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An *Arabidopsis* T-DNA mutant affected in *Nrt2* genes is impaired in nitrate uptake

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Abstract Expression analyses of *Nrt2* plant genes have shown a strict correlation with root nitrate influx mediated by the high-affinity transport system (HATS). The precise assignment of NRT2 protein function has not yet been possible due to the absence of heterologous expression studies as well as loss of function mutants in higher plants. Using a reverse genetic approach, we isolated an *Arabidopsis thaliana* knock-out mutant where the T-DNA insertion led to the complete deletion of the *AtNrt2.1* gene together with the deletion of the 3′ region of the *AtNrt2.2* gene. This mutant is impaired in the HATS, without being modified in the low-affinity system. Moreover, the de-regulated expression of a *Nicotiana plumbaginifolia Nrt2* gene restored the mutant nitrate influx to that of the wild-type. These results demonstrate that plant NRT2 proteins do have a role in HATS.

Key words: Nitrate transport; T-DNA mutant; Complementation; *Arabidopsis*

1. Introduction

Nitrate is one of the major sources of nitrogen for higher plants. It is taken up from the soil by active transporters in the plasma membranes of root cells. Measurements of NO_3^- influx kinetics have shown that the roots possess at least three NO_3^- transport systems (see for review [1,2]): (i) a low-affinity transport system (LATS) which is active at external NO_3^- concentrations >0.5 mM and displays a linear kinetics; (ii) a constitutive high-affinity transport system (cHATS) with K_m values of 6–20 μM and V_{max} values of 0.3–0.82 μmol h^{-1} g^{-1} root fresh weight and (iii) an inducible high-affinity transport system (iHATS), which is activated within the first hours of exposition to NO_3^- (K_m and V_{max} values of 13–79 μM and 3–8 μmol h^{-1} g^{-1} root fresh weight, respectively). In recent years, many genes or cDNAs involved in these NO_3^- transport systems have been isolated (see for reviews [3,4]). On the basis of their deduced amino acid sequences, the corresponding proteins have been categorised into two distinct families, NRT1 and NRT2.

The primary source of information about the function of the NRT1 and NRT2 proteins came from mutant phenotype analyses from, respectively, *Aspergillus nidulans* and *Arabidopsis thaliana* [5,6]. The latter mutant was first shown to be specifically impaired in the LATS function, a result which was further supported by heterologous expression studies of the corresponding gene, *AtNrt1.1*, in *Xenopus* oocytes [7,8]. Subsequently, two different studies have demonstrated that the ATNRT1.1 protein is a dual-affinity nitrate transporter, involved in multiple phases of nitrate uptake [9,10]. Most of the functional characteristics of the NRT2 family have been obtained for the algae *Chlamydomonas reinhardtii*. Using functional complementation of various mutant strains, it has been shown that at least three NO_3^- and/or NO_2^- uptake systems, specified by the *CrNrt2.1*, *CrNrt2.2* and *CrNrt2.3* genes, are active with distinctive kinetic and regulatory properties [11,12]. Phenotype analyses of complemented mutants revealed that nitrate uptake activity was restored only when either one of the *Nrt2* gene and the *Nar-2* gene, located in the same cluster, were present [13]. Until recently, the *CrnA* transporter gene from *A. nidulans* was the only member of the NRT2 family that gave an electrophysiological response to nitrate after injection in *Xenopus* oocytes. This year, in agreement with the genetic evidence, recent heterologous expression analyses in oocytes shows that the *CrNrt2.1* and *nar-2* gene products are required for functional nitrate uptake and that this process does not involve a transcriptional regulation mediated by the NAR-2 protein [14].

At least four *Nrt2* genes are present in the genome of *Arabidopsis*: *AtNrt2.1* and *AtNrt2.2* are located in opposite orientations on chromosome I [15,16] while *AtNrt2.3* and *AtNrt2.4* are in a tail to tail orientation on chromosome V [4]. Due to the similarity between the AtNRT2 proteins and the fungal and algal components of the iHATS system [3], these genes are thought to encode functionally similar elements in higher plants. Various evidence reinforces this hypothesis, at least for the *AtNrt2.1* gene. Its expression has been found to take place mainly in roots, up-regulated by NO_3^- and down-regulated by reduced forms of nitrogen such as NH_4^+ or glutamine [15]. Zhuo et al. have reported substantially lower root levels of *AtNrt2.2* transcript when compared to *AtNrt2.1* [16]. Recent experiments with *A. thaliana* and transgenic *Nicotiana plumbaginifolia* plants have shown a strict correlation between *Nrt2* mRNA levels and nitrate uptake mediated by iHATS activity [17–19]. However, nothing is known about the regulation of the expression of the

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two others *AtNrt2* genes and their functional significance is presently unknown.

In order to give additional evidence of NRT2 function in iHATS and indication of the individual role of the *Nrt2* genes, we describe in this paper the isolation of an *Arabidopsis* T-DNA mutant affected in the two genes *AtNrt2.1* and *AtNrt2.2*. A comparative study of NO_3^- influx in mutant plants with that of the wild-type or mutants complemented with a *N. plumbaginifolia Nrt2* gene has been performed.

2. Materials and methods

2.1. Plant material and growth conditions

Seed stocks of *Arabidopsis thaliana* (L.) Heynh from Wasselewskija ecotype were used for all experiments.

