Nitrate transport in plants: which gene and which control?

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Abstract
Nitrate uptake by root cells is a key step of nitrogen metabolism and has been widely studied at the physiological level and, more recently, at the molecular level. Two classes of genes, NRT1 and NRT2, have been found to be potentially involved in the high and low affinity nitrate transport systems (HATS and LATS, respectively). The complexity of the molecular basis of nitrate uptake has been enhanced by the finding that in many plants both NRT1 and NRT2 classes are represented by multigene families. Furthermore, recent studies demonstrate that the control mechanisms that lead to an active protein at the plasma membrane act on gene transcription, modulating the steady-state levels of mRNA, and on the activation of the protein, possibly by a phosphorylation/dephosphorylation process. This is a review of recent progress in the characterization of the NRT2 nitrate transporters, the composition of this family in Arabidopsis, their possible role in nitrate acquisition, and some aspects of their regulation in plants.

Key words: Mutant, multigene family, nitrate transporter, regulation.

Introduction
For most of the cultivated crops, nitrate (NO₃⁻) is the major source of nitrogen. As the first step of the NO₃⁻ assimilation pathway, NO₃⁻ uptake by root cells is considered to be a key step of N metabolism. Once inside, NO₃⁻ can be redirected out of the root cell, either by extrusion in the external medium or by unloading in the xylem vessel to reach the aerial organs (Forde and Clarkson, 1999). The reduction of NO₃⁻ takes place in roots or in leaves (Faure et al., 2001), depending on the species and on nitrogen conditions. Nitrate is first reduced to nitrite by nitrate reductase (NR). Nitrite is then translocated to the chloroplast where it is reduced into ammonium by nitrite reductase (NiR). The third possible fate for NO₃⁻, in roots as well as in leaves, is its uptake by the vacuole where it participates in the general osmoticum or serves as a reservoir to sustain the growth process when the external nitrogen supply becomes limiting (der Leij et al., 1998). All this traffic requires the transport of nitrate or nitrite through different cellular membranes and thermodynamic calculations clearly show that NO₃⁻ influx is an active process (Glass and Siddiqi, 1995). The identification of the proteins and the genes that are responsible for NO₃⁻ movements within the plant is a prerequisite to a better understanding of the mechanisms that control NO₃⁻ absorption and distribution in the whole plant. This review will focus on the identification of a class of genes possibly involved in these processes and the different regulatory controls that lead to the presence of an active transporter at the membrane.

A number of other reviews, dealing with various aspects of NO₃⁻ transport, have been published recently (Daniel-Vedele et al., 1998; Crawford and Glass, 1998; Forde, 2000; Galvan and Fernandez, 2001; Williams and Miller, 2001; Glass et al., 2001).

Root nitrate uptake: physiological properties and molecular aspects
Among all the fluxes in the whole plant, the passage of NO₃⁻ through the plasma membrane of root cells has been intensively studied. Three systems seem to coexist within
the cell (Forde and Clarkson, 1999), each showing different characteristics with respect to NO$_3^-$ inducibility or the concentration range of nitrate in the external medium (NO$_3^{ext}$) at which they operate. The first system operates at low NO$_3^{ext}$, in the range of 0.2 mM, even if the plants have never been supplied with nitrate. This constitutive high-affinity transport system (cHATS) is completed by another high-affinity system which is inducible by very low NO$_3^{ext}$ (iHATS) (Beyl et al., 1988; Aslam et al., 1992). When the NO$_3^{ext}$ reaches higher values (> 1 mM), a low-affinity transport system takes place (Siddiqi et al., 1990; Glass et al., 1992). This LATS activity is expressed in barley without prior exposure of the plant to NO$_3^-$. However, in some species, the activity of LATS is enhanced by NO$_3^-$ supply (Kronzucker et al., 1995). Thus, in some cases, NO$_3^-$ acts as a regulator for its own uptake, a specific property which is not shared with other ion transport systems such as phosphate or sulphate (Smith et al., 2000). In addition to nitrate, reduced nitrogen sources are involved in the regulation of NO$_3^-$ uptake. Ammonium, for example, has strong inhibitory effects which can be divided into short-term (within minutes of exposure) or longer-term effects. The former control occurs at the plasma membrane by inhibiting the influx rather than stimulating the efflux and is shown to affect the iHATS rather than the cHATS or LATS (Kronzucker et al., 1999). Providing amino acid as the sole nitrogen source for plant growth also exerts a strong inhibition of NO$_3^-$ uptake (Muller and Touraine, 1992). This observation raises the question of whether ammonium or a product of its accumulation is involved in the feedback regulation of HATS.

