Regulation and function of ammonium carriers in bacteria, fungi, and plants

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Abstract

The ammonium transport (Amt) family of proteins comprises a unique and ubiquitous group of integral membrane proteins found in all domains of life. They are present in bacteria, archaea, fungi, plants, and animals, including humans where they are represented by the Rhesus proteins. The Amt proteins have a variety of functions. In bacteria and fungi, they act to scavenge ammonium and to recapture ammonium lost from cells by diffusion across the cell membrane. In fungi, they have also been proposed to act as ammonium sensors to control filamentous growth. In plants, they make a major contribution to nitrogen nutrition and in higher animals; they are involved in ammonium fluxes in kidney and liver. In this paper, we review current knowledge of the biology of Amt proteins in bacteria, fungi, and plants with particular attention to the different functions of the proteins and their modes of regulation.

1 Introduction

Ammonium[1] plays a key role in the nitrogen metabolism of most cells. For many organisms, notably bacteria and eukaryotic microbes, ammonium is the preferred nitrogen source and although organisms can often acquire a variety of nitrogen sources most of these are transformed into ammonium before they are utilised in biosynthetic pathways. The importance of ammonium as a nitrogen source also means that a number of microbes show chemo-attraction towards ammonium, which raises the concept of ammonium sensors in both bacteria and fungi. Ammonium is important in plant metabolism not only as a major nitrogen source for plant growth and development, but also as a major form for nitrogen retrieval in leaves. Since ammonium is generated by photorespiration in the mitochondria and subsequently assimilated in the chloroplast, significant ammonium fluxes are likely to occur across a number of plant cell membranes. Finally, in animals, transmembrane fluxes of ammonium have been studied primarily in the mammalian kidney where excretion of ammonium constitutes the major component of acid

[1] The term ammonium is used to denote both NH_3 and NH_4^+ and chemical symbols are used when specificity is required.

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excretion to maintain acid/base balance. However, a flux of ammonium from neurons to glial cells is also found in most nervous tissues implicating ammonium transport in brain function.

The beneficial effect of ammonium as the key nitrogen form for acquisition, intracellular transport and retrieval of nitrogen is reversed at high ammonium concentrations when ammonium becomes harmful. Sensitivity to ammonium is a universal phenomenon and occurs in animals, plants, and microorganisms, although toxicity levels depend strongly on the type of organism and can vary largely between closely related species (Bai et al. 2001; Britto et al. 2001a). Several explanations have been provided for the toxic effect of ammonium, such as the dissipation of proton gradients across membranes, the acidification of the external medium in response to ammonium uptake, or a disequilibrium in the acid/base balance (Gerendas et al. 1997). However these explanations can at best only partially explain the observed ammonium toxicity syndromes and in plants membrane transport of ammonium, in particular efflux from the most sensitive cellular compartments, appears to be crucial for alleviating ammonium toxicity (Britto et al. 2001a).

Despite the fact that these varied biological systems all involve flux of ammonium across cell membranes the view was held for many decades that these fluxes can be satisfied by the free diffusion of NH_3 and that there is no requirement for specific ammonium transport systems. Quantitative determinations of the permeability coefficient for NH_3 have been carried out for a variety of membranes from bacterial, plant, and animal cells as well as for artificial bilayers. The accumulated data suggest that NH_3 is indeed capable of permeating through membranes at a rate similar to that for water. However as ammonium has a pK_a of 9.25, both NH_3 and NH_4^+ will normally be present in biological systems and at a physiological pH of around 7 (or below) 99% will be present as NH_4^+. Consequently, both the diffusion of NH_3 and the specific transport of NH_4^+ can potentially occur in biological systems.

In plants, attempts have been made to predict the likelihood and direction of NH_4^+ and NH_3 transport across membranes by measuring ammonium concentrations in different compartments and by calculating concentration gradients according to compartmental pH and membrane potential (Britto et al. 2001b; Howitt and Udvardi 2000). Assuming ammonium concentrations in the cytosol in the millimolar range, thermodynamic considerations argue in favour of cytosolic ammonium import in form of NH_4^+, which requires secondary active transport, as long as external concentrations are below millimolar. By contrast, loading of the plant vacuole with NH_3 might be thermodynamically more favourable though it could occur with NH_4^+ when vacuolar acidification is inhibited (Britto et al. 2001b; Plant et al. 1999). Thus, cells may have adapted to varying ammonium concentrations by evolving transporters for both substrates, NH_4^+ and NH_3 thereby allowing cellular import and compartmentalisation of ammonium whilst minimising energy demand.

The first proposal for the existence of active ammonium transport was obtained in 1970 in the fungus *Penicillium chrysogenum* and since then evidence for ammonium carriers has been reported in a wide range of organisms, mostly bacteria.

In neutral environments most bacteria maintain a higher intracellular than extracellular pH, which would promote NH_3 efflux down an NH_3 gradient. Hence, in many organisms, the measurement of ammonium gradients with significantly higher concentrations inside the cell than outside strongly argues that transport of either NH_3 or NH_4^+ should take place. Studies in a number of organisms have suggested the presence of more than one ammonium uptake system. In prokaryotes, kinetic studies in both proteobacteria e.g. *Rhodobacter sphaeroides* (Cordts and Gibson 1987) and cyanobacteria e.g. *Anacystis nidulans* (Cordts and Gibson 1987) could be interpreted in this way. However, these data could also reflect kinetic differences between ammonium uptake and assimilation. In plants, ammonium depletion studies indicated that concentration-dependent ammonium uptake into *Lemna* cells followed bi- or multiphasic kinetics (Ullrich et al. 1984), and uptake studies using ^{13}N-labelled ammonium showed that substrate affinity and maximum transport capacity of rice roots varied significantly depending on the nitrogen status (Wang et al. 1993).

Initial genetic approaches to the identification of potential ammonium transporters in bacterial systems were unsuccessful. Early reports of an ammonium transport gene (*amtA*) in *E. coli* later proved to be incorrect and the gene was correctly identified as *cysQ*, which plays a role in sulphite synthesis (Fabiny et al. 1991; Neuwald et al. 1992). Indeed it was not until 1994 that the cloning of two genes, one from *Saccharomyces cerevisiae* and one from *Arabidopsis thaliana*, led to the recognition of the first two members of the ammonium transporter (Amt) family of proteins. In this review, we will discuss the very considerable progress that has been made since 1994 in characterising this family of proteins from a wide variety of organisms and in beginning to understand the ways in which Amt proteins function.

2 The ammonium transport protein family

2.1 Distribution and phylogeny

The cloning of the first two genes that encode ammonium transport proteins was facilitated by the availability of a novel mutant of *S. cerevisiae* that grew very slowly on minimal medium containing only 1mM ammonium as sole nitrogen source (Marini et al. 1994; Ninnemann et al. 1994). Genomic and cDNA libraries from both *S. cerevisiae* and *A. thaliana*, respectively, were used to complement the growth defect of the mutant leading to the isolation of *S. cerevisiae MEP1*[2] and *A. thaliana AMT1;1*. A number of potential homologous proteins were identified from the, then relatively sparse, gene databases and these included proteins from *Bacillus subtilis, Rhodobacter capsulatus, Corynebacterium glutamicum,* and *Caenorhabditis elegans* suggesting that comparable transporters were present in

[2] The designation MEP derives from methylammonium permease because the ammonium analogue $^{14}[C]$ – methylammonium is used as a substrate for transport assays.

bacteria and in nematode worms. With the exponential growth of the databases, Amt homologues have now been identified in all the domains of life. Representatives are found in eubacteria, archaea, fungi, nematode worms, insects, fish, and primates.

