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Review

Nitrate transporters in plants: structure, function and regulation

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Abstract

Physiological studies have established that plants acquire their NO_3^- from the soil through the combined activities of a set of high- and low-affinity NO_3^- transport systems, with the influx of NO_3^- being driven by the H^+ gradient across the plasma membrane. Some of these NO_3^- transport systems are constitutively expressed, while others are NO_3^--inducible and subject to negative feedback regulation by the products of NO_3^- assimilation. Here we review recent progress in the characterisation of the two families of NO_3^- transporters that have so far been identified in plants, their structure and their regulation, and consider the evidence for their roles in NO_3^- acquisition. We also discuss what is currently known about the genetic basis of NO_3^- induction and feedback repression of the NO_3^- transport and assimilatory pathway in higher plants.

Keywords: Nitrate transporter; Major facilitator superfamily; Nitrogen regulation

1. Introduction

Nitrate (NO_3^-) is the most important source of mineral N for plants growing in aerobic soils. Plants acquire their NO_3^- from the soil solution by absorbing it across the plasma membrane (PM) of epidermal and cortical cells of the root. Once inside, the NO_3^- is reduced to ammonium (NH_4^+) by nitrate reductase (NR) and nitrite reductase (NiR) and the NH_4^+-N is then assimilated into organic N *via* the GOGAT cycle [1].

In most plant species only a proportion of the absorbed NO_3^- is assimilated in the root, the remainder being transported upwards through the xylem for assimilation in the shoot. In situations of excess NO_3^- supply, high concentrations of NO_3^- can accumulate in the vacuole and some of the NO_3^- may also be lost to the soil solution by efflux across the PM [2]. The vacuolar store of NO_3^- may be used to help maintain the concentration of the cytosolic NO_3^- pool, which has been reported to be held relatively constant under a wide range of external NO_3^- concentrations [3]. Thus an essential element in the process of NO_3^- assimilation is the trafficking of the NO_3^- ion across membranes. This review will focus on the transporters that mediate NO_3^- influx across the PM in roots; as yet little is known of those that are responsible for NO_3^- efflux from the cell, for its

Abbreviations: GOGAT, glutamate synthase; HATS, high-affinity nitrate transport system; iHATS, inducible high-affinity nitrate transport system; cHATS, constitutive high-affinity nitrate transport system; LATS, low-affinity nitrate transport system; MFS, major facilitator superfamily; MSX, methionine sulfoximine; NR, nitrate reductase; NiR, nitrite reductase; PCR, polymerase chain reaction; PM, plasma membrane

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uptake and release by the vacuole or for its loading and unloading in the xylem.

2. Kinetics and energetics of NO_3^- uptake

The physiological aspects of NO_3^- uptake in plants have recently been reviewed in detail [2], so only a brief summary will be given here. Thermodynamic considerations indicate that NO_3^- uptake will require an active transport system, even at the highest NO_3^- concentrations likely to be found in the soil [4–6] and physiological studies have provided evidence that NO_3^- influx is proton-coupled and therefore dependent on the H^+ pumping activity of the PM H^+-ATPase.

The accumulated evidence from kinetic studies indicates that roots have at least three distinct NO_3^- uptake systems, two of which have a high affinity for NO_3^-, while the third has a low affinity. One of the high-affinity systems is strongly induced in the presence of an external NO_3^- supply and is known as the inducible high-affinity transport system (or iHATS), while the second high-affinity system (the cHATS) is constitutively expressed [7,8]. The cHATS has the higher affinity for NO_3^- (K_m values of 6–20 μM compared to 13–79 μM for the iHATS) [2], but the iHATS has a much greater capacity for NO_3^- uptake: in barley the V_{max} for iHATS activity following induction with 100 μM NO_3^- was over 25-fold higher than the uninduced cHATS activity [9]. The low-affinity system (or LATS), which appears to be constitutively expressed, is most important at external NO_3^- concentrations >1 mM, and despite displaying linear kinetics it too appears to be an active H^+-dependent transport system [7,10,11].

The physiological data indicate that the regulation of the individual components of the NO_3^- uptake system is complex [2]. Thus although the cHATS is constitutive in the sense that its expressed in the absence of NO_3^-, its activity in at least some plant species is stimulated several fold by NO_3^- treatment [7,11]. Furthermore, the iHATS as well as being nitrate-inducible is also negatively feedback-regulated by the products of N assimilation [12–14] and a similar negative feedback regulation of the LATS has been noted in barley [10].

The picture that is emerging from studies of the molecular biology of NO_3^- transporters in plants suggests that there are more components to the NO_3^- uptake system than the three that were recognised from the physiological studies. Nitrate transporters belonging to two different families (the NNP and the PTR families) have now been identified in plants, each of which is represented by multiple genes that are differentially regulated and which may encode transporters with different regulatory or kinetic properties. What is currently known about the structure, function and regulation of these two classes of NO_3^- transporter will be reviewed below.

3. NO_3^- transporters of the NNP family

3.1. Structure and phylogeny

The NNP (nitrate-nitrite porter) family of NO_3^- and NO_2^- transporters, which has members from both prokaryotes and eukaryotes, is one of seventeen families of transporters currently assigned to the Major Facilitator superfamily (MFS) [15]. The MFS is a divergent group of proteins that are typically 500–600 amino acids in length and with a membrane topology in which two sets of six transmembrane helices are connected by a cytosolic loop [15,37,38]. A well-characterised prokaryotic member of the NNP family, the *Escherichia coli narK* gene product, is thought to act as a NO_2^- extrusion system [28], but the role of eukaryotic members of the family generally appears to be in NO_3^- and NO_2^- influx.

The first eukaryotic member of the family to be cloned was the *crnA* gene from *Aspergillus (Emericella) nidulans*, a mutation in which confers resistance to chlorate (ClO_3^-) and a partial defect in NO_3^- uptake [39,40]. Subsequently it was discovered that high-affinity NO_3^- and NO_2^- uptake in the green alga *Chlamydomonas reinhardtii* is under the control of at least three *crnA*-related genes, *CrNRT2.1*, *CrNRT2.2* and *CrNRT2.3* [22–24] (see Fig. 1). (*NRT2* is the accepted nomenclature for genes of the NNP family in algae and higher plants, with a two-letter prefix or suffix denoting the species of origin).

NRT2 genes have now been cloned from a wide range of higher plant species, including barley (*Hordeum vulgare*) [21], soybean (*Glycine max*) [20], *Nico-*

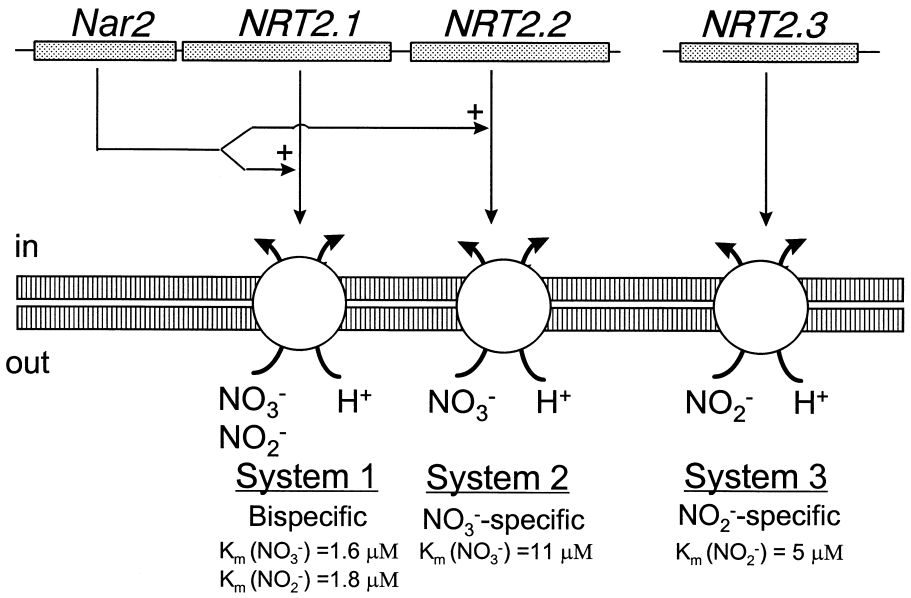

Fig. 1. Molecular genetics of the NO_3^- and NO_2^- transport systems in *C. reinhardtii*. The *Nar2* gene which is closely linked to the *NRT2.1* and *NRT.2* genes is required for the synthesis or activity of systems 1 and 2, but its precise function is unknown. The *NRT2.3* gene which specifies system 3 is unlinked to the other three genes and does not have a requirement for *Nar2*; it is not known whether another *Nar2*-related gene has a role in the biosynthesis of system 3. See text for further details.

tiana plumbaginifolia [18] and *Arabidopsis* (*Arabidopsis thaliana*) [16,17] (see Table 1), but in each case the cloning has been based not on their functional properties but on their homology to other *NRT2* genes or, in one instance, their pattern of gene expression [16]. Nevertheless, some direct evidence for their role in high-affinity NO_3^- transport is now emerging and will be discussed in Section 3.2.

