lower than that of the cytoplasm. In practice, the difference is often greater than one pH unit (see Section 3). Roberts and Pang [93] recorded an increase in vacuolar pH associated with ammonium influx into maize root tip cells, using NMR spectroscopy. In a study of isolated tonoplast vesicles from barley, Garbarino and Dupont [98] showed that ammonium collapsed the tonoplast $\Delta$pH. Both results are consistent with passive influx of $NH_3$ into the vacuole.

Despite the important role of chloroplasts and the possible role of mitochondria in ammonium assimilation, little is known about the transport of ammonium into these organelles. Yamaya et al. [23] found concentrations of ammonium in mitochondria isolated from corn shoots of between 3.6 and 5.0 mM. They also showed that such concentrations of ammonium were able to support glutamate synthesis via GDH in these mitochondria (see Section 4). Ammonium concentrations are likely to be significantly lower than this in the cytoplasm [93]. Bearing this in mind, and taking into account the data in Table 1 and the discussion in Section 2, we suggest that ammonium transport into mitochondria is likely to occur via passive uniport of $NH_4^+$, perhaps even via a member of the AMT1 or AMT2 families.

Ammonium efflux from mitochondria is an important process, at least in photorespiring cells where ammonium generated in these organelles is transported to the chloroplasts for assimilation by GS [21]. Although it has not been studied to our knowledge, we speculate that the mechanism of efflux involves passive diffusion of $NH_3$. This hypothesis is compatible with the analysis presented in Table 3, and the scenario is analagous to that which occurs in nitrogen-fixing bacteroids which apparently export ammonium as $NH_3$ [17]. An interesting question then remains: Do mitochondria from photorespiring cells express $NH_4^+$ transporters? The answer may be no, for the same reason that bacteroids repress the expression of their $NH_4^+$ transporter, AmtB prior to nitrogen fixation [17,52]. If an ammonium transporter were active, a futile cycle that could uncouple the bacteroids or mitochondria would be created by the influx of $NH_4^+$, via the ammonium transporter and efflux of $NH_3$. Bacteroids avoid this by repressing *amtB* at the transcriptional level. It will be interesting to test whether the same is true for mitochondria from photorespiring cells, if indeed mitochondria possess $NH_4^+$ transporters. Clearly, the molecular tools that will enable us to answer questions like these are now becoming available.

It is surprising, given the paramount role of chloroplasts in assimilating photorespiratory ammonium [21], that virtually nothing is known about ammonium transport in these organelles. Because no photorespiratory mutants affected in ammonium transport have been isolated, one might speculate that ammonium influx in chloroplasts is not protein-mediated. However, this is probably too simplistic given that both $NH_3$ and $NH_4^+$ can be transported independently across many membranes. The *Arabidopsis* AtAMT1;2 protein has a predicted chloroplast/plastid transit peptide (see Section 5.1), although it is not expressed in leaves [35]. It will be interesting to see if any members of the AMT1 or AMT2 families are chloroplast ammonium transporters.

The physiological data on competition between $K^+$ and $NH_4^+$ for transport into roots of different plant species has been reviewed recently [11]. The picture that emerges from this data is one in which the two ions compete for transport via low-affinity systems but not high-affinity systems. In other words, it appears that plants have evolved separate, high-affinity transport systems for $NH_4^+$ and $K^+$ that are able to discriminate between these ions. These systems are induced or activated when the ion that they transport becomes limiting, and they enable the plant to extract these ions from the soil when they are present in micromolar concentrations. The low-affinity transport system(s) appear to be constitutively expressed and discriminate less well between $K^+$ and $NH_4^+$ which, therefore, compete with each other. The physiological properties of these high- and low-affinity transport systems are not unlike the properties of the recently cloned high- and low-affinity $K^+$ transporters, and the high-affinity $NH_4^+$ transporter that were discussed above. Indeed, it seems that we are not too far from having all of the molecular pieces that will enable us to explain the competition between $K^+$ and $NH_4^+$ for transport across plant membranes. Putting the pieces together and solving the puzzle will, no doubt, be aided greatly by reverse genetics approaches such as that used recently to show a role for AKT1 in $K^+$ nutrition in *Arabidopsis* [99].

## 9. Future prospects

It is an exciting time to be working on ammonium transport in plants. The completion of the *Arabidopsis* genome-sequencing project is in sight, and this will enable us to identify all of the possible molecular players involved in transporting ammonium into and around the plant. In parallel, the genome of *Arabidopsis* is being saturated with DNA-insertion mutations that, together with PCR- or Southern-based methods, will enable us identify a mutation in any gene we care to study. Combining reverse genetics with careful physiological, cell-biological, biochemical and biophysical measurements will enable us to determine the physiological role of many plant proteins, including ammonium transporters. Also, we now have some of the molecular tools, in the form of genes encoding ammonium transporter genes, that will enable us to modify ammonium transport processes in plants of agricultural interest. Such work may lead to plants that are better able to compete for and utilise ammonium that may be naturally present in the soil or which is added in the form of fertiliser. This, in turn, may help to reduce our reliance on fertiliser nitrogen which will benefit not only farmers, but also the environment.

Continued work on ammonium transport at the molecular level offers other rewards. Apart from obtaining a more satisfying picture of the detailed mechanisms of ammonium transporters, we are bound to obtain a better understanding of how proteins interact to form functional units, and how these units are integrated and regulated to allow plants to respond to changes in the supply of, and demand for nitrogen. Of course, ammonium transport and nitrogen metabolism are only a part of a much larger picture that encompasses all of plant growth and development. Fitting ammonium transport into a holistic molecular picture of the living plant, using functional genomics and bioinformatics amongst other approaches, remains perhaps the most exciting challenge of all.

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