Amongst published genomes Amt proteins are notably absent in certain bacterial pathogens many of which rely on their hosts for the provision of nitrogen-containing compounds and consequently do not need systems for ammonium uptake (Thomas et al. 2000a). Nevertheless, these organisms are the exceptions and Amt proteins are encoded in nearly all genomes such that in excess of 200 members of the family can now be identified. Furthermore, in very many cases multiple copies of *amt* genes are present. Two or more *amt* genes are found in many bacteria and archaea, three copies are present in *S. cerevisiae* and ten putative *amt* genes are found in rice (Marini et al. 1997a; Suenaga et al. 2003; Thomas et al. 2000a).

A major extension of the Amt family occurred with the recognition that the human Rhesus (Rh) proteins, that are found both in erythrocytes and in the kidney and liver, show significant homology at the primary amino acid sequence level to Amt proteins (Marini et al. 1997b). Furthermore the human RhAG and RhGK genes can rescue the growth of a *mep* deficient strain of yeast on medium with low ammonium (Marini et al. 2000a). Phylogenetic analysis shows that the Rh proteins constitute a discreet subfamily of the Amt proteins (Liu et al. 2000; Ludewig et al. 2001). Furthermore, Rh proteins are not restricted to primates but are also found in fish, insects, slime moulds, and marine sponges. It is interesting to note that *C. elegans* and *Drosophila melanogaster* encode both Amt and Rh homologues in their genomes (Ludewig et al. 2001).

2.2 Membrane topology

The ammonium transport proteins were expected to be integral membrane proteins and computer analyses of the first cloned sequences revealed them to encode highly hydrophobic proteins with a molecular mass of 50-55 kDa. Hydrophathy plots have been reported for a number of homologues and have identified between 9 and12 putative transmembrane (TM) regions (Javelle et al. 2003a; Marini et al. 1994; Monahan et al. 2002a; Montanini et al. 2002; Ninnemann et al. 1994; Siewe et al. 1996; Thomas et al. 2000b; Vermeiren et al. 2002). The variation in the predicted number of TM helices arises in part because of differences between the various algorithms used but may also reflect some real differences between Amt proteins (see below). The majority of analyses predict that the C-terminal region of Amt proteins constitutes a discreet cytoplasmic region of at least 30 amino acids.

A more definitive model of Amt topology can be derived from a combination of empirical analysis and multiple alignment of sequences derived from databases. A detailed empirical analysis of the *Escherichia coli* Amt protein (AmtB) used fusions between AmtB and the reporter proteins alkaline phosphatase (which is active in the periplasm) and β-galactosidase (which is active in the cytoplasm) to

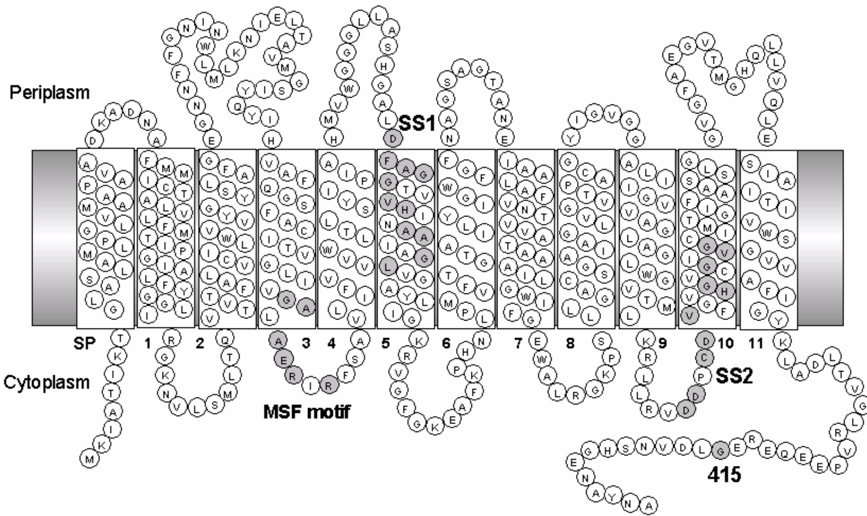

Fig. 1. Topology model for Amt proteins. Model based on empirical analysis of the topology of the E. coli AmtB protein. The signal peptide (SP) is apparently only present in Gram negative bacteria and is cleaved off in the mature protein suggesting that all Amt proteins could have a common membrane topology with an extracytoplasmic N-terminus and a cytoplasmic C-terminus. The Amt signature sequences (SS1 and SS2) are highlighted as are the region showing homology to the MSF motif and the highly conserved glycine residue (G415 in E. coli AmtB) in the C-terminal tail.

map the topology. This analysis concluded that the protein contained 12 TM helices with both the N and C-termini located in the cytoplasm (Thomas et al. 2000b) (see Fig. 1). Multiple sequence alignment was then used to map this model onto other Amt proteins leading to the conclusion that many Amt proteins e.g. *S. cerevisiae* MEP2 and *A. thaliana AMT1;1*, would have only 11 TM helices with the N-terminus being extracytoplasmic. The N-terminus of *S. cerevisiae* MEP2 was confirmed as being extracytoplasmic by mapping of an N-glycosylation site to residue N4 (Marini and Andre 2000). One analysis arrived at a different model with ten TM helices and both the N and C-termini extracellular (Howitt and Udvardi 2000). These authors also proposed that the proteins exhibit internal symmetry and could have evolved from an ancestral “six plus six” topology and that certain eukaryotic Amt proteins had “lost” one or more helices. There is currently no empirical data to support this model.

The apparent difference in topology between certain Amt proteins (11 or 12 TM helices) was resolved by purification of the *E. coli* AmtB protein. Amino-terminal sequencing of this protein revealed that the first 22 residues of AmtB are cleaved off in the mature protein such that the N-terminus would then be predicted to be extracellular (Blakey and Merrick, unpublished). It would therefore appear that the putative first TM helix of *E. coli* AmtB is actually a signal sequence and indeed it has all the characteristics of such a signal (van Dommelen et al. 2001). Furthermore, multiple sequence alignments suggest that this may be a common

feature of all Amt proteins from Gram negative bacteria. Hence, the currently available data are consistent with a common membrane topology for Amt proteins with 11 TM helices, an extracytoplasmic N-terminus and a cytoplasmic C-terminus (Fig. 1). This C-terminal region is minimally 30 residues but is predicted to comprise more than 150 residues in some cases.