During the last decade, molecular components have been proposed to be involved in the LATS and iHATS. The picture that is emerging from studies of these putative NO$_3^-$ transporters reveals the existence of two classes of proteins (Forde, 2000). The NRT2 and NRT1 genes encode the NNP (nitrate–nitrite porter) and the PTR (peptide transporter) families, respectively. The NRT1 and NRT2 proteins share the same structural topology, with 12 transmembrane domains, distributed in two sets of six helices connected by a cytosolic loop. Beside their common structure, no primary sequence homology is of six helices connected by a cytosolic loop. Beside their common structure, no primary sequence homology is found between the NRT1 and NRT2 classes.

The Chl1 line (Tsai et al., 1993), affected in the AtNRT1.1 gene from Arabidopsis and the crna clone of Aspergillus nidulans (Unkles et al., 1991) were the first mutants characterized by a defect in NO$_3^-$ uptake mediated, respectively, by the LATS and the HATS systems. The conclusion that NRT1 and NRT2 genes code for proteins respectively involved in LATS and HATS was somewhat hasty. Indeed, recent studies demonstrate that AtNRT1.1, initially proposed to encode a LATS component according to heterologous expression experiments in Xenopus oocytes (Huang et al., 1996; Tsai et al., 1993), is a dual-affinity NO$_3^-$ transporter, involved in multiple phases of NO$_3^-$ uptake (Wang et al., 1999; Liu et al., 1999). NRT1 and NRT2 classes contain at least two members inside the same family and sometimes more, depending on the plant species, and recent results obtained on different NRT2 families will be described.

### The multigene NRT2 families

As stated above, the first member of the NNP/NRT2 class was identified in a lower eukaryote, the A. nidulans fungus. The crna mutant was isolated on the basis of its resistance to chlorate and was further shown to be affected in NO$_3^-$ uptake in the conidiospore and young mycelium stages (Unkles et al., 1991, 1995). Later on, the first gene involved in nitrate/nitrite transport in the alga Chlamydomonas reinhardtii, CrNRT2.1, was identified by complementation of a deletion mutant (Quesada et al., 1994). Amino acid sequence comparison with crna revealed highly conserved regions. This feature was further exploited to derive degenerated oligonucleotides that allow the cloning of most of the NRT2 genes (Table 1) identified so far in higher plants, from barley (Trueman et al., 1996), Nicotiana plumbaginifolia (Quesada et al., 1997), Glycine max (Amarasinghe et al., 1998) and, recently, from tomato (Ono et al., 2000).

This PCR approach followed by Southern studies revealed that in most species, the NRT2 genes are present in the genome in at least two copies (e.g. N. plumbaginifolia (Quesada et al., 1997) and in tomato (Ono et al., 2000)). The size of these multigene families is very high in barley where at least seven NRT2 genes are supposed to be distributed throughout the genome including four genes that have been isolated and sequenced (Trueman et al., 1996; Vidmar et al., 2000a). In the model species Arabidopsis, the NRT2 genes are also organized in a rather complex multigenic family. In fact, shortly after the first AtNRT2.1 gene was isolated on the basis of its differential expression under NO$_3^-$ versus glutamine nutrition (Fillleur and Daniel-Vedele, 1999), a second one, AtNRT2.2 was identified at the genomic and cDNA levels (Zhuo et al., 1999). The systematic sequencing of the whole genome increased to seven the number of the AtNRT2 genes. They are distributed on three chromosomes as represented in Fig. 1. Four among the seven genes are organized in tandem: in tail-to-tail configuration for AtNRT2.1 and AtNRT2.2 on chromosome I and in head-to-tail configuration for AtNRT2.3 and AtNRT2.4 on chromosome V. A fifth gene, AtNRT2.5, is located at the bottom of chromosome I, the sixth gene AtNRT2.6 is on the bottom of the chromosome III and the last gene, AtNRT2.7, is at the top of the chromosome V. When the seven amino acid sequences are compared, the seven corresponding genes can be subdivided in three subgroups (Fig. 2). The first group