It has been estimated that there are up to ten closely related *NRT2* genes in the barley genome [21], and the sequences of four of these are now available (see Table 1). Indications are that other plant species such as soybean [20] and *N. plumbaginifolia* [18] may have much smaller *NRT2* gene families. In *Arabidopsis*, initial studies suggested that there were just two *NRT2* genes (*AtNRT2.1* and *AtNRT2.2*) located near the top of chromosome 1 just 1.5 kb apart in a tail-to-tail configuration [16,17]. Recently, however, the large-scale *Arabidopsis* genome sequencing project has uncovered two further *NRT2* genes (*AtNRT2.3* and *AtNRT2.4*) located on chromosome V (GenBank accession no. AB015472). *AtNRT2.3* and *AtNRT2.4* are spaced about ~4 kb apart in a head-to-tail configuration. Surprisingly, this second pair of *NRT2* genes was not detected in genomic Southern blots using an *AtNRT2.1* sequence as probe ([16,17]; H. Zhang, B.G. Forde, unpublished) but their predicted amino acid sequences are respectively 83% and 69% identical to AtNRT2.1.

The dendrogram presented in Fig. 2 shows that *AtNRT2.4* is the most divergent member of the higher plant *NRT2* family so far identified, being as distantly related to the other three *Arabidopsis NRT2* genes as to the monocot *NRT2* genes. This finding raises the possibility that there may be further undiscovered *NRT2* genes in the *Arabidopsis* genome and that *NRT2* gene families in other plant species may be larger and more diverse than had been appreciated on the basis of hybridisation studies.

The plant and algal *NRT2* gene products are about 30% identical to their fungal homologues and are predicted to possess a similar 12 transmembrane domain structure [41]. On the basis of their hydropathy profiles the members of the NNP family can be classified into three main groups (Fig. 2) [42]. Transporters of type I (represented in Fig. 2 by the *E. coli* NarK protein) are the smallest and have a minimal amount of sequence outside the 12 transmembrane domains, the fungal members of the family (type II) have a large hydrophilic central loop of 90 amino acids located between transmembrane domains 6

Table 1
Cloned sequences of known or putative NO_3^-/NO_2^- transporters belonging to the NNP and PTR families and referred to in this review

Gene	Alternative name	Species	Accession no.	NO_3^- inducible	Transport activity	Ref.
1. NNP Family						
AtNRT2.1	*ACH1*	*A. thaliana*	Z97058	Yes	NO_3^- transporter (high affinity)	[16,17]
AtNRT2.2	*ACH2*	*A. thaliana*	AF019749	Yes	?	[16,17]
AtNRT2.3		*A. thaliana*	AB015472	?	?	–
AtNRT2.4		*A. thaliana*	AB015472	?	?	–
NpNRT2		*N. plumbaginifolia*	Y08210	Yes	?	[18,19]
GmNRT2		*G. max*	AF047718	Yes	?	[20]
HvNRT2.1	*BCH1*	*H. vulgare*	U34198	Yes	?	[21]
HvNRT2.2	*BCH2*	*H. vulgare*	U34290	Yes	?	[21]
HvNRT2.3	*BCH3*	*H. vulgare*	AF091115	?	?	–
HvNRT2.4	*BCH4*	*H. vulgare*	AF091116	?	?	–
LjNRT2[a]		*Lotus japonicus*	–	Yes	?	–
OsNRT2		*O. sativa*	AB008519	?	?	–
CrNRT2.1	*Nar3*	*C. reinhardtii*	Z25438	Yes	NO_3^-/NO_2^- transporter (high affinity)	[22–24]
CrNRT2.2	*Nar4*	*C. reinhardtii*	Z25439	Yes	NO_3^--specific transporter (high affinity)	[22–24]
CrNRT2.3		*C. reinhardtii*	AJ223296	Yes	NO_2^--specific transporter (high affinity)	[23,24]
crnA		*A. nidulans*	U34382	Yes	NO_3^-/NO_2^- transporter (high affinity)	[25]
YNT1		*H. polymorpha*	Z69783	Yes	NO_3^- transporter (high affinity)	[26]
NarK		*E. coli*	X15996	Yes	NO_2^- extrusion system	[27,28]
II. PTR Family						
AtNRT1.1	*CHL1*	*A. thaliana*	L10357	Yes	NO_3^-/ClO_3^- transporter (low affinity)	[29–31]
AtNRT1.2	*NTL1*	*A. thaliana*	AF073361	?	NO_3^- transporter (low affinity)	[30,32]
AtNTP2		*A. thaliana*	AJ011604	?	?	[33]
AtNTP3		*A. thaliana*	AJ131464		?	[33]
BnNRT1.2	*RCH2*	*B. napus*	U17987	Yes	NO_3^- and his transporter (low affinity)	[34,35]
CsNiTR1		*C. sativus*	Z69370	?	chloroplast NO_2^- transporter?	–
LeNRT1.1	*NIT1*	*L. esculentum*	X92853	No	?	[36]
LeNRT1.2	*NIT2*	*L. esculentum*	X92852	Yes	?	[36]

[a]The *LjNRT2* cDNA was cloned and sequenced by G. Leggewie, I. Onyeocha and B.G. Forde (unpublished).

and 7, and the algal and higher plant members of the family (type III) have an extended C-terminal domain of ~70 amino acids. The type III sequences can be further sub-divided by virtue of the presence (type IIIb) or absence (type IIIa) of an additional N-terminal sequence of ~20 amino acids. This N-terminal domain is highly conserved amongst the dicot members of the NRT2 family but is absent in the algal and barley sequences. Unexpectedly, the N-terminal extension is also found in a rice (*Oryza sativa*) NRT2 sequence (GenBank accession no. AB008519), showing that it is not dicot-specific and suggesting that the NRT2 family in barley and other monocots may have sequences of both type IIIb and IIIa. The N-terminal extension has none of the characteristics of known protein-sorting or localisation signals as determined by the PSORT program [43].