It is worth noting that one aspect in which the Rh proteins are distinguished from other Amt proteins is that the topology of Rh proteins has been empirically determined to fit a twelve TM helix model with both the N and C termini being cytoplasmic (Avent et al. 1996). Indeed multiple sequence alignments suggest that the homology between Amt and Rh is not conserved at their N-termini and that TM helices 3 to 11 of the Amt proteins are homologous to TM helices 4 to12 of the Rh proteins (M. Merrick, unpublished).

2.3 Structure-function relationships

The models derived by bioinformatic and empirical analyses provide a starting point from which to develop hypotheses and experimental approaches to an understanding of the structure/function relationships within the Amt family. Bioinformatic analysis shows that the Amt proteins constitute a novel family of transport proteins with two distinctive signature sequences located in TM helix 5 and TM helix 10 respectively (see Fig. 1) (Saier Jr et al. 1999).

SS#1 D (F Y W S) A G (G S C) X2 (L I V) (E H)X2 (G A S) (G A) X2 (G A S) (L F)
SS#2 D D X (L I V M F C) (E D G A) (L I V AC) X3 H (G A L I V) X2 (G S) X (L I V A W) G

In a study of a large number of inactive mutants of one of the two *Aspergillus nidulans* Amt proteins (MeaA), two independent mutations affected glycine167, which is located in the cytoplasmic loop between TM helices 3 and 4. This led to the recognition of a motif (GAVAERxK/R) in this region of Amt proteins that shows similarity to part of the G-X-X-X-D/E-R/K-X-G-R/K-R/K motif found in the major facilitator superfamily (MSF) of secondary transporters, where it is located in the cytoplasmic loop between TM helices 2 and 3 (Monahan et al. 2002b). This motif has been studied in detail in the *E. coli* LacY permease and shown to be important for facilitating conformational changes necessary for substrate translocation across the membrane. Apart from this particular motif, the distinctive nature of the proteins means that predictions concerning their structures and mode of action cannot obviously be derived from other transporters. Hence, insights are most likely to be derived from *in vivo* analysis of wild type and mutant proteins and from *in vitro* studies of purified proteins.

Relatively few mutant forms of Amt proteins have yet been analysed in detail. The most detailed analysis relates to a highly conserved glycine residue in the C-terminal tail of the protein. Following the recognition that *S. cerevisiae* encodes three Amt proteins the genotype of the mutant strain initially used to isolate *S. cerevisiae MEP1* and *A. thaliana AMT1-1* was determined. The strain contains a wild type copy of *MEP3*, a complete deletion of *MEP2* (*mep2Δ*) and a single point

mutation in *MEP1* (*mep1-1*) that converts glycine 412 to aspartate. This mutation is trans-dominant such that its presence inhibits the activity of Mep3 causing a defect in ammonium transport (Marini et al. 2000b). The equivalent mutation was introduced into the *A. nidulans meaA* gene and the MeaA G447D protein was also transdominant to either a wild type copy of MeaA or to the second *A. nidulans* Amt protein, MepA (Monahan et al. 2002b). These data strongly suggest interactions between Amt monomers in both *S. cerevisiae* and *A. nidulans* and indicate that such interactions may involve the C-terminal tail. Although hetero-oligomers may form naturally in species such as *S. cerevisiae* they are clearly not a prerequisite for ammonium transport as strains expressing just a single gene are transport proficient (Marini et al. 1997a, 2000b; Monahan et al. 2002a).

The equivalent amino acid exchange, G458D, has also been analysed in LeAMT1;1 from tomato. It resulted in loss of function when expressed in either oocytes or yeast, even though the G458D-GFP fusion protein still localised to the plasma membrane when expressed in yeast or plant cells (Ludewig et al. 2003). Coexpression of G458D with wild type LeAMT1;1 in oocytes provoked a dominant negative inhibition of ammonium transport suggesting homo-oligomerisation. A physical interaction between LeAMT1;1 proteins was further supported using a split-ubiquitin yeast two-hybrid system. Surprisingly, coexpression of G458D with wild type LeAMT1;2 also inhibited ammonium transport in a dominant negative manner whilst a coexpressed amino acid permease was functionally not affected in the presence of G458D (Ludewig et al. 2003). These data provide evidence for the formation of heteromeric complexes between both LeAMTs in oocytes but whether AMTs also interact in plants remains to be shown.

Recent studies suggest that oligomerisation is a general feature of secondary transport proteins although the relationship between oligomeric structure and function has only been established in a few cases (Veenhoff et al. 2002). The *E. coli* AmtB protein is the only Amt protein to have been purified so far and this protein purifies as a stable trimer that does not dissociate in SDS but is denatured by boiling (Blakey et al. 2002). Immunoblots of all three of the *S. cerevisiae* Mep proteins also show signals compatible with the existence of homomultimers. It may therefore be the case that the native state of all Amt proteins is oligomeric and indeed the Rh proteins have been proposed to associate in tetrameric complexes in the erythrocyte membrane (Eyers et al. 1994).

The C-terminal cytoplasmic region of Amt is only highly conserved within the first 30 residues and any extensions beyond this appear to be species-specific. This region has been deleted from the Amt proteins of both *E. coli* and *Aquifex aeolicus* and in both cases the proteins retain a methylammonium transport activity that is reduced to around 25% of the wild type value (Coutts et al. 2002; Soupene et al. 2002b). Hence, the C-terminus is not absolutely required for transport activity.

Of the three *S. cerevisiae* Mep proteins, only one, Mep2, is glycosylated but mutation of the glycosylation site did not inactivate the protein and only increased the K_m for methyl-ammonium by a factor of 2 (Marini and Andre 2000). A number of other point mutations that affect Amt activity have been reported in *A. nidulans* MeaA and in *Lotus japonicus* AMT1;1 (Monahan et al. 2002b; Salvemini et

al. 2001) though in each case there was no evidence that the mutant proteins were actually expressed and/or stable in the membrane.

2.4 Mode of action

As ammonium exists as a mixture of the two species, NH_3 and NH_4^+, the ratio of which is dependent on pH there has been an intensive controversy on the molecular species transported by AMT/MEP/Rh proteins. Initially, uptake studies with ^{14}C-labelled methylammonium in *C. glutamicum* and *AtAMT1*-transformed yeast showed that inhibitors of the plasma membrane ATPase and protonophores strongly inhibited methylammonium transport, arguing in favour of NH_4^+ being transported (Meier-Wagner et al. 2001; Ninnemann et al. 1994; Siewe et al. 1996). Furthermore the K_m of AmtB for methylammonium transport in *C. glutamicum* (Meier-Wagner et al. 2001; Siewe et al. 1996) and in *E. coli* (Thomas and Merrick, unpublished) is essentially independent of pH, which again favours $CH_3NH_3^+$ transport or CH_3NH_2/H^+ cotransport. The conclusions drawn from these observations have been questioned on the basis that rapid assimilation of methylammonium to methylglutamine by glutamine synthetase, or compartmentation to the vacuole in the case of fungi, might create an internal sink, which subsequently would accelerate inward-directed diffusion of CH_3NH_2 (Soupene et al. 1998, 2001). However, the K_m of AmtB for methylammonium transport in *C. glutamicum* and in *E. coli*, is around 100-fold lower than that of glutamine synthetase for methylammonium assimilation (Meier-Wagner et al. 2001; Thomas and Merrick, unpublished) suggesting that in bacteria glutamine synthetase is not the driving force for (methyl)ammonium uptake by AmtB.