For expression studies, the plants were grown on sand at 25°C and 70% relative humidity for 40 days under 8 h light/16 h dark. Cultures were supplied with a complete solution described by Lejay et al. [19] containing 0.2, 1, 5 or 10 mM NO_3^-.

For hydroponic cultures, the wild-type and transgenic plants were grown under non-sterile conditions on mineral nutrient solutions as described previously [19]. 1 week after germination on tap water, the plants were supplied with a nutrient solution containing 1 mM NH_4NO_3, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 0.25 mM $CaCl_2$, 0.25 mM K_2SO_4, 0.1 mM FeNa–EDTA, 50 μM KCl, 30 μM H_3BO_3, 5 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$ and 0.1 μM $(NH_4)_6Mo_7O_{24}$. At the age of 5 weeks, and 7 days before the experiments, the plants were transferred to a nutrient solution where N was supplied as 0.5 mM KNO_3 and 0.25 mM $Ca(NO_3)_2$ instead of 1 mM NH_4NO_3. To keep K^+ and Ca^{2+} concentrations constant as compared to the nutrient solution used for growth, $CaCl_2$ and K_2SO_4 were omitted in the NO_3^- solution.

2.2. Isolation of a T-DNA plant disrupted in the Atnrt2 genes

The primary polymerase chain reaction (PCR) screen was performed on pooled chromosomal DNA from ~30 000 individual lines of a collection of *Arabidopsis* T-DNA insertion mutants (constructed by Laboratoire de Génétique et Amélioration des Plantes, INRA, Versailles, France). The oligonucleotide primers used corresponded to the right border of the T-DNA (Tag 11: 5′-ATCGAAACGCAGCACGATACGCTGG-3′) and to both *AtNrt2.1* and *AtNrt2.2* genes (Nrt3: 5′-CACCATAGCCACAACGGCAGTTACAAGGG-3′). Each PCR reaction contained in 25 μl: 25 ng DNA, 25 pmol of each primer, 1 U Taq polymerase (Gibco BRL, Life Technologies), 2.5 μl 10× PCR buffer and 0.2 mM dNTPs. The following PCR program was used: 94°C for 2 min, 10 cycles of 94°C for 15 s, 65°C for 30 s (−1°C/cycle) and 72°C for 2 min, 35 cycles of 94°C for 15 s, 55°C for 15 s and 72°C for 1 min. The PCR products were separated on 1% agarose gels. After the migration, the gel were immersed in 0.4 N NaOH. DNA was transferred onto nylon membranes (Hybond N^+ Amersham) on each side of gel. Blots were hybridised with radiolabelled *AtNrt2.1* cDNA probe or T-DNA right and left borders and washed at high stringency [20]. One positive line was detected and the position of the T-DNA within the *AtNrt2* genes was determined by sequencing the PCR amplified fragments.

2.3. Complementation of the atnrt2 mutant

In this experiment two constructs were used, containing the *NpNrt2.1* gene under the control of the 35*S* promoter of the *CaMV* or the *rolD* promoter of *Agrobacterium rhizogenes* [17]. Binary vectors were introduced into *Agrobacterium tumefaciens* strain C58C1 (pMP90). The *atnrt2-A* mutant was transformed by the in planta method using the surfactant Silwet L-77 [21] and transformants were selected on 20 μg/ml hygromycin B.

2.4. Northern blot analysis

Total RNAs were phenol-extracted from 0.5 g of root tissues as described by Filleur and Daniel-Vedele [15]. The RNAs (8 μg) were fractionated on 1.2% agarose gels containing formaldehyde and transferred to Genescreen membrane following the manufacturer's instructions (DuPont). Blots were hybridised with a radiolabelled *AtNrt2.1* cDNA [15] or *NpNrt2.1* cDNA [17] probe and washed at high stringency (0.2× standard sodium citrate at 65°C). Equal RNA loading was verified by ethidium bromide-stained rRNA bands.

2.5. $^{15}NO_3^-$ influx studies

Measurements of $^{15}NO_3^-$ influx in the roots were performed as described by Delhon et al. [22]. The plants were transferred first to 0.1 mM $CaSO_4$ for 1 min, then to complete nutrient solution containing $^{15}NO_3^-$ (99 at% ^{15}N) for 5 min, and finally to 0.1 mM $CaSO_4$ for 1 min. The $^{15}NO_3^-$ concentration in the labelling solution was between 0.005 and 20 mM for the determination of the influx kinetics, and 0.2 mM for the experiment with the mutant plants complemented with *NpNrt2.1*. Roots were separated from shoots immediately after the final transfer to $CaSO_4$, and dried at 70°C for 48 h. Influx of $^{15}NO_3^-$ was calculated from the ^{15}N content of the roots, analysed mass spectrometrically, using the ANC-MS system (PDZ Europa, Crewe, UK).

3. Results and discussion

3.1. Isolation of an AtNrt2 T-DNA insertion mutant

A reverse genetic approach was used to isolate a T-DNA mutant [23] affected in *AtNrt2.1* or *AtNrt2.2* genes from an *Arabidopsis* library of 30 000 lines independently transformed by the *A. tumefaciens* strain MP5–1 [24,25]. An oligonucleotide primer, Nrt3, located in the 3′ region of *AtNrt2* genes,