The membrane topologies of some members of the MFS have been established experimentally [44,45] and are consistent with the models shown in Figs. 2 and 3 where the N- and C-termini and the central loop are all predicted to lie on the cytosolic side of

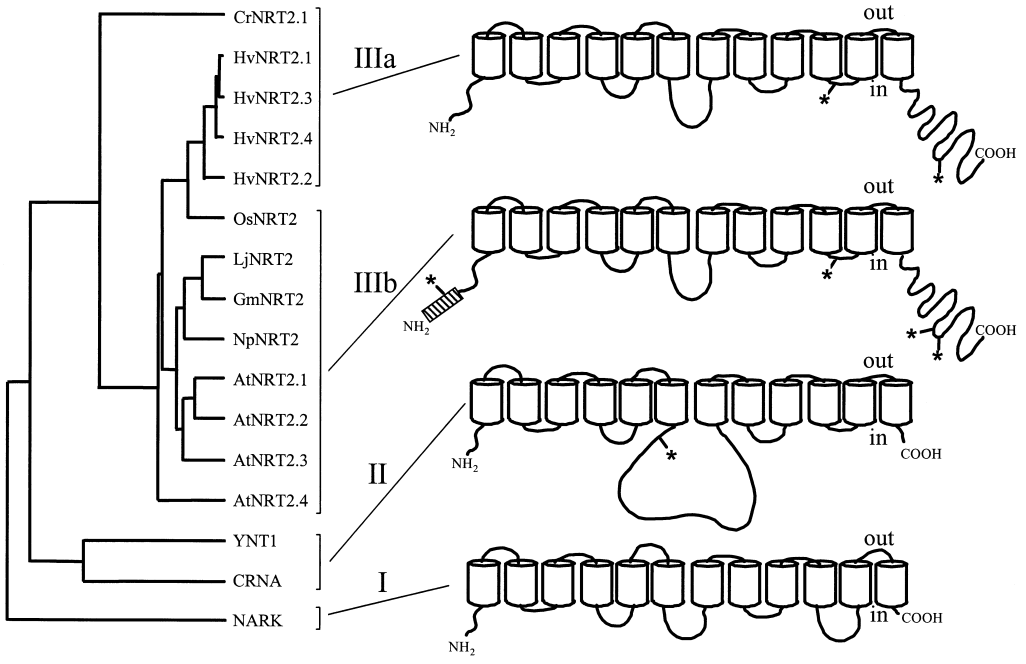

Fig. 2. Sequence relationships and comparisons of the predicted two-dimensional structures of members of the NNP family of NO_3^- and NO_2^- transporters. The amino acid sequences were aligned and the tree of sequence relationships generated using PILEUP [42] (only full-length sequences were used). See Table 1 for further details of the sequences used in the analysis and their GenBank accession codes. The diagrammatic representation of the membrane topologies of the transporters is based on previous predictions [21]. The domain indicated by a shaded rectangle represents a conserved N-terminal sequence that is specific to type IIIb NNP transporters. Asterisks indicate the location of conserved protein kinase C recognition motifs (S/T-x-R/K). The majority of the type IIIb sequences have two potential protein kinase C motifs within the C-terminal domain which are located nine amino acids apart (the exceptions being AtNRT2.1, which lacks the more C-terminal of the two motifs, and AtNRT2.2 which has neither). Amongst the type IIIa sequences, only the algal CrNRT2.1 and CrNRT2.2 [22] transporters have both motifs.

the membrane. The function of these domains in those members of the NNP family that possess them is unknown, but by analogy with examples from elsewhere in the MFS it is possible that they have some kind of regulatory role. In the GLUT family of mammalian glucose transporters there is evidence that the C-terminal domain has a key role in regulation of glucose transport through its involvement in insulin-regulated subcellular targeting of the transporter [46]. In the case of the *E. coli* lactose permease it has been shown that the central loop participates in interactions with a soluble protein responsible for allosteric regulation of the permease [47]. It has recently been shown that the C-terminal domain of an ATP-binding subunit of the ABC-type NO_3^-/NO_2^- transporter from the cyanobacterium *Synechococcus* PCC 7942 has a regulatory role in determining the sensitivity of the transporter to inhibition by NH_4^+ [48].

Indicated in Fig. 2 are the locations of a number of conserved protein kinase C recognition motifs (S/T-x-R/K) which are present in the N- and C-terminal domains of NRT2 transporters and in the central loop of the fungal NO_3^- transporters. The existence of these motifs could indicate that phosphorylation and dephosphorylation reactions play a part in the regulation of the activity of the NNP transporters.

The most highly conserved regions of the NRT2 transporters are found within the predicted transmembrane domains (Fig. 3). One sequence of note is the highly conserved motif found within transmembrane domain 5. This sequence (A-G-W/L-G-N-M-G) also occurs in the *E. coli* NarK protein and was proposed as a signature motif for the NNP family [21]. Based on a comparison that included a larger number of prokaryotic NNP sequences, an alternative signature sequence (located in the same region of the protein) has been proposed: F/Y/K-x_3-I/L/Q/R/K-x-G/A-x-V/A/S/K-x-G/A/S/N-L/I/V/F/Q-$x_{1,2}$-G-x-G-N/I/M-x-G-G/V/T/A [15]. A related

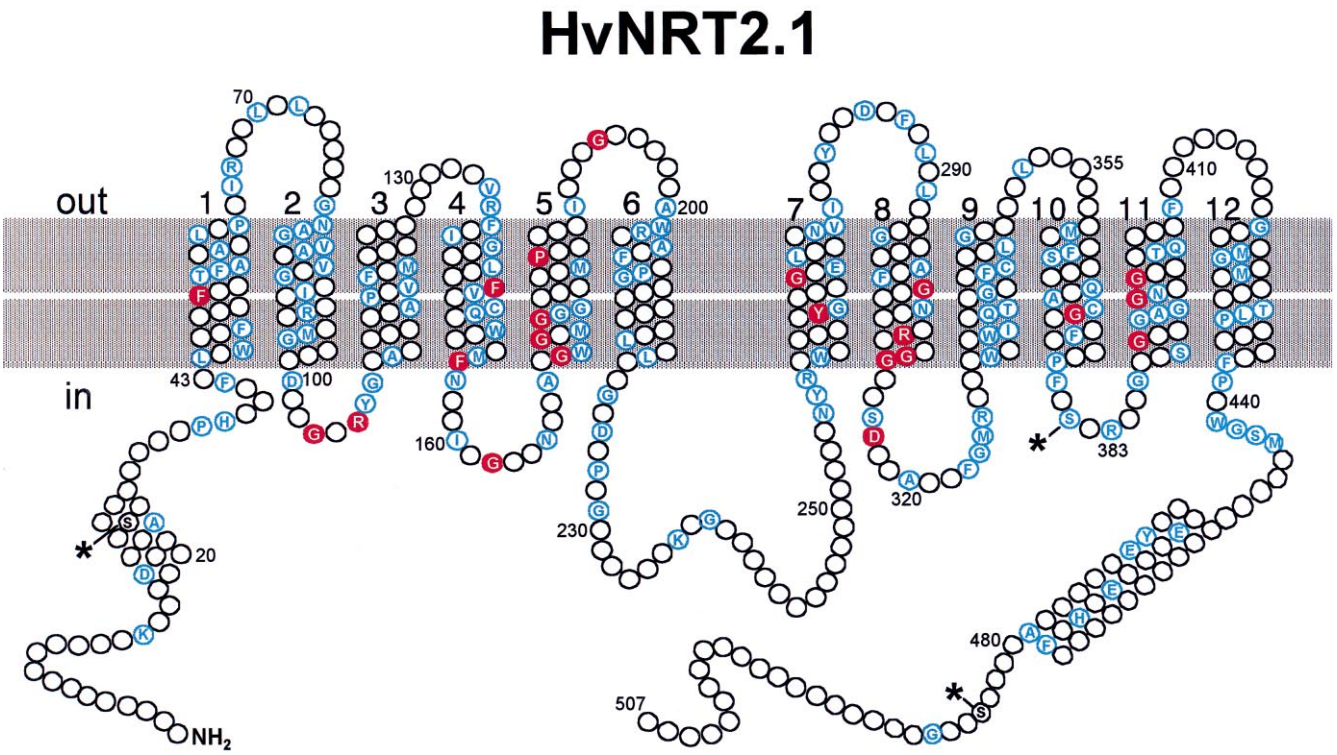

Fig. 3. Predicted membrane topology of HvNRT2.1. The assignment of the 12 predicted transmembrane domains was based on an analysis of multiply aligned HvNRT2.1, CrNRT2.1, CRNA and NARK sequences using the TMAP program [49], together with examination of the hydrophobicity profile of HvNRT2.1 [41] and comparisons with the experimentally determined topologies of other members of the MFS [44,45]. The topology and the boundaries of the transmembrane helices should not be considered as definitive but rather as a model which needs to be experimentally verified. Residues conserved in an alignment of all the bacterial, fungal, algal and plant sequences in Fig. 2 are indicated in red and residues conserved in all the plant and algal sequences in blue. Three serine residues which are located within conserved protein kinase C motifs are marked by asterisks: Ser-28 is part of a S/T-x-R motif found in all HvNRT2 sequences and in OsNRT2; Ser-381 (between transmembrane helices 10 and 11) is part of a S-x-R motif found in all the plant and algal sequences; Ser-484 (in the C-terminal domain) is part of a S-x-R motif found in the algal and the majority of the higher plant sequences. Two regions of predicted α-helix which appear to be conserved in similar locations in the hydrophilic N- and C-terminal domains of other higher plant NRT2 polypeptides are also shown.

sequence is found in transmembrane domain 11, consistent with evidence from examination of other MFS sequences that an early event in the evolution of the MFS family involved the internal duplication of a sequence with six transmembrane domains [15,38]. The many conserved glycine residues throughout the protein sequence could have a structural role, while certain conserved charged or polar residues within transmembrane domains (for example, the arginine residues conserved within transmembrane domains 2 and 8) might have a catalytic function.