At concentrations of less than 1mM ammonium, growth of AmtB- or Mep-defective *E. coli* and yeast, respectively, is impaired at low but not at neutral pH. This has been taken as evidence that AmtB or MEPs are only required if the pH-dependent concentration of uncharged NH_3 drops below a critical value (Soupene et al. 1998, 2001). This rationale implies that AmtB- or Mep-mediated transport will increase with pH as the concentration of NH_3 increases, whilst that of NH_4^+ remains largely unchanged. However, electrophysiological studies on the tomato transporter LeAMT1;1 do not support this view. The properties of LeAMT1;1 were examined by expressing the protein in *Xenopus laevis* oocytes and using two-electrode voltage clamp to study the transport mechanism. Micromolar concentrations of external ammonium induced concentration- and voltage-dependent currents that remained constant over a pH range of 5.5 to 8.5 (Ludewig et al. 2002). This is in agreement with NH_4^+ being the transported species because if NH_3 was the transported species the K_m should increase by tenfold for each unit increase in pH.

A phenomenon, which has not received appropriate attention by Soupene et al. (Soupene et al. 1998, 2001, 2002a), is that ammonium transport at neutral and acid pH is accompanied by membrane depolarisation. In intact plants, membrane depolarisation was immediately induced after ammonium supply and then reversed slowly at continuing ammonium supply (Ayling 1993). This reversal is likely to

be due to an enhanced activity of the H^+-ATPase or membrane fluxes of other ions to compensate for the influx of positive charge and to reinstate the negative membrane potential. In oocytes expressing LeAMT1;1, membrane depolarisation was immediately induced after addition of ammonium to the bath solution and quickly reversed after ammonium withdrawal (Ludewig et al. 2002), which is again clearly indicative of the charged species being transported.

The situation may differ for Rhesus proteins in mammalian cells, where membrane potentials are smaller and controlled by Na^+ and K^+ gradients. Based on uptake studies with ^{14}C-labelled methylammonium in oocytes expressing the Rhesus protein RhAG, transport increased with external pH but decreased with increasing internal pH of the oocyte (Westhoff et al. 2002). It was therefore suggested that transport of methylammonium might be coupled with an antiport of H^+. However, as the transporter was not characterised by electrophysiology, and as the use of methylammonium as a substrate analogue only allows monitoring of substrate accumulation in minutes rather than seconds, further experiments are definitely required to substantiate this exceptional transport mechanism.

Functional expression of RhAG and RhAK in the yeast triple *mep* background permitted not only import of ammonium but also export (Marini et al. 2000a). Export could only be observed when yeast cells were fed with arginine, in which case internal degradation of this amino acid enhances internal ammonium pools. This experiment was reproduced in *Salmonella typhimurium*, where AmtB enhanced ammonium leakage upon arginine supply; the ammonium being detected by growth of a co-cultivated strain defective in arginine catabolism (Soupene et al. 2002a). Although neither approach could define the transported ammonium species, these feeding studies indicated that Amt and Rh proteins can act bidirectionally. If NH_3 was transported, the transport direction would simply follow the concentration gradient. If NH_4^+ was transported, the transport direction would be determined by the electrochemical gradient (Ludewig et al. 2002), which in plant cells is certainly oriented outwards when cytosolic ammonium concentrations are in the millimolar range and external concentrations are approximately 100-fold lower (Britto et al. 2001b).

In conclusion, as experienced from the characterisation of transporters and channels for other substrates, complementation and tracer uptake studies in model organisms alone cannot provide conclusive information on a transport mechanism (Ward 1997). This is of particular importance if the real substrate must be substituted by a traceable analogue and if different substrate species occur at the same time, as in case of NH_3 and NH_4^+. In this case, only electrophysiological studies allow clear differentiation of the charged and non-charged species, which at least for LeAMT1;1 clearly indicated NH_4^+ uniport as the transport mechanism. Future electrophysiological studies of Amt and Mep proteins should include the use of mutant derivatives as controls to provide better assessments of the degree to which alternative routes for ammonium transport may contribute to overall transport (see 6.6).

3 Bacterial ammonium transporters

The extensive sequencing of eubacterial and archaebacterial genomes (over 100 completed and more than 300 in progress) allows the distribution of Amt proteins to be assessed. From this it is apparent that *amt* genes are present in nearly all prokaryotic genomes and there are quite often multiple paralogues e.g. three in *Archaeoglobus fulgidus* and two in *C. glutamicum*. The exceptions that have no *amt* genes include a number of intracellular and extracellular pathogens such as *Rickettsia prowazekii, Chlamydia trachomatis, Borrelia burgdorferi, Helicobacter pylori*, and *Haemophilus influenzae.* These organisms either rely on their hosts for the provision of nitrogenous compounds or they utilise very specific nitrogen compounds e.g. urea in the case of *Helicobacter* and glutamate in the case of *Haemophilus* (Thomas et al. 2000a).

3.1 Regulation of Amt activity in bacteria

The prokaryotic *amt* genes are remarkable in one particular respect, namely that within the eubacteria (other than the cyanobacteria) and the archaea they are almost invariably found associated with a second gene (*glnK*) that encodes a small signal transduction protein belonging to the P_{II} protein family (Thomas et al. 2000a).

P_{II} proteins act as transducers of the cellular nitrogen status in prokaryotes and are also present in plants where they are located in the chloroplast (Arcondéguy et al. 2001). The P_{II} proteins have been studied in most detail in the proteobacteria, especially in *E. coli* which expresses two P_{II} proteins, GlnK and GlnB. GlnK is highly homologous to GlnB and both proteins adopt very similar tertiary and quaternary structures (Xu et al. 1998). They are trimers and take the form of a squat barrel approximately 50 Å in diameter and 30 Å high, above the surface of which three loops (the T-loops) protrude. In response to nitrogen deprivation, the P_{II} proteins of proteobacteria are covalently modified by uridylylation of a tyrosine residue (Y51) at the apex of the T-loop and this process is reversed in nitrogen sufficiency. A similar situation occurs in cyanobacteria excepting that in this case the proteins are modified by phosphorylation of a serine residue (S49), again at the apex of the T-loop. Hence, the modification state of the P_{II} protein is an indicator of the intracellular nitrogen status. P_{II} proteins typically regulate the activities of other proteins by protein-protein interaction as exemplified by the role of GlnB in modulating the activity of the transcriptional activator NtrC by interaction with the histidine protein kinase NtrB.