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comprises the \textit{AtNRT2.3} and \textit{AtNRT2.6} genes, the second one corresponds to the \textit{AtNRT2.1}, \textit{AtNRT2.2} and \textit{AtNRT2.4} genes and the last group contains the \textit{AtNRT2.5} and \textit{AtNRT2.7} genes (Fig. 2). Taking the amino acid sequence of \textit{AtNRT2.1} as a reference, the \textit{AtNRT2.2}, \textit{AtNRT2.3}, \textit{AtNRT2.4}, \textit{AtNRT2.5}, \textit{AtNRT2.6}, and \textit{AtNRT2.7} proteins exhibit 91\%, 77\%, 88\%, 69\%, 77\%, and 57\% similarity, respectively. When lower eukaryotic or prokaryotic sequences of NNP are used to generate a phylogenetic tree, this last group appears to belong to the same class as genes from \textit{C. reinhardtii} or \textit{E. coli} (M Orsel et al., unpublished results).

The evolution of multigenic families involves multiple mechanisms, from the duplication of an ancestral gene to the functional specification of each protein. Despite the indications that such comparative analysis gives on their specific role, complementary approaches are necessary to elucidate the functions of each member of a multigene family.

\textbf{The function of NRT2 genes}

There seems to be no clear borderline between the HATS/NRT2 and the LATS/NRT1 systems. As \textit{AtNRT1.1} is involved in both systems, one could wonder whether or not certain \textit{NRT2} members might play a role in LATS activities. Furthermore, physiological studies unravelled a constitutive component of the HATS, but the existence of the corresponding locus in \textit{Arabidopsis} is only supported by genetic data (Wang and Crawford, 1996).

\begin{table}
\centering
\begin{tabular}{lll}
\hline
\textbf{Nrt2 gene} & \textbf{Organism} & \textbf{Genomic DNA}\textsuperscript{a} & \textbf{mRNA}\textsuperscript{b} \\
\hline
\textit{HvNRT2.1} & \textit{BCH1} & \textit{Hordeum vulgare} & U34198 \\
\textit{HvNRT2.2} & \textit{BCH2} & \textit{Hordeum vulgare} & U34290 \\
\textit{HvNRT2.3} & \textit{BCH3} & \textit{Hordeum vulgare} & AF091115 \\
\textit{HvNRT2.4} & \textit{BCH4} & \textit{Hordeum vulgare} & AF091116 \\
\textit{AtNRT2.1} & \textit{ACH1} & \textit{Arabidopsis thaliana} & AC026875 \\
\textit{AtNRT2.2} & \textit{ACH2} & \textit{Arabidopsis thaliana} & AC026875 \\
\textit{AtNRT2.3} & & \textit{Arabidopsis thaliana} & AB015472 \\
\textit{AtNRT2.4} & & \textit{Arabidopsis thaliana} & AB015472 \\
\textit{AtNRT2.5} & & \textit{Arabidopsis thaliana} & AC012187 \\
\textit{AtNRT2.6} & & \textit{Arabidopsis thaliana} & AL353992 \\
\textit{AtNRT2.7} & & \textit{Arabidopsis thaliana} & AL163792 \\
\textit{NpNRT2.1} & & \textit{Nicotiana plumbaginifolia} & Y08210 \\
\textit{BnNRT2} & & \textit{Brassica napus} & AJ293028 \\
\textit{OsNRT2} & & \textit{Oryza sativa} & AB008519 \\
\textit{GnNRT2} & & \textit{Glycine max} & AF047718 \\
\textit{TaNRT2.1} & & \textit{Triticum aestivum} & AF332214 \\
\textit{TaNRT2.2} & & \textit{Triticum aestivum} & AF288688 \\
\textit{TaNRT2.3} & & \textit{Triticum aestivum} & AF153602 \\
\textit{LeNRT2.1} & & \textit{Lycopersicon esculentum} & AF092655 \\
\textit{LeNRT2.2} & & \textit{Lycopersicon esculentum} & AF092654 \\
\textit{LjNRT2} & & \textit{Lotus japonicus} & AJ292342 \\
\textit{CrNRT2.1} & \textit{Nar3} & \textit{Chlamydomonas reinhardtii} & Z19584 \\
\textit{CrNRT2.2} & \textit{Nar4} & \textit{Chlamydomonas reinhardtii} & Z25438 \\
\textit{CrNRT2.3} & & \textit{Chlamydomonas reinhardtii} & Z255439 \\
\textit{CnM} & & \textit{Aspergillus nidulans} & A223296 \\
\textit{YNT1} & & \textit{Hansenula polymorpha} & M61125 \\
\textit{NarK} & & \textit{Escherichia coli} & Z69783 \\
\textit{napA} & & \textit{Thermoplasma volcanium} & X15996 \\
\textit{napA} & & \textit{Synechococcus sp. WH7803} & AF299255 \\
\textit{napA} & & \textit{Synechococcus sp. WH8102} & AF299254 \\
\hline
\textsuperscript{a}Refers to the number of the BAC that contains the gene. \\
\textsuperscript{b}Refers to the number of the EST.
\end{tabular}
\caption{Nitrate transporter genes from the NRT2 group}
\end{table}
Fig. 2. Sequence alignment and comparison of *Arabidopsis* NRT2 proteins. The alignment was performed using the pileup program, identities of at least five residues among seven are boxed and shaded, gaps generated in the alignment are indicated by dots.
The localization and identity of the gene have not yet been established and one of the seven AtNRT2 genes might be a good candidate. Moreover, beside the different root-nitrate uptake system, nothing is known about the possible implication of NRT2 genes in long-distance transport of NO$_3^-$ in the whole plant.