Also worthy of note is a conserved sequence located in the vicinity of the putative cytosolic loop between transmembrane domains 2 and 3. This sequence (G-x-x-x-D-x-x-G-x-R) is closely related to a motif (G-x-x-x-D/N-R/K-x-G-R-R/K) which is highly conserved in other members of the MFS and which is specific to members of this superfamily [15,37]. This motif frequently occurs twice in the MFS transporter sequences, once in loop 2/3 and again in loop 8/9, although in the case of the NRT2 transporters the copy of the motif in the second half of the protein is less well conserved (note the conserved G-313 and D-317 residues in Fig. 3). Experimental evidence for the structural and functional significance of this motif has been obtained for both the *E. coli* lactose permease [50,51] and the *E. coli* Tn10-encoded tetracycline carrier [52].

3.2. Function

It is from *C. reinhardtii* that most information about the functional properties of NRT2 transporters is available [22,23]. *C. reinhardtii* has three separate NO_3^- or NO_2^- uptake systems, each with distinctive kinetic and regulatory properties (Fig. 1): system 1 (specified by the *CrNRT2.1* gene) has a very high-affinity for both NO_3^- and NO_2^- (K_ms = 1.6 and 1.8 μM, respectively), system 2 (specified by *CrNRT2.2*) is specific for NO_3^- (K_m = 11 μM), while system 3 (specified by *CrNRT2.3*) is NO_2^--specific (K_m = 3.4 μM) [23,24].

The *A. nidulans* CRNA transporter is the only member of the NNP family whose kinetic properties have been characterised in detail using a heterologous expression system. The *crnA* mRNA was injected into *Xenopus* oocytes and electrophysiological techniques were used to establish that *crnA* encodes a high-affinity H^+/NO_3^- co-transporter with a H^+:NO_3^- stoichiometry of 2:1 and K_ms for NO_3^- and NO_2^- of 2.5 μM and 96 μM, respectively (J.-J. Zhou, L.J. Trueman, K.J. Boorer, F.L. Theodoulou, B.G. Forde, A.J. Miller, unpublished). No significant currents were obtained when oocytes expressing *crnA* were treated with other possible substrates such as Cl^-, HCO_3^-, SO_4^- or cyanate. Surprisingly, despite the fact that the *crnA* mutant was isolated on the basis of its ability to confer ClO_3^- resistance, the CRNA transporter expressed in oocytes failed to transport ClO_3^-. However, it did transport chlorite (ClO_2^-) with a K_m of 16.9 μM, and since ClO_3^- solutions are often contaminated with ClO_2^- it is possible that the *crnA* mutation actually conferred resistance to ClO_2^- rather than ClO_3^-. Alternatively, it cannot be ruled out that the substrate specificities of CRNA when expressed in this heterologous system may differ from those of the endogenous *A. nidulans* protein.

Because similar attempts to express a variety of plant *NRT2* genes in oocytes have proved unsuccessful (J.-J. Zhou, A.J. Miller, personal communication), much of the evidence implicating NRT2 transporters in NO_3^- transport in higher plants is circumstantial, being based on their homology to the fungal and algal high-affinity NO_3^- transporters and an expression pattern that closely matches the one observed for the iHATS (see below). Attempts have been made to complement the *ynt1* NO_3^- uptake mutant of the yeast *Hansenula polymorpha* by heterologous expression of the barley *HvNRT2.1* and *HvNRT2.2* cDNAs (N. Brito, L.J. Trueman, J. Siverio, B.G. Forde, unpublished). These experiments established that both NRT2 transporters were able to increase the ability of the mutant to grow on NO_3^- as sole N source and to stimulate NO_3^- uptake from low external NO_3^- concentrations. Furthermore, deletion of the hydrophilic C-terminal domain had no significant effect on the activity of the HvNRT2.1 transporter, supporting the idea that this domain may have a regulatory rather than a catalytic role. However, for reasons that are still unclear, the NO_3^- uptake activity of the transformants was very low compared to the wild type, so that more detailed analysis of the properties of the NRT2 transporters was not possible.

Recently, direct experimental evidence that *NRT2* gene products do contribute to high-affinity NO_3^- uptake has been obtained using transgenic *Arabidopsis* lines carrying an *AtNRT2.1* antisense construct: lines in which *AtNRT2.1* expression was strongly suppressed displayed a 40% reduction in the rate of NO_3^- uptake from medium containing 250 μM KNO_3 (H. Zhang, A. Jennings, B.G. Forde, unpublished).

From genetic evidence, the *Arabidopsis CHL8* gene appears to encode a component of the cHATS [53], but its chromosomal location and DNA sequence are unknown, leaving open the question of whether it belongs to the *NRT2* gene family.

3.3. Regulation

3.3.1. NO_3^- induction

It is well established that the high-affinity NO_3^- uptake systems in higher plants are rapidly induced by the presence of external NO_3^- [2,54,55]. This inducibility sets high-affinity NO_3^- uptake systems apart from high-affinity uptake systems for other mineral nutrients (such as SO_4^-, inorganic phosphate or NH_4^+) which are not induced by their substrates but only derepressed by a deficiency in the corresponding ion [56]. The maximum rates of high-affinity NO_3^- uptake are usually seen several hours after the start of the period of NO_3^- induction, with rates then declining significantly due, it is thought, to feedback inhibitory effects resulting from the accumula-

tion of the products of NO_3^- assimilation [57,58] and perhaps also the internal pool of NO_3^- itself [14]. Use of inhibitors of RNA and protein synthesis provided early evidence that induction of the iHATS involves the synthesis of new transporter protein [59–61].

It has been demonstrated in barley [21], *N. plumbaginifolia* [18], soybean [20] and *Arabidopsis* ([16,17] that the abundance of *NRT2* mRNAs rapidly increases when N-starved roots are treated with NO_3^-, even at concentrations as low as 10–50 μM [16,19]. In *Arabidopsis*, both the *AtNRT2.1* and the *AtNRT2.2* genes are NO_3^--inducible, but the latter gene is expressed at very much lower levels than the former ([17]; H. Zhang, B.G. Forde, unpublished). Western blots using antibodies raised against the C-terminus of a barley NRT2 polypeptide have established that the NRT2 transporters are also nitrate-inducible at the protein level (M. Hansen, S. Dunn, B.G. Forde, unpublished).

Although the iHATS for NO_3^- in barley is reported to be inducible by a pre-treatment with NO_2^- [60], a 3-h exposure of *Arabidopsis* roots to 1 mM NO_2^- failed to induce *AtNRT2.1* expression [17]. However, this experiment should perhaps be repeated with a lower concentration of NO_2^- since previous studies in barley found that the induction of NO_3^- uptake activity by NO_2^- was maximal at 10 μM and was markedly reduced at higher concentrations, apparently due to toxicity of the NO_2^- [60].