Conservation of gene linkage such as that found with *amtB* and *glnK* has often been found to reflect situations where the gene products physically interact (Dandekar et al. 1998) and studies in both *Escherichia coli* and *Azotobacter vinelandii* suggest that this is the case for AmtB. GlnK binds to the cell membrane in an AmtB-dependent manner and this interaction is dependent on the nitrogen status of the cell such that binding is maximal in nitrogen-sufficient conditions (Coutts et al. 2002). This response suggests that the sequestration of GlnK is

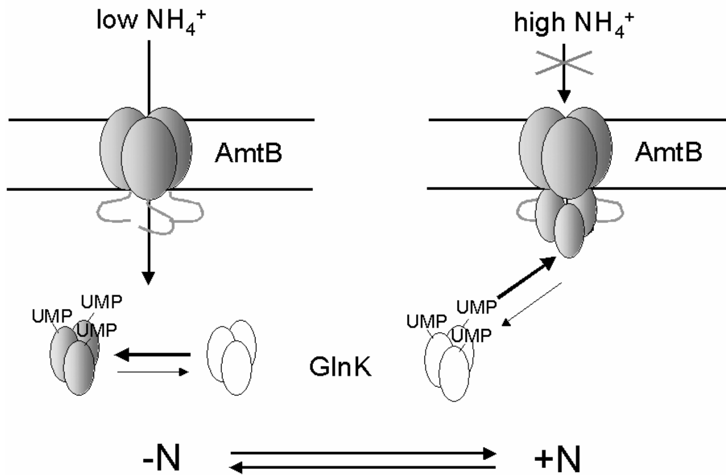

Fig. 2. Model for regulation of AmtB activity by GlnK. In conditions of nitrogen limitation (-N) GlnK is predominantly in its fully uridylylated state and is not strongly membrane-associated (non-shaded GlnK indicates the minority species in each situation). AmtB is active and will effectively scavenge ammonium from the surrounding medium. In the event of a marked rise in the availability of extracellular ammonium (+N), GlnK is rapidly deuridylylated and the unmodified form of the protein associates tightly with AmtB in the inner membrane. This association inhibits the activity of AmtB and ammonium is no longer transported into the cell until the cellular N-status drops when the inhibition is rapidly reversed.

regulated by its uridylylation state and indeed a non-uridylylatable mutant of GlnK (GlnKY51F) is bound to the membrane even in nitrogen-limiting conditions (Javelle and Merrick, unpublished). Detailed studies have shown that GlnK sequestration is rapid (being detectable after only 30 seconds exposure to elevated ammonium in the medium) and reversible. It can also be induced by as little as 50μM ammonium conditions (Javelle and Merrick, unpublished). The role of GlnK appears to be to provide rapid and sensitive regulation of AmtB activity (Fig. 2) and this is supported by experiments that demonstrate that GlnKY51F completely inhibits the transport activity of AmtB. In the event that the cellular nitrogen status remains high then transcription of the *glnKamtB* operon ceases because in *E. coli*, and probably in many other organisms, expression of the genes is controlled by the global nitrogen regulation system.

It seems likely that this mechanism will be found to be conserved throughout prokaryotes and indeed the membrane-association of GlnK has also been reported in *Azoarcus* (Martin and Reinhold-Hurek 2002) and in *Klebsiella pneumoniae* (Klopprogge et al. 2002). Of particular interest in this context are the archaea, where although *amtB* and *glnK* are linked there is presently no evidence for covalent modification of GlnK. In a broader context, the control of AmtB activity by GlnK raises the question as to whether similar post-translational control of Amt

proteins might occur in eukaryotes and if so which proteins might effect that regulation.

The conservation of the *glnK amtB* operon also suggests that P_{II} proteins might have originally evolved together with the Amt proteins and that the primary role of the P_{II} protein was to regulate the activity of the ammonium transporter. Based on this model, the P_{II} paralogues that are now found in the bacteria and the archaea, namely the GlnB proteins of the proteobacteria and the NifI proteins in the nitrogen fixation gene clusters of some archaea (Arcondéguy et al. 2001), are likely to be the result of subsequent gene duplication and specialisation. Of particular interest in this context is the fact that both the GlnK and AmtB proteins are trimers. To date, the reason for the trimeric nature of P_{II} proteins has not been apparent, particularly as none of their known targets has trigonal symmetry. However, if GlnK evolved in concert with AmtB its trimeric structure may reflect a symmetry required for optimal interaction between the two proteins.

3.2 The role of Amt in ammonium sensing

In prokaryotes, there is relatively little evidence for ammonium sensing involving Amt proteins. However, the sequestration of GlnK does imply that elevation of external ammonium could lead to a rapid depletion of the cytoplasmic GlnK pool and consequently for any cytoplasmic system that is regulated by GlnK the AmtB protein would essentially comprise a part of an ammonium sensing mechanism. Two possible examples of this have been described.

In *Klebsiella* pneumoniae, GlnK is required to relieve the inhibitory effects of NifL on the activator protein NifA and, somewhat surprisingly, uridylylation of GlnK is not required to regulate this process (He et al. 1998; Jack et al. 1999). This leads to the question as to how NifL-mediated inhibition is restored when ammonium is added back to a nitrogen-limited medium and it was originally suggested that GlnK might be proteolysed or covalently modified by a mechanism other than uridylylation upon replenishment of ammonium (He et al. 1998). An alternative explanation is that the sequestration of deuridylylated GlnK by AmtB would rapidly lower the cytoplasmic GlnK pool and thereby release NifL to inhibit NifA activity.

In *Rhodobacter capsulatus* (and a number of other diazotrophs), the nitrogenase enzyme is rapidly covalently modified and inactivated by ADP-ribosylation in response to an extracellular ammonium shock. The process is reversible and the signal transduction pathway involved has yet to be completely elucidated but it appears to involve AmtB. *R. capsulatus* encodes two *amt* genes, *amtB* (which is linked to *glnK*) and *amtY* (which is monocistronic). An *amtB* mutant (but not an *amtY* mutant) is totally defective in ADP-ribosylation in response to ammonium addition (Yakunin and Hallenbeck 2002). *R. capsulatus* encodes two P_{II} proteins, GlnB and GlnK, and both of them appear to be involved in regulating ADP-ribosylation. A *glnB, glnK* double mutant is completely defective in nitrogenase inactivation in reponse to ammonium (Hallenbeck et al. 2002). Hence, it is cer-

tainly possible that the effects of AmtB could be explained in terms of GlnK sequestration.

4 Fungal ammonium transporters

Ammonium transporters appear to be ubiquitous in fungi and are found in all fungal genomes sequenced to date. The biological roles of the fungal Amt proteins have yet to be fully determined but in the case of the ectomycorrhizal fungi studies have suggested that they can make a significant contribution to the nitrogen nutrition of their plant hosts (Smith and Read 1997). Amt genes have been cloned and characterised from *S. cerevisiae, A. nidulans, Hebeloma cylindrosporum*, and *Tuber borchii* (Javelle et al. 2001; Marini et al. 1994, 1997a; Monahan et al. 2002a; Montanini et al. 2002). Fungi frequently encode multiple Amt proteins, there being three in *S. cerevisiae* and *H. cylindrosporum* and four in *Neurospora crassa.* Studies in *S. cerevisiae* and *H. cylindrosporum* suggest that they can be divided into two groups according to their affinity for ammonium. The high-affinity transporters ScMep1, ScMep2, HcAmt1, and HcAmt2 have a K_m for NH_4^+ in the range of <1 to 10μM whilst ScMep3 and HcAmt3 have values of >10μM to 2mM (Javelle et al. 2001, 2003b, Marini et al. 1997a). These groupings appear to be reflected in a phylogenetic analysis of fungal Amt proteins suggesting that transporters in the same affinity class may have common features (Javelle et al. 2003a).