The organ specificity of expression of a gene can give some indications of its role and can be easily tested by northern or, particularly in case of multigenic families, by RT-PCR analyses. In higher plants, the NRT2 genes isolated so far are preferentially expressed in roots. In tomato, no LeNRT2 expression is observed in whole shoots or leaves (Ono et al., 2000) while in N. plumbaginifolia, NpNRT2 transcripts are also detected at low levels in leaves, petioles, buds, flowers, and seeds (Quesada et al., 1997). In Arabidopsis, RT-PCR using specific primers for AtNRT2.1 and AtNRT2.2 reveals that the expression of the two genes in young seedlings appears 10 d after sowing, but is not detectable earlier (Zhuo et al., 1999). The same approach has been applied to RNA extracted from different organs of mature plants using specific primers for each of the seven Arabidopsis genes (M Orsel et al., unpublished results). These experiments give a picture in which genes like AtNRT2.1, AtNRT2.2 and AtNRT2.4, although expressed at different levels, show similar patterns of root predominant expression. In contrast, it seems that AtNRT2.7 is preferentially expressed in shoots, suggesting a role in leaf NO$_3^-$ uptake. The cellular localization of NRT2 transcripts has been done only in N. plumbaginifolia. The accumulation of mRNA levels is maximum in epidermal and endoderm cells near the root tip while it remains at detectable level only in epidermal or lateral root primordia when moving upward along the root axis (Krapp et al., 1998). It is noteworthy that for NRT1 genes, cellular localization has been performed only on AtNRT1.1 in Arabidopsis (Huang et al., 1996). One explanation for this lack of data could be the low sensitivity of the technique, together with the difficulty of designing specific probes for each member of the family.

Chlorate resistance screens allowed the isolation of a NO$_3^-$ transport defective mutant in A. nidulans followed by the identification by complementation of Crna as the first gene coding for a high-affinity nitrate transporter (Unkles et al., 1991). In the alga Chlamydomonas, mutant strains defective in NO$_3^-$ transport and displaying an active NR have been genetically constructed. Complementation of the NO$_3^-$ non-utilizing phenotype is directly achieved by co-transformation with the CrNRT2 and Nar2 genes (Quesada et al., 1994). These experiments lead to the identification of four genes coding for mono- or bispecific nitrate/nitrite high-affinity carriers (Galvan and Fernandez, 2001). Unfortunately, the chlorate resistance screen used on higher plants results only in the isolation of mutants affected in nitrate reductase activity, LATS or HATS activities or a regulatory mutant (Hoff et al., 1994; Lin and Cheng, 1997; Tsay et al., 1993; Wang and Crawford, 1996). iHATS system might not be involved in chlorate uptake in plants (Touraine and Glass, 1997) and this would explain why no NRT2 mutant has been isolated using this screen. In fact, overexpression of a NpNRT2.1 in wild-type N. plumbaginifolia plants (Fraisier et al., 2000) did not increase the sensitivity of transgenic plants to chlorate (V Fraisier, personal communication).