Most studies show that the abundance of *NRT2* mRNAs reaches a peak a few hours after the start of the NO_3^- induction and then falls [16–20], following a similar time-course to the kinetics of induction of the *NIA* and *NII* genes (for NR and NiR, respectively) [19] and consistent with the existence of some form of feedback regulatory mechanism (see below). The peak in the abundance of the *AtNRT2.1* mRNA about 5 h after induction was seen to coincide with a peak in the activity of the iHATS as measured by the rate of ^{13}N influx [17].

3.3.2. Feedback repression

It is well established that the iHATS in plants is feedback regulated according to the plant's demand for N (reviewed in [2]). Which N pool(s) are responsible for exerting these feedback effects is unknown, but by analogy with what is known of N regulation in microorganisms the key metabolite is usually thought to be glutamine or possibly the glutamine/2-oxoglutarate ratio. Unfortunately experimental support for this is still lacking in plants, and there are even reports suggesting that other amino acids such as arginine may be more important [58,62].

There is evidence that higher plant *NRT2* genes are feedback repressible by downstream metabolites. For example, when *N. plumbaginifolia* plants were supplied with glutamine or NH_4Cl (at 5 mM) there was a rapid decline in the abundance of the *NpNRT2* mRNA, with glutamine having the stronger effect [18,19]. Similar repressive effects of NH_4^+ treatment on the accumulation of *NRT2* mRNA were observed in soybean [20].

A number of inhibitors of N assimilation were used in a recent attempt to identify the N metabolites responsible for down-regulating *NRT2* gene expression in *Arabidopsis* [17]. When NR activity was inhibited by tungstate, there was little effect on *AtNRT2.1* expression, leading to the suggestion that the internal NO_3^- pool is not important for feedback regulating this gene (at least at the transcriptional level). A similar conclusion was reached from studies using NR-deficient mutants, which are known to accumulate NO_3^- and have smaller pools of amino acids: in both *Arabidopsis* [16] and *N. plumbaginifolia* [19] the abundance of the *NRT2* mRNA was found to be elevated in the NR-deficient lines. One cautionary note on the interpretation of these experiments is that while the particular treatments used may have led to accumulation of NO_3^- in the (major) vacuolar pool, there is evidence from studies with NO_3^--selective microelectrodes that the cytosolic NO_3^- pool may be highly regulated by a homeostatic mechanism [3], which would make it more difficult to manipulate. If this is so, then it becomes difficult to rule out the possibility that there are some conditions under which the iHATS and/or the *NRT2* genes are subject to feedback regulation by an internal NO_3^- pool.

When methionine sulfoximine (MSX) was used to inhibit NH_4^+ assimilation, externally applied NH_4^+ had a stronger inhibitory effect on the accumulation of *AtNRT2.1* mRNA and on $^{13}NO_3^-$ influx, suggesting that the tissue NH_4^+ pool may be an important regulator of both *AtNRT2.1* gene expression and iHATS activity in *Arabidopsis*. This is in contrast to two earlier studies in which an MSX treatment

was shown to relieve the inhibitory effect of NH_4^+ on the activity of the iHATS in dwarf bean [63] and maize roots [64], but is more consistent with a third study in which MSX failed to relieve the inhibition of NO_3^- influx caused by a high rate of NO_3^- supply [14]. Inhibitors of aspartate aminotransferase and GOGAT provided evidence that tissue concentrations of glutamate and glutamine may also be important in feedback regulation of the iHATS and *AtNRT2.1* [17]. On the other hand, in the same study, arginine and asparagine (applied externally at 1 mM) were much more effective at down-regulating *AtNRT2.1* than was glutamine. However, as pointed out by the authors, the interpretation of these results is not straightforward as the individual amino acids may be absorbed and/or assimilated at different rates.

When NO_3^--grown *Arabidopsis* roots are transferred to N-free medium there is a transient increase in the abundance of the *AtNRT2.1* mRNA, which reaches a maximum after 24–48 h [16] and parallels fluctuations in HATS activity [65]. In view of the evidence for feedback repression of *AtNRT2.1*, this response to NO_3^- withdrawal would be explained by an initial derepression of *AtNRT2.1*, followed by a de-induction of the gene as the tissue is depleted of NO_3^-.

It is noteworthy that with respect to their ability to be derepressed by substrate deprivation, the *NRT2* genes behave in a very similar way to transporter genes for sulphate [66] and phosphate [67]. The difference lies in the ability of the *NRT2* genes to also be induced by the presence of their substrate, something that has not been reported for other nutrient transporter genes. It is not clear why NO_3^- transporters should be regulated in a more complex way than other types of nutrient transporter.

3.3.3. Diurnal regulation

In a number of plant species there is clear evidence for diurnal regulation of NO_3^- uptake, with the uptake rates generally peaking during the light period and reaching a minimum in the dark [68–70], and there is circumstantial evidence linking these diurnal changes with the rate of photosynthesis in the shoot [71]. Similar diurnal changes in the rate of NO_3^- influx were seen in *Arabidopsis* and it was shown that parallel changes occurred in the abundance of the *AtNRT2.1* transcript [65]. Furthermore the decline in both HATS activity and *AtNRT2.1* mRNA abundance could be delayed by supplying 1% sucrose in the nutrient solution [65]. These results suggest that the diurnal regulation of HATS activity in the roots is at least partly mediated by the supply of C metabolites from the shoot, acting through changes in the expression of the *AtNRT2.1* gene at the transcriptional or mRNA level.

3.3.4. Spatial and developmental regulation

Consistent with their postulated role in high-affinity NO_3^- uptake, the available evidence indicates that *NRT2* genes are expressed more strongly in roots than in aerial tissues, at least in *N. plumbaginifolia* [18] and *Arabidopsis* [17]. In *Arabidopsis*, the abundance of the *AtNRT2.1* transcript in shoots was estimated to be ~1% of that in roots [17]. In *N. plumbaginifolia*, *NRT2* transcripts were also detected at low levels in leaves, petioles, buds, flowers and seeds [18]. The expression of *AtNRT2.1* in *Arabidopsis* roots is reported to be developmentally regulated: its mRNA was undetectable by the sensitive technique of RT–PCR (reverse transcriptase–polymerase chain reaction) in 2-day-old or 5-day-old plants, was first detected around day 10 and had increased substantially by day 15 [17].

The results of in situ hybridisations to *NRT2* mRNAs in *N. plumbaginifolia* roots present a picture in which *NRT2* expression is highest in epidermal and endodermal cells close to the root tip, while in mature root tissue its expression is highest in the epidermis and in lateral root primordia [19].

4. NO_3^- transporters of the PTR family

4.1. Structure and phylogeny

The *Arabidopsis AtNRT1.1 (CHL1)* gene is up to now the only NO_3^- transporter gene from a higher plant to be cloned on the basis of its function. Mutants in the *AtNRT1.1* gene (*chl1* mutants) were originally isolated in the early 1970s in screens for ClO_3^--resistance [72,73] and later shown to be defective in both ClO_3^- and NO_3^- uptake [74–76]. When the *AtNRT1.1* gene was eventually cloned by T-DNA tagging it was found to encode a hydrophobic 65

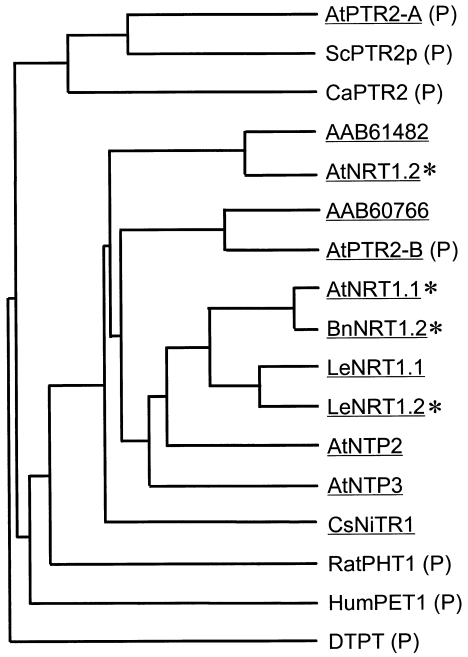

Fig. 4. Dendrogram showing sequence relationships between selected members of the PTR family. The amino acid sequences were aligned and the tree of sequence relationships generated using PILEUP [42]. Sequences indicated by an asterisk have been implicated in NO_3^- transport by functional analysis or by virtue of their NO_3^--inducibility (see Table 1), while sequences known to be oligopeptide transporters are indicated by a (P). Plant sequences are underlined. Two of the plant sequences are known only by their GenBank accession numbers; the accession numbers of the other plant sequences can be found in Table 1. Accession numbers for the non-plant sequences are: ScPTR2 (P32901), CaPTR2 (P46030), RatPHT1 (BAA20489), HumPET1 (P46059) and DTPT (O07380).

kDa protein with the characteristic features of a typical membrane transporter [29].