4.1 Regulation of Amt/Mep protein activity in fungi

Regulation of protein activity may occur because of interactions with other proteins or may be caused by post-translational modification. In *S. cerevisiae*, one of the three Mep proteins has been shown to be post-translationally modified but the functional significance of this remains to be elucidated. By a combination of Western analysis, protein deglycosylation and site-directed mutagenesis Mep2 was shown to be N-glycosylated at residue N4 whereas Mep1 and Mep3 were not modified (Marini and Andre 2000). Time-dependent ammonium depletion from the external medium by a glycosylation-defective variant (Mep2 N4Q) was not significantly affected, nor was the Mep2-dependent signalling that triggers pseudohyphal growth (see Section 4.2).

As described earlier, studies of a trans-dominant mutation of *mep1* that results in a single amino acid substitution (G413D) located in the cytoplasmic C-terminus of Mep1 indicated that Mep1 can interact with Mep3 (Marini et al. 1997a, 2000b). This interaction is not necessary for transport activity of either protein as they are active individually when expressed alone. However the sum of the individual activities is higher than the transport activity of wild type cells suggesting cross-regulation between the Mep proteins (Marini et al. 2000b).

4.2 Amt proteins as ammonium sensors in fungi

In *S. cerevisiae*, nitrogen nutrition regulates pseudohyphal differentiation, a filamentous growth form in diploid cells induced upon nitrogen starvation or low ammonium supply. Pseudohyphal cells are more elongated than vegetative cells, employ a bipolar budding pattern and thus can efficiently invade a growth substrate. This morphological response is controlled by two interconnected signalling pathways, one of which involves a pheromone-responsive MAP kinase cascade, while the second involves Mep2 and the Gα-protein GPA2 (Lorenz and Heitman 1997). Since pseudohyphal differentiation and activation of GPA2 under nitrogen limitation were absent in a *mep2Δ* strain and mutational activation of GPA2 could rescue pseudohyphal growth in this strain, Mep2 was proposed to function upstream of GPA2 (Lorenz and Heitman 1998). Thus, Mep2 apparently fulfils a sensing function by linking low ammonium availability with an adaptive growth response. The ability of ectomycorrhizal Amt proteins to carry out this sensing function was investigated by complementing a *S. cerevisiae mep2Δ* strain and complementation was found to be restricted to the high-affinity transporters HcAmt1, HcAmt2, and TbAmt1 (Javelle et al. 2003a). It was proposed that the sensing function of fungal Amt proteins is a distinct property of the high-affinity transporters.

By the construction of chimeric proteins, it was concluded that the pseudohyphal regulatory function of *S. cerevisiae* Mep2 could be mapped to the N-terminal region of the protein (Lorenz and Heitman 1998). The authors used a topology model with only 10 TM helices and an incorrectly located N-terminus but on the current 11 TM topology model (Marini and Andre 2000) the region defined equates to the first three TM helices. Attempts were made to refine the region further, but although the authors reported that the introduction into Mep1 of a region from Mep2 encompassing TM helices 2 and 3 and the intervening extracytosolic loop was sufficient to confer the pseudohyphal regulatory function. The control reciprocal swap did not have the expected phenotype, consequently the precise features of Mep2 that determine its regulatory function remain to be defined (Lorenz and Heitman 1998).

Ammonia is also involved in long-distance signalling between neighbouring yeast colonies. Neighbouring colonies exhibit growth inhibition of the facing parts of both colonies, a phenomenon that is presumed to orient growth so as to minimise competition for nutrients. Colonies produce pulses of ammonia that are apparently perceived by neighbouring colonies which produce reciprocal pulses in response (Palková et al. 1997). However in this case, the Mep permeases are not involved in sensing of the signal and extracellular amino acids are believed to serve as the source for volatile ammonia production (Zikánová et al. 2002). Three membrane proteins, designated Ato1, Ato2, and Ato3, that are members of the YaaH family have been reported to be involved in ammonia production in *S. cerevisiae* and have been suggested to be ammonium/H^+ antiporters that extrude NH_4^+ from yeast cells and import protons (Palková et al. 2002).

5 Plant ammonium transporters

Ammonium transporters are widely distributed in plants being found in both mono- and di-cotyledonous plants and being expressed in most tissues. Most detailed studies have been carried out in two model systems, namely *Arabidopsis* and tomato but symbiotic legumes (*Lotus japonicus* and *Glycine max*) and rice are also being analysed. Plants encode multiple Amt proteins with six being encoded in the *Arabidopsis* genome and ten in the rice genome. With this level of complexity, there is clearly scope for specific physiological roles to be carried out by distinct homologues e.g. being located in specific cell types or tissues.

5.1 The function of Amt proteins in plants

A T-DNA insertion in the 5'-region of *AtAMT1;1* led to a complete loss of *AtAMT1;1* mRNA as determined by Northern analysis. Short-term uptake studies using ^{13}N-labelled ammonium showed that at micromolar concentrations of external ammonium, influx into roots of *amt1;1-1 (AtAMT1;1:T-DNA)* was decreased by 30% compared to the wild type, when plants were precultured under nitrogen deficiency (Kaiser et al. 2002). By contrast, at molar concentrations of ammonium influx by *atamt1;1-1* was even higher suggesting that low-affinity uptake was enhanced as a result of a compensatory overexpression of other AMTs in the absence of *AtAMT1;1* expression. In addition, the insertion line was lethal under exclusive ammonium nutrition and showed an altered leaf morphology (Kaiser et al. 2002). Although these observations might point to a role of *AtAMT1;1* in ammonium-dependent growth, they certainly require substantiation by investigations on the recomplemented insertion line or on further allelic insertion lines before firm conclusions can be drawn.

Similar to the *atamt1;1-1* insertion line, RNAi inhibition of *AtAMT2*, which also led to a loss of *AtAMT2* gene expression, did not provoke an altered phenotype of soil-grown plants (Sohlenkamp et al. 2002). An *AtAMT2* promoter-reporter gene fusion showed that *AtAMT2* expression was confined to the vascular system in leaves, to root tips and flowers, which at least partially explains the low expression levels detected by Northern analysis (Sohlenkamp et al. 2000). Determination of the substrate affinity in yeast and transient expression of GFP-fused *AtAMT2* indicated that *AtAMT2* encodes a high-affinity transporter that resides in the plasma membrane. Its physiological function, however, remains unclear.

Isolation of a closely related AMT2 homologue from *Lotus japonicus* showed expression throughout the whole plant, including all major tissues of root nodules. Transient expression of LjAMT2-GFP fusion proteins in plant cells also indicated plasma membrane localisation. Thus, a role of LjAMT2 in ammonium retrieval from nodules and other plant cells has been suggested (Simon-Rosin et al. 2003).