For many transmembrane proteins, heterologous expression is one of the most suitable approaches to elucidate their functions. This could involve the expression in a yeast mutant, which is defective for an analogous transport system (Gazzarrini et al., 1999). In this way, the barley HvNRT2.1 and HvNRT2.2 cDNAs were used to complement a ynt1 NO$_3^-$ uptake mutant of the yeast Hansenula polymorpha. This complementation is only partial because, for unknown reasons, the NO$_3^-$ uptake activity of the transformants is very low compared to the wild type (Forde, 2000). The injection into insect cells or Xenopus oocytes followed by electrophysiological measurements has been successfully used to establish the kinetic properties of different carriers, including NRT1 proteins (Huang et al., 1996; Zhou et al., 1998). However, positive results for NRT2 transporters were achieved only with the Crna or CrNRT2 genes from A. nidulans and C. reinhardtii. In the latter case, the co-injection in oocytes of CrNRT2.1 and Nar2, a gene belonging to the same nitrate-regulated gene cluster, is required to obtain detectable electrophysiological signals and to produce a functional HATS system (Zhou et al., 2000). There are already several examples of transport systems that require two genes for functional activity. A Nar2 homologous gene might exist in plants that would be required for successful heterologous expression and essential for transport activity in plants.

Reverse genetics, as part of the toolbox for functional genomics by abolishing gene function (i.e. by knockout), was recently the matter of intensive research (Bouchez and Hofte, 1998). Because of many illegitimate recombination events, the control of homologous recombination in plants has proven to be very difficult, although some success has been reported (Kempin et al., 1997). At a large scale, homologous recombination in plants is only available in the moss Physcomitrella patens (Schaefer, 2001), so that gene-disruption methods were designed and many large populations mutagenized with an insertion element (transposons or T-DNA) have been derived from Arabidopsis (Bouchez and Bouchez, 2001). Using an oligonucleotide located in the 3’ region of the AtNRT2.1 in combination with a T-DNA primer, one single positive plant was identified among 30 000 lines independently transformed by Agrobacterium tumefaciens (Filleur et al., 2001). This mutant is a null mutant both
for \textit{AtNRT2.1} and \textit{AtNRT2.2} genes, because a large deletion of 25 kb occurs, leading to the entire loss of the \textit{AtNRT2.1} gene and of a part of the \textit{AtNRT2.2} gene. In this \textit{Atnrt2-A} mutant, the \textit{AtNRT2.1} and \textit{AtNRT2.2} mRNAs are thus no longer detectable whatever the \([\text{NO}_3^-]_{\text{ext}}\) or the nutritional status of the plant. As a consequence, the activity of HATS is strongly reduced in the mutant as compared to the wild type, with a calculated \(V_{\text{max}}\) in the mutant representing only 27\% of that of the wild type. By contrast, once this influx corresponding to HATS activity was subtracted from total \(\text{NO}_3^-\) influx measurements, no significant difference was found between the two genotypes. This result demonstrates that LATS activities are not disturbed in the mutant and that both \textit{AtNRT2.1} and \textit{AtNRT2.2} genes are probably not involved in the LATS system, at least under these experimental conditions. Such a mutant constitutes a valuable tool to assign a precise function to plant \textit{NRT2} genes in the global \(\text{NO}_3^-\) transport process. A cDNA coding for \textit{N. plumbaginifolia} \textit{NpNRT2.1} protein, driven by the 35S or the more root-specific \(\text{RolD}\) promoters, was introduced by transformation in this \textit{Atnrt2-A} mutant. Root influxes were measured in three independent transformants showing different levels of the transgene expression. Wild-type influx is nearly fully restored in the genotype exhibiting the highest level of \textit{NpNRT2.1} expression, but decreases and is partially restored in other genotypes following the steady-state levels of \textit{NpNRT2.1} mRNA (Filleur \textit{et al}., 2001). These results do not, however, demonstrate that the NRT2 protein is directly involved in the transport process. It could, for example, activate another unknown gene or participate to the stability/activity of the transporter itself in association with another protein, as is the case for CrNRT2/NAR2 proteins. Nonetheless, this approach brings out a strong correlation between the inactivation of certain genes and the defect of one component of the HATS or LATS systems.