AtNRT1.1 is a member of an unusual family of transporters (the PTR or POT family) that is widely distributed in both prokaryotes and eukaryotes, most members of which function as H^+/oligopeptide co-transporters in the PM [77]. In common with the NNP family, there is evidence that the PTR family is a member of the MFS [2,15], although its designation as the 18th cluster of this superfamily is still considered to be unconfirmed [15].

At least five other members of the PTR family have so far been identified in *Arabidopsis* (Fig. 4). Two of these (PTR2A and PTR2B) are oligopeptide transporters which are 25% and 39% identical respectively to the *Arabidopsis* NRT1 transporter [78]. The remaining four have emerged from the EST (expressed sequence tag) database or the genome sequencing project. One of these (AtNRT1.2 or NTL1) has been reported from *Xenopus* oocyte expression studies to be a low-affinity NO_3^- transporter [32] (see Section 4.2), but the functional properties of the NRT1-related proteins encoded by *AtNTP2* and *AtNTP3* [33] and GenBank accession no. AAB61482 are unknown. From the tree of sequence relationships shown in Fig. 4 it is evident that it is not a straightforward matter to predict the likely substrate specificity of the uncharacterised members of the PTR family simply from their sequence similarities. For example, the oligopeptide transporter AtPTR2B clusters more closely with AtNRT1.1 than does AtNRT1.2. A signature motif for the PTR family (F-Y-x-x-I-N-x-G-S-L), which is located within transmembrane domain 5 [77], is equally well conserved in PTR family members that are known or putative NO_3^- transporters as amongst the peptide transporters. Nevertheless, it is clear from the dendrogram in Fig. 4 that there is a need to investigate the potential NO_3^- transporter activity of the products of some of the still uncharacterised plant members of the PTR family, particularly the *AtNTP2* and *AtNTP3* genes (which respectively encode proteins with 54% and 51% amino acid identity to AtNRT1.1).

Like most other PTR transporters AtNRT1.1 is predicted to have 12 transmembrane domains consisting of two sets of six hydrophobic segments flanking a central hydrophilic region (residues 239–335) that contains a large number of positively and negatively charged residues [79,80]; both the N- and C-terminal domains are quite short (18 and 28 residues, respectively). The membrane topology of a bacterial member of the family, the DtpT transporter of *Lactococcus lactis*, has been determined experimentally and shown to consist of 12 transmembrane domains and N- and C-termini that are located on the cytoplasmic side of the membrane [81]. Extrapolating this to AtNRT1.1 it appears that it has an overall secondary structure that is remarkably similar to those predicted for the fungal high-affinity NO_3^- transporters, CRNA and YNT1 (see Fig. 2). Alignment of AtNRT1.1 with its closest relatives (including BnNRT1.2, LeNRT1.1, LeNRT2.2, AtNTP2, AtNTP3 and AtNRT1.2) shows a high degree of se-

quence identity and co-linearity amongst them (not shown), indicating that they are all likely to have a similar membrane topology. The sequence divergence between AtNRT1.1 and AtNRT1.2 is greatest in the central loop. In a further striking similarity to CRNA and YNT1, the AtNRT1.1, BnNRT1.2, LeNRT1.1, LeNRT2.2, AtNTP2 and AtNTP3 sequences also contain a conserved protein kinase C recognition motif (T-x-R/K) at the start of the central loop (see Fig. 2).

4.2. Function

Analysis of the phenotype of *chl1* mutants of *Arabidopsis* has been the primary source of information about the function of the AtNRT1.1 transporter. The original mutant (known as B1) absorbs NO_3^- at wild-type rates at concentrations in the high-affinity range (up to 1 mM NO_3^-), but the activity of the LATS is markedly reduced [75]. The conclusion that *chl1* mutants are specifically impaired in the LATS was confirmed in a later study in which $^{13}NO_3^-$ was used to measure influx rates [31]. A complicating factor in the analysis of *chl1* mutants has been that the defect in the LATS is only apparent under certain growth conditions, the previous N nutrition of the plants apparently being critical to the phenotype [30,31,74]. One explanation for this is that there is at least one additional component of the LATS which is able to compensate for the *chl1* deficiency under one set of growth conditions, but under different conditions (usually when plants have been grown on NH_4^+) this second LATS is down-regulated and the effects of the deficiency in AtNRT1.1 are revealed [30,31]. One candidate which has been suggested as a second component of the LATS is the *AtNRT1.2* gene product [30] which, despite its distant relationship to AtNRT1.1 (38% amino acid identity) has recently been shown to have low-affinity NO_3^- transport activity in a heterologous expression system [32] (see below).

Consistent with the genetic evidence that *AtNRT1.1* encodes a component of the LATS, heterologous expression studies in *Xenopus* oocytes have indicated that AtNRT1.1 is a H^+-dependent NO_3^- transporter with a K_m for NO_3^- of 8.5 mM [29,30]. Similar studies on the closely related BnNRT1.2 transporter from *Brassica napus* confirmed that its K_m for NO_3^- was similarly in the low-affinity range [35]. However, in this case it was shown that the K_m was voltage-dependent, increasing from 4 mM at a membrane potential of −40 mV to 14 mM at −180 mV.

In addition to its NO_3^- transport activity, BnNRT1.2 was also found to be able to transport L-histidine, with the amino acid generating even larger currents than NO_3^- [35]. The K_m for histidine was also voltage-dependent, decreasing from 25 mM at −100 mV to 1.4 mM at −180 mV (measured at pH 8.5). D-Histidine, NO_2^-, cyanate, ClO_3^- and the dipeptide His–Leu were all tested and found to give no significant currents. The inward cation currents obtained with both NO_3^- and histidine were again consistent with a H^+-coupled system. Curiously the pH optima for the two substrates were quite different, with histidine transport being favoured at alkaline pH and NO_3^- transport being favoured at acidic pH.

The ability of *BnNRT1.2* to transport two such different substrates as NO_3^- and histidine is surprising, but is not unique amongst members of the PTR family. This family of transporters is recognised as being exceptional in both the variety of different substrates which its members can mobilise (oligopeptides, amino acids, NO_3^-, ClO_3^-) and in the ability of individual transporters to handle substrates of very different sizes and charges. Another example of this is the rat PHT1 transporter which is able to transport both peptides and histidine (K_m for histidine = 17 μM) [82]. Whether the ability of BnNRT1.2 to transport histidine is physiologically significant is a question that will have to await the generation of *B. napus* lines defective in *BnNRT1.2* expression.

In view of the weight of evidence that *NRT1* genes specify a component of the LATS, two recent reports that AtNRT1.1 also has a role in NO_3^- uptake at low external NO_3^- concentrations [32,83] have come as something of a surprise. When *Arabidopsis* seedlings were grown on NH_4NO_3 and their NO_3^- depletion rates assayed in liquid culture containing a range of KNO_3 concentrations, it was found that the *chl1* mutant showed reduced net rates of uptake not only in the low-affinity range but also at NO_3^- concentrations below 500 μM [32,83]. An estimate for the contribution of AtNRT1.1 to NO_3^- uptake at low external concentrations was obtained by subtracting the

NO_3^- depletion rates of the *chl1* mutant from those of the wild type, and although Michaelis–Menten kinetics for this NRT1-dependent activity were not demonstrated, the existence of a saturable high-affinity component of AtNRT1.1 with a K_m for NO_3^- of 38 μM was claimed [83]. The defect in *chl1* in high-affinity NO_3^- uptake was sensitive to the pH of the medium, being most evident at pH 5.5 and least at pH 7, and was also dependent on the previous N nutrition of the seedlings, being absent if plants were grown on KNO_3 rather than NH_4NO_3 [83]. The conditional nature of this aspect of the *chl1* phenotype presumably accounts for why it was not previously observed [31,75].