Taken together, studies on *Arabidopsis* lines with repressed expression of *AMT* genes indicate so far that there is considerable redundancy among the AMTs caused by at least partially overlapping physiological functions of the individual transporters. Thus, the generation of multiple *AMT* insertion lines or multiallelic

RNAi lines might be important strategies in future to uncover the importance of individual AMTs for ammonium transport in plants.

5.2 Biochemical transport properties of AMTs

Heterologous expression of AtAMT1;1 in yeast and short-term uptake studies of ^{14}C-labelled methylammonium as a substrate analogue showed high sensitivity against proton uncouplers (Ninnemann et al. 1994). This can be explained by the strong dependency of AMT1;1-mediated ammonium transport on the membrane potential gradient (Ludewig et al. 2002). Changes in external pH between 5.5 and 8.5 had little or no effect on the K_m for ammonium further supporting the concept that NH_4^+ and not NH_3 is the substrate for AMT1;1-like transporters (Ludewig et al. 2002; Sohlenkamp et al. 2002). AtAMT1;1 exhibited high selectivity towards ammonium, while potassium, despite its similar ion radius, could not compete for uptake by AMT1;1 paralogues (Ludewig et al. 2002; Ninnemann et al. 1994).

In yeast AtAMT1;1, AtAMT1;2, and AtAMT1;3 transported methylammonium at half-maximal velocity at external concentrations between 8 and 24 µM (Gazzarrini et al. 1999). The determination of inhibition constants further suggested that AtAMT1;1 has the highest affinity for ammonium (K_i < 0.5 µM) compared to AtAMT1;2 and 1;3 (K_i = 25 and 40 µM, respectively). This high affinity of AtAMT1;1 was not confirmed when substrate affinity was determined directly using ^{13}N-labelled ammonium for uptake studies in yeast (Sohlenkamp et al. 2002). On the other hand, a discrimination between ammonium and methylammonium transport was also observed for LeAMT1;1 when expressed in oocytes, where ammonium-induced currents were approximately fivefold higher than those of methylammonium (Ludewig et al. 2002). Such a prominent substrate discrimination at the level of AMT1;1-like transporters might explain the lower substrate affinity for methylammonium generally measured in nitrogen-deficient plant roots (Kosola and Bloom 1994; Shelden et al. 2001).

AtAMT2, the most distantly related AMT member, was initially described as having a unique ability to discriminate sharply between ammonium and methylammonium because it allowed ammonium uptake in yeast but did not confer resistance to methylammonium (Sohlenkamp et al. 2000). Uptake of ^{13}N-labelled ammonium by AtAMT2 in yeast revealed a similar K_m (approx. 20 µM) to that of AtAMT1;1 (Sohlenkamp et al. 2002) suggesting that both the AMT1 and AMT2 transporters, at least in *Arabidopsis*, encode high-affinity transporters with similar K_m values. These substrate affinities in the lower micromolar range closely match the relatively low ammonium concentrations in the soil solution, which rarely exceed 50 µM (Marschner 1995).

5.3 Transcriptional regulation of AMT transporters in plants

Given the large number of AMT proteins encoded by plants and the potential for complex tissue-specific expression it is not surprising that transcriptional

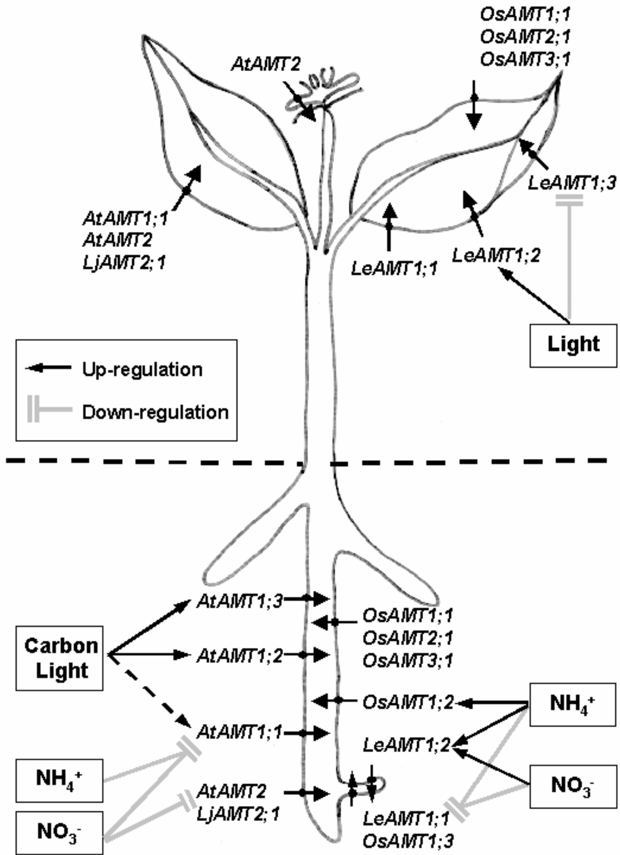

Fig. 3. Model summarising the transcriptional regulation of AMT genes in plants. Arrows indicate promoting effects on transcript levels, while capped lines represent repressive effects. The model is based on data cited in the text. Transcriptional regulation of OsAMT genes from rice is based on Suenaga et al. (2003) and Sonoda et al. (2003). Another report on OsAMT gene expression based on RT-PCR analysis found that *OsAMT1;1*, *1;2*, and *1;3* were commonly upregulated after transfer from high to low ammonium concentrations in the medium or vice versa (Kumar et al. 2003).

regulation of *AMT* genes in plants is highly complex. The complexity of the regulation revealed in studies to date is summarised in Figure 3. The plant N nutritional status and the external availability of ammonium or nitrate are two major components in the transcriptional regulation of AMT genes. In *Arabidopsis* and in tomato, transcript levels of *AMT1;1* steeply increased already after several hours of N deficiency coinciding with a concomittant increase in ammonium influx into roots (Gazzarrini et al. 1999; Rawat et al. 1999; von Wirén et al. 2000). *AtAMT1;1* expression was predominantely dependent on the local nitrogen status of the roots, because it was upregulated mainly in the root part that directly experienced nitro-

gen starvation when plants were grown in a split-root system (Gansel et al. 2001). Following resupply of nitrogen, root glutamine concentrations correlated best with decreasing influx, suggesting that glutamine might be the metabolic trigger down-regulating *AtAMT1;1* at the transcriptional level (Rawat et al. 1999; von Wirén et al. 2000). However, *AtAMT1;1* also responded rapidly to nitrate and was even downregulated when supplying nitrate to a nitrate reductase-deficient mutant, indicating that nitrate *per se* might act as a signal repressing *AtAMT1;1* (Gansel et al. 2001; Wang et al. 2000).