### Regulation of NRT2 genes

The effect of external factors such as \([\text{NO}_3^-]_{\text{ext}}\) on \(\text{N}\) absorption led to the identification of two physiological processes, iHATS and cHATS (Behl \textit{et al}., 1988). The use of inhibitors of protein synthesis demonstrate that independent components set up these two systems and that iHATS components were not derived from some cHATS element expressed at the basal level prior to induction by \(\text{NO}_3^-\) (Lee and Drew, 1986). There is overwhelming evidence that \(\text{NO}_3^-\) uptake by plant roots depends not only on external factors but also on the \(\text{N}\) status of the whole plant (Glass and Siddiqi, 1995). The rate of \(\text{N}\) acquisition is controlled by these internal factors so that it matches tightly to \(\text{N}\) demand during plant growth (Ismande and Touraine, 1994). The key questions that have to be addressed for a better understanding of this complex regulation is, first, what are the metabolic signals reflecting the environmental conditions and internal status of the plant and, second, do they act directly on the genes involved in \(\text{N}\) absorption.

The expression of the NRT2 genes identified so far have been demonstrated to be \(\text{NO}_3^-\) responsive, showing a rapid accumulation of mRNA following \(\text{NO}_3^-\) induction. The NRT2 genes in \textit{N. plumbaginifolia} and \textit{Arabidopsis} were shown to be induced by very low levels of \(\text{NO}_3^-\) (10–50 \(\mu\)M) (Krapp \textit{et al}., 1998; Filleur and Daniel-Vedele, 1999) to a transient maximum, while in barley, NRT2 mRNAs accumulated to the highest levels when the \([\text{NO}_3^-]_{\text{ext}}\) is maintained at 50 \(\mu\)M under quasi steady-state conditions (Vidmar \textit{et al}., 2000b). The role of the internal \(\text{NO}_3^-\) pool on \(\text{NO}_3^-\) influx has been proposed in \textit{Lemma} by Ingemarsson (Ingemarsson \textit{et al}., 1987). They found a reverse correlation between the increase of internal \(\text{NO}_3^-\), following tungstate treatment, and the decrease of \(\text{NO}_3^-\) influx. By contrast, the increase of the \(\text{HvNRT2}\) transcript level in response to tungstate treatment is not consistent with a possible repressive effect of a high \(\text{NO}_3^-\) internal pool. Moreover, this result in barley is in perfect agreement with the overexpression of \textit{NpNRT2} genes in \textit{N. plumbaginifolia} \textit{NR}-deficient mutants although internal \(\text{NO}_3^-\) pools are high in these plants. The fact that \textit{NRT2} genes are \(\text{NO}_3^-\) inducible in various plants does not imply that they are the components of the iHATS. When non-induced wild type and \textit{Atnrt2-A} mutants, however, are \(\text{NO}_3^-\) supplied, the wild-type plants show a marked induction of \textit{AtNRT2.1} expression and HATS-mediated influx while the mutant exhibits only a very limited response (Cerezo \textit{et al}., 2001). These data provide the first functional evidence that \textit{AtNRT2.1} and/or \textit{AtNRT2.2} (that is also deleted in the mutant) are strictly required for the activity of the iHATS. Thus, it seems that the control of the \(\text{NO}_3^-\) inducibility of iHATS occurs at the transcriptional levels ruling out the possibility of an \(\text{NO}_3^-\) activation of a pre-existing NRT2 protein.