Confirmatory evidence that *AtNRT1.1* is active in both the high-affinity and low-affinity ranges has come from oocyte expression studies [32]. The high-affinity phase of activity was found to elicit an electrical response too small to be easily monitored, so it was necessary to measure rates of NO_3^- accumulation within the oocytes and/or rates of NO_3^- depletion from the medium bathing the oocytes. The results obtained from measurements done over a wide range of external [NO_3^-] indicated that there was a saturable high-affinity component with a K_m for NO_3^- of 50 μM, in addition to the low-affinity system ($K_m = 4$ mM). The oocyte data, taken together with the in vivo analysis of the *chl1* mutants, therefore suggest that AtNRT1 is a dual-affinity transporter with two distinct K_ms for NO_3^-, perhaps analogous to the AtKUP1 dual-affinity K^+ transporter from *Arabidopsis* [84]. Analysis of the kinetic properties of the *AtNRT1.2* gene product in oocytes showed that although it had similar activity to AtNRT1.1 in the low-affinity range it lacked any activity in the high-affinity range [32], indicating that this NRT1 transporter is not dual-affinity.

One unexplained feature of the *chl1* phenotype in the high-affinity range is that it primarily affects the cHATS rather than the iHATS [32,83]. While expression of *AtNRT1.1* is strongly NO_3^--inducible at the mRNA level [16,29], the high-affinity component of AtNRT1.1 appeared to be present constitutively in NO_3^--starved roots and was only moderately stimulated by NO_3^- treatment [83]. This discrepancy might be explained if there were additional levels of regulation of the high-affinity phase of AtNRT1.1 which operated on, say, translation or on the protein itself.

4.3. Expression

4.3.1. NO_3^- induction

Like the *NRT2* genes, *AtNRT1.1* and its close homologue in *B. napus*, *BnNRT1.2*, are both strongly and rapidly induced by NO_3^- [29,35]. On the other hand, the two *NRT1* genes in tomato are differentially responsive to NO_3^-: while expression of *LeNRT1.2* requires the presence of NO_3^-, *LeNRT1.1* is expressed constitutively and is unaffected by the addition of NO_3^- [36]. (When considering the significance of these data in relation to NO_3^- uptake, it should be borne in mind that there is as yet no published evidence that either of the tomato *NRT1* genes do encode a NO_3^- transporter).

Even NO_3^- concentrations < 100 μM are sufficient for induction of both *AtNRT1.1* and *BnNRT1.2* [35,83]. This would be consistent with these *NRT1* genes having a role in high-affinity uptake [83], but could also simply be a consequence of plants not having evolved a separate signal transduction pathway for gene induction by high concentrations of NO_3^-. The *AtNRT1.1* gene can also be induced in the absence of external NO_3^- by a sudden reduction in the external pH: the abundance of *AtNRT1.1* mRNA increased within 2 h after the pH of the medium was decreased from 6.5 to 5.5 [29].

4.3.2. Feedback regulation

Recent evidence indicates that *AtNRT1.1* expression is much less susceptible to feedback repression than are members of the *NRT2* family [16]. When NO_3^--grown *Arabidopsis* seedlings were deprived of N, the abundance of the *AtNRT2.1* transcript (which was initially very low) increased markedly by day 2 of N starvation, while the abundance of the *AtNRT1.1* transcript (which was initially high) actually declined by about 40% over the same period. Furthermore, while the *AtNRT2.1* transcript was generally much more abundant in an NR-deficient mutant than in the wild type after prolonged N starvation or in the first few hours of NO_3^- induction (the same response as seen for the *NIA* gene in this mutant), the abundance of the *AtNRT1.1* transcript in the mutant was similar to the wild type [16].

Differences between the regulation of *AtNRT1.1* and *AtNRT2.1* were also found in a separate study using the same NR-deficient mutant [65]. In plants

grown continuously on 1 mM NH_4NO_3 and then transferred to 1 mM KNO_3 for 1–3 days, the removal of the NH_4^+ supply led to an apparent derepression of *AtNRT2.1* which was more pronounced in the NR mutant than the wild type. In the case of the *AtNRT1.1* gene, there was no increase in mRNA abundance following NH_4^+ removal in either the mutant or the wild type. Consistent with the findings of Filleur and Daniel-Vedele [16], the authors therefore concluded that *AtNRT1.1* is not feedback regulated by the N status of the plant. However, the additional observation that *AtNRT1.1* was overexpressed in the NR mutant even under N-sufficient conditions (i.e., during growth on NH_4NO_3) led to the suggestion that *AtNRT1.1* is somehow negatively regulated by the presence of active NR [65], although other possibilities such as an effect of the tissue NO_3^- concentration should perhaps also be considered.

In view of its apparent insensitivity to feedback repression, it becomes more difficult to explain why the abundance of *AtNRT1.1* mRNA reaches a peak a few hours after NO_3^- induction and then declines, with very similar kinetics to those of *AtNRT2.1* [16].

One consequence of the differential sensitivity of *AtNRT1.1* and *AtNRT2.1* to feedback repression may be that when plants are grown at high NO_3^- concentrations (the conditions where the LATS is likely to be most important), the gene for the low-affinity transporter would be preferentially expressed compared to the gene for the HATS [16].

4.3.3. Diurnal regulation

As is the case with *AtNRT2.1* (see Section 3.3), the expression of *AtNRT1.1* is also strongly diurnally regulated and the changes in the abundance of the transcripts during the light/dark cycle are remarkably similar [65]. Furthermore, as was the case with *AtNRT2.1*, the decline in the abundance of the *AtNRT1.1* transcript in the dark period could be delayed by adding sucrose to the medium. However, while the effects of sucrose on *AtNRT2.1* mRNA abundance were correlated with a similar stimulation of HATS activity, there was no such correlation between the effects on *AtNRT1.1* expression and LATS activity. This may be explained by the existence of another component of the LATS which is regulated in a different way (see Section 4.2).

The finding that *AtNRT1.1* and *AtNRT2.1* are similarly responsive to the sucrose supply, but differentially responsive to feedback regulation by the N supply (see above), indicates that we cannot evoke a common mechanism for the two regulatory phenomena based on effects on the N/C balance [85]. It appears that the regulation of the NO_3^- transporter genes by sucrose operates in a way that is distinct from the regulation by N status.

4.3.4. Spatial pattern of expression

In situ hybridisation studies have shown that close to the root tip *AtNRT1.1* is expressed mainly in the epidermal cell layer, while in the more mature part of the root its expression shifts to the cortex and the endodermis [30]. However, other *NRT1* genes in *Arabidopsis* may well show a different pattern of distribution: the NO_3^--inducible *LeNRT1.2* gene in tomato is root-hair specific, while the constitutively expressed *LeNRT1.1* gene is expressed both in root hairs and in the main part of the root [36].

5. Regulatory genes

There is a shortage of N regulatory mutants in higher plants on which to build an understanding of the genetics of the system. The only clearly defined N regulatory mutant to have been isolated to date is the *mea*115 mutant of *N. plumbaginifolia* mutant which was selected for resistance to methylammonium (a toxic analogue of NH_4^+) and has a defect in a gene that may be involved in the feedback regulation of NO_3^- uptake [86]. Whereas NO_3^- uptake in the wild type was inhibited by methylammonium, NO_3^- uptake in the mutant was less strongly inhibited, and this was correlated with a less pronounced repression of the *NpNRT2* gene at the mRNA level [19]. However, NO_3^- uptake in the mutant was still equally susceptible to inhibition by NH_4^+.