By contrast, transcript levels of *LeAMT1;2* were low under nitrogen-deficient growth conditions, but sharply increased with resupply of ammonium (Lauter et al. 1996; von Wirén et al. 2000). *LeAMT1;2* induction was highest at 5 – 50 µM ammonium but absent when ammonium assimilation was blocked by methionine sulphoxamine (MSX) (Becker et al. 2002). Besides ammonium, nitrate also induced *LeAMT1;2* (Lauter et al. 1996), which might reflect the requirement for ammonium retrieval after nitrate-stimulated efflux of ammonium. Interestingly, *LeAMT1;2* was also induced in the presence of wild type cells of the root-associated bacterium *Azospirillium brasiliense*. Induction was lower in presence an nitrogen fixation-deficient *Azospirillium* mutant, which points to a possible contribution of LeAMT1;2 in the uptake of ammonium from root-associated N_2-fixing bacteria (Becker et al. 2002).

Functional ammonium transporters have also been isolated from rice. While two of these root-expressed transporter genes were constitutively expressed (*OsAMT1;1*) or upregulated under N deficiency (*OsAMT1;3*), *OsAMT1;2* was induced under ammonium supply, even if external concentrations were lower than 200 nM (Sonoda et al. 2003). *In-situ* RNA detection confined *OsAMT1;2* gene expression to the cell surface of root tips as well as to the central cylinder, implying that one transporter might participate in different physiological functions, such as ammonium uptake from the soil solution and long distance transport. Using RT-PCR Kumar et al. observed that all three genes, *OsAMT1;1, 1;2*, and *1;3*, were negatively regulated by external ammonium and only differed in their expression levels (Kumar et al. 2003).

Three ammonium transporters in *A. thaliana* roots showed diurnal regulation with highest transcript levels at the end of the light period followed by a sharp decline in the dark (Gazzarrini et al. 1999). *AMT* transcript levels increased with light intensity and were relieved from repression after the supply of sucrose during the dark period, indicating that sugars also participate in transcriptional regulation of *AMT* genes (Lejay et al. 2003). By contrast, malate or 2-oxoglutarate could not stimulate *AMT* gene expression. Parallel investigations on the nitrate transporter gene *AtNRT2;1*, which showed a similar dependency on photosynthesis as *AtAMT1;2* and *AtAMT1;3*, suggested that hexokinase or its downstream metabolites from glycolysis might trigger induction of *AtNRT2;1* (Lejay et al. 2003). Whether this also applies to *AMT*s, however, remains to be demonstrated.

Under natural growth conditions, sugars regulating *AMT* expression are expected to mainly derive from photoassimilates. In fact, growing plants under elevated atmospheric CO_2 concentrations increased the uptake capacity of tobacco roots for ammonium versus nitrate (Matt et al. 2001). In wheat, nitrate reduction

was impaired under elevated CO_2, favouring nitrogen assimilation and plant growth when ammonium nitrogen was supplied (Bloom et al. 2002). This might well be due to a CO_2-dependent regulation of AMT transporters. Transcriptional regulation of *AMT*s by CO_2 is of additional importance in leaves. Transcript levels of *LeAMT1;2* and *LeAMT1;3* decreased under elevated atmospheric CO_2, but showed an almost inverse diurnal expression pattern with highest mRNA levels for *LeAMT1;2* after onset of light and for *LeAMT1;3* during the dark. This might point to an involvement of LeAMT1;2 in ammonium retrieval, which serves to compensate for ammonium losses due to photorespiration at low CO_2 (von Wirén et al. 2000).

6 Ammonium/ammonia transport by other (than AMT-type) transporters in plants

Since the yeast triple *mep* mutant still exhibits a low ammonium uptake capacity in particular at high external supply (Marini et al. 1997a), it has been concluded that transporters other than AMT/MEPs should contribute to overall ammonium transport. Electrophysiological studies on root plasma membranes from *Arabidopsis* allowed the characterisation of so-called non-selective cation channels (NSCC) as transporters with a remarkably high selectivity for ammonium (Demidchik and Tester 2002). NSCCs can account for a major component of Na^+ influx at a relatively low dependence on membrane voltage and are inhibited by high Ca concentrations (Tyerman et al. 2002). However, the molecular identity of these transporters, that also exist in yeast (Bihler et al. 1998), has not yet been uncovered.

The chemical similarity and identical ionic radii of NH_4^+ and K^+ suggest that K^+ channels might permeate NH_4^+. Indeed, root-expressed AtKAT1 permitted ammonium-inducible inward currents (Moroni et al. 1998) and single amino acid substitutions in AtKAT1 could even increase ammonium permeability dramatically in oocytes (Uozumi et al. 1995), indicating that the transport of both substrates is not easily discriminated by this type of K^+ channel. However, ammonium uptake studies in *akt1* knockout plants must be awaited before a more precise idea is obtained about the contribution of AKT1 to overall ammonium uptake.

Ammonium transport is also of central importance at the symbiosome membrane, which surrounds N_2-fixing bacteroids and mediates the import of ammonium into the host plant cytoplasm in exchange for the export of dicarboxylates. Initially, it was believed that simple diffusion of uncharged NH_3 across the bacteroid and symbiosome membranes provided the main pathways for symbiotic nitrogen delivery to the host (Streeter 1989). Then, patch-clamp recordings with soybean symbiosome membranes proposed a voltage-activated cation channel to transport ammonium from the symbiosome space into the cytosol (Roberts and Tyerman 2002; Tyerman et al. 1995), again without knowing the molecular identity of these NH_4^+-permeable channels.

The mutant *S. cerevisiae* strain originally used to clone *S. cerevisiae MEP2* was used to isolate potential *AMT* genes from a soybean cDNA library by complementation of the yeast growth phenotype on limiting ammonium. This led to the isolation of *GmSAT1* (*Glycine max* symbiotic ammonium transporter), which was proposed to encode an ammonium transporter located on the soybean peribacteroid membrane that could be involved in the transfer of fixed nitrogen from the bacteroid to the host (Kaiser et al. 1998). However, later studies revealed that *GmSAT1* could not complement the triple *MEP* deletion strain, that *GmSAT1* caused overexpression of Mep3 and that GmSAT1 has features of a transcriptional regulatory protein including a DNA-binding domain. It was subsequently concluded that GmSAT1 is not an ammonium transporter but rather a protein that interferes with the Mep1(G413D)-dependent inhibition of Mep3 (Marini et al. 2000b). Although interference between *MEP1* and *MEP3* at the transcriptional level could not be ruled out, a direct interaction between the two Mep proteins was suggested, reminiscent of the hetero-oligomers formed between Rhesus proteins (Hartel-Schenk and Agre 1992).

The view of passive NH_3 diffusion across the symbiosome bilayer was further revised by the observation that facilitated ammonium diffusion could be inhibited by mercurials (Niemietz and Tyerman 2000) and that Nodulin 26, which represents a major intrinsic protein from the symbiosome membrane, is a mercurial-sensitive water and solute channel (Dean et al. 1999; Rivers et al. 1997). In parallel, expression of the mammalian aquaporin AQP1 in oocytes provoked an internal acidification of the oocytes in response to ammonium supply at high pH, indicating that NH_3 might be a transported substrate by aquaporins (Nakhoul et al. 2001). Thus, due to their high density in any membrane, major intrinsic proteins (MIPs) could in general provide an efficient pathway for a pH-dependent equilibration of ammonium concentrations between cellular compartments.

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