With the exception of \textit{NR}-deficient mutants (Fraiser \textit{et al}., 2001), the induction by \(\text{NO}_3^-\) peaks approximately 1 h after the addition of \(\text{NO}_3^-\) and thereafter \textit{NRT2} mRNA steady-state levels decrease (Krapp \textit{et al}., 1998; Filleur and Daniel-Vedele, 1999; Zhuo \textit{et al}., 1999). \(\text{NO}_3^-\) influx shows the same transient induction followed by a down-regulation when plants are starved of nitrogen (Lejay \textit{et al}., 1999). These regulations are thought to be due to the repression of \(\text{NO}_3^-\) transport by reduced \(\text{N}\) metabolites accumulating in root tissues when plants are under non-limiting nitrogen nutrition. In contrast to the involvement of \(\text{NO}_3^-\) in its own uptake, the identification of the signal(s) responsible for feedback regulation of iHATS has been the matter of debate. Root
internal NO₃⁻, amino acid or ammonium concentrations, have all been proposed as candidates (King et al., 1993; Muller and Touraine, 1992; Siddiqi et al., 1989). The use of NO₃⁻ accumulating mutants (see above) has ruled out the first putative factor. Regarding ammonium or amino acids effects, they both have been demonstrated to inhibit NO₃⁻ influx (Kronzucker et al., 1999; Muller and Touraine, 1992) without any agreement on which amino acid is most active. NpNRT2 transcript abundance declines when glutamine is fed to roots tissues (Krapp et al., 1998) while in Arabidopsis, arginine is more effective than asparagine or glutamine, reducing AtNRT2.1 expression levels to 18, 38 and 77%, respectively (Zhuo et al., 1999). In barley, both NO₃⁻ influx and HvNRT2 mRNAs levels decrease in root tissues in response to glutamate, asparagine, arginine, and glutamine, this latter amino acid being the least effective in both processes (Vidmar et al., 2000b). Furthermore, treatment of root tissue with aza-serine, which inhibits glutamate synthase and led to the accumulation of glutamine within the cell, decreases the HvNRT2 transcript levels by 97% (Vidmar et al., 2000b). This treatment, however, also affects other parameters such as root ammonium content that increases by 130% and it is well known that exogenous ammonium supply dramatically decreases the NO₃⁻ influx as well as the level of NRT2 expression in various species (Krapp et al., 1998; Lejay et al., 1999; Amarasinghe et al., 1998). In the Atnrt2-A mutant, ammonium influx and regulatory mechanisms responsible for ammonium repression are still operating. Despite this, the NO₃⁻ influx is no longer repressed by exogenous ammonium supply in the mutant, suggesting that the HATS component under feedback regulation by reduced nitrogen metabolites is the iHATS (Cerezo et al., 2001).

In some cases, however, this correlation between NO₃⁻ influx and NRT2 mRNA abundance was not so clear. Thus, NRT2 transcript levels were found to be high in Glycine max plants continuously fed with high concentrations of NO₃⁻ although HATS-mediated NO₃⁻ influx appears to be low (Amarasinghe et al., 1998). In the same way, when N-fed plants are treated with tungstate, an inhibitor of nitrate reductase, root HvNRT2 transcript levels increase by 20–30% although NO₃⁻ influx is reduced by 44% (Vidmar et al., 2000b). Evidence for post-transcriptional regulation by a reduced nitrogen source came from physiological studies of transgenic N. plumbaginifolia plants. Indeed, despite a high level of NpNRT2.1 mRNA, driven by the 35S or RdID promoters, exogenous ammonium addition still exerts its repressive effects on root NO₃⁻ influx in transgenic plants (Fraisier et al., 2000). Whether this control occurs at the level of NRT2 protein synthesis (translational control) or in NRT2 protein activity (post-translational control) remains to be established. However, a possible phosphorylation/dephosphorylation mechanism has been proposed, taking into account the rapid and reversible inhibition by ammonium of NO₃⁻ influx and the presence of a S/T-X-R/K motif, a target sequence for protein kinase C, located in the hydrophilic C-terminal domain of all NRT2 sequences available so far (Forde, 2000). Moreover, evidence of post-translational changes that alter the electrophoretic mobility of HvNRT2 proteins on SDS-PAGE have been found and these are related to changes in N nutrition (M Hansen, BG Forde, personal communication). The complementation of Atnrt2-A mutant with the wild type or modified HvNRT2 protein will help to evaluate the role of such post-transcriptional modifications on iHATS-mediated NO₃⁻ influx.

Concluding remarks

Plants have gathered together in a single organism many different NO₃⁻ transport systems that allow them to cope rapidly and easily with the large variations of N availability and to adjust N uptake to growth demand. The complexity of these systems is based on the multiplicity of the structural genes involved and on the different levels of controls that lead from the gene to the presence of an active protein at the membranes (Fig. 3). The cloning of NRT1 and NRT2 genes has made it possible to address some of the questions that were raised by physiological studies. However, a better understanding of the complex network of these transporters requires answers to more questions: what is the role of each gene? How are they coordinately regulated? What are the signalling molecules and how are they sensed? What are the regulatory genes that are involved in the transduction pathway?

The use of a knockout mutant in Arabidopsis or over-expressing plants should help to formulate the beginning of an answer in the near future.

![Regulation of NO₃⁻ transport](image)

**Fig. 3.** Scheme for regulation by N metabolites of NO₃⁻ transport mediated by NRT2 proteins.
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