The genetics of N regulation are best worked out in the fungal species *A. nidulans* and *Neurospora crassa* [87]. In *A. nidulans*, NO_3^--inducibility of the 1.8 kb transcript of the *crnA* NO_3^- transporter is under the control of NIRA, the pathway-specific regulatory factor that is also responsible for the induction of the NR and NiR genes, while NH_4^+ repression is under the control of AREA, the global regulatory protein for N metabolite repression [25].

Homologues of AREA involved in global N metabolite repression are also found in *Saccharomyces cerevisiae* (Gln3p) and in *N. crassa* (NIT2), while the *N. crassa* homologue of NIRA is NIT4 [87]. AREA, Gln3p and NIT2 are members of the GATA-binding family of transcription factors, which have in common the possession of one or two zinc fingers that participate in sequence-specific binding to *cis*-acting elements containing a core GATA motif [87]. The pathway-specific regulatory factors (NIRA and NIT4) belong to the large GAL4 family of regulatory proteins that have a single Cys_6/Zn_2 type of binuclear zinc cluster [87]. While the GATA-binding family is phylogenetically widely dispersed, the GAL4 family has so far only been found in fungi. Recently, the *YNA1* gene in the yeast *H. polymorpha*, which encodes a NIRA/NIT4 homologue, has been shown to be required for the NO_3^--inducible transcription of the NO_3^- transporter and the NO_3^- assimilatory genes [88].

The strongest evidence that GATA-binding factors have a role in N-regulated gene expression in plants comes from an analysis of *cis*-acting elements in the promoter of the spinach *NII* gene for NiR [89,90]. These studies identified a region of the *NII* promoter (between −230 and −200) that was required for NO_3^--inducibility [89] and using an in vivo footprinting technique it was shown that a NO_3^--inducible DNA-binding activity recognised a GATA motif located between −230 and −181 [90].

A cDNA for a GATA-binding protein (NTL1) has been cloned from tobacco using degenerate primers and PCR [91], but the role of NTL1 in N regulation has not been established. In an alternative approach to cloning plant homologues of the *areA/gln3/nit-2* genes, an *Arabidopsis* cDNA library was used to try to complement a *gln3gdh1* mutant of *S. cerevisiae* [92]. This led to the identification of two related *Arabidopsis* genes, *RGA1* and *RGA2*, which are members of a multigene family that also includes *SCARECROW*, a gene involved in regulating pattern formation in roots [93]. Since RGA1 and RGA2 are not members of the GATA-binding family it is unclear how they were able to complement the *gln3* mutant and whether they have any N regulatory function in plants. Expression of both genes appears to be constitutive with respect to both the N supply and the plant tissue [92]. A recent attempt to repeat the complementation experiments of Truong and colleagues on a larger scale (screening 800 000 transformants) led to the isolation of a number of different complementing cDNAs, including *RGA1* and *RGA2* and some putative transcription factors, but again no *GLN3* homologues were identified (N. Muttucumaru, G. Leggewie, H. Jones, B.G. Forde, unpublished). In view of a report that a mammalian member of the GATA-binding family (mGATA-1) can substitute for AREA in *A. nidulans* [94], it is perhaps surprising that these complementation experiments have so far failed to uncover a plant *areA/gln3/nit-2* homologue.

A phylogenetic analysis of type IV zinc-finger proteins (to which the GATA-binding proteins belong) has revealed that they fall into two main subfamilies, designated IVa and IVb [95]. The two subfamilies differ in the structure of the zinc-finger DNA-binding domains: type IVa proteins have a $C\text{-}x_2\text{-}C\text{-}x_{17}\text{-}C\text{-}x_2\text{-}C$ motif, while type IVb have $C\text{-}x_2\text{-}C\text{-}x_{18}\text{-}C\text{-}x_2\text{-}C$, and there are other differences in the consensus sequences within these motifs. AREA, Gln3p, NIT2 and mGATA-1 all belong to type IVa, whereas the tobacco NTL1 protein [91] is a type IVb protein. No examples of type IVa proteins have so far been identified in plants, suggesting the possibility that this sub-family does not exist in plants. If so, this might account for the difficulties encountered in cloning *GLN3* homologues from plants by yeast complementation.

A number of genetic loci responsible for N regulation of NO_3^- transporters and the NO_3^- assimilatory pathway have been identified in *C. reinhardtii*. *Nit2* is a positive regulatory gene required for NO_3^- induction of the NO_3^- assimilatory genes [24] and *Nit9*, which is closely linked to *Nit2*, is another positive regulatory locus required for NO_3^- induction of *NRT2.3* and possibly other NO_3^--inducible genes [96]. The *NIT2* gene is reported to encode a protein of 1196 amino acids with no homology to any known transcription factors, but with some of the structural features associated with regulatory proteins (an acidic domain, long glutamine repeats and glutamine-rich regions) [97]. The *C. reinhardtii Nrg1* and *Nrg2* genes are involved in NH_4^+ repression of the NO_3^- assimilatory pathway, but they differ from the fungal *nirA/gln3/areA* genes in that they are pathway-specific [98].

The *C. reinhardtii Nar2* gene, which is contained within the same gene cluster as the *CrNRT2.1,*

CrNRT2.2 and *Nia1* genes, is required for functional expression of both *CrNRT2.1* and *CrNRT2.2* [22]. The precise function of NAR2 is still not known, although there is preliminary evidence that it is not a transcription factor (see [2]). Other possibilities are that it is a second (regulatory) subunit of the membrane transporter itself or that it is a cofactor involved in the processing and/or targeting of the NRT2 proteins to the PM. The related CRNA transporter from *A. nidulans* seems not to require a second gene product for its activity since, as discussed above, it can be functionally expressed on its own in a heterologous expression system.

Thus, although there may be superficial similarities between the algal and fungal N regulatory circuits, there are early indications that there could be fundamental differences between them. This may have implications for our embryonic understanding of N regulation in plants, which might be expected to be more closely allied to the algal model than to the fungal or bacterial ones. Having said that, a recent paper [99] reports the identification of an *Arabidopsis* homologue of the bacterial P_{II} (GlnB) protein that is part of the regulatory pathway controlling the transcription of a number of N assimilatory genes in response to cellular N status [100]. However, the *Arabidopsis* P_{II}-like protein is a chloroplast protein and is most closely related to cyanobacterial P_{II} sequences [99]. Since chloroplasts are thought to have evolved from a symbiotic association with cyanobacteria, it would not be surprising if they had inherited the cyanobacterial N regulatory circuit. If (as seems plausible) N regulation in plants is to some degree compartmentalised within the cell, it then remains possible that separate N regulatory pathways of eukaryotic origin exist in the cytosol.

6. Concluding remarks

The identification of two families of NO_3^- transporter genes in higher plants has proved to be a major step forward in unravelling the complexities of the NO_3^- uptake system. Nevertheless there is still much to learn before we can say that we have a clear idea of all the components that go to make up the cHATS, the iHATS and the LATS, how they function and, importantly, how they are regulated.

A number of key questions remain to be answered. How many different *NRT1* and *NRT2* genes contribute to NO_3^- transport and to what extent do the various members of each gene family encode proteins with different kinetic properties, tissue-specificities and physiological roles? How can we account for the pleiotropic effects of mutations in the *AtNRT1.1* gene on high-affinity NO_3^- uptake and on the transport of K^+ and Cl^- ions? What is the role of the *NAR2* gene product in the biosynthesis of functional NRT2 NO_3^- transporters in *C. reinhardtii*, and do homologues of NAR2 exist in higher plants? What are the key metabolites that are used by the plant to monitor its N status and what is the molecular pathway by which changes in these key metabolites cause feedback repression of the NO_3^- assimilatory pathway? Are the NRT1 and NRT2 transporters also regulated at the post-translational level (possibly through phosphorylation/dephosphorylation mechanisms)? What is the NO_3^- sensor, where in the cell is it located and what is the signal transduction pathway leading to induction of NO_3^--regulated genes? Finding answers to these questions will be an exciting challenge for the future and one which should be greatly assisted by the immense power of molecular genetics being unleashed through the current plant genomics programmes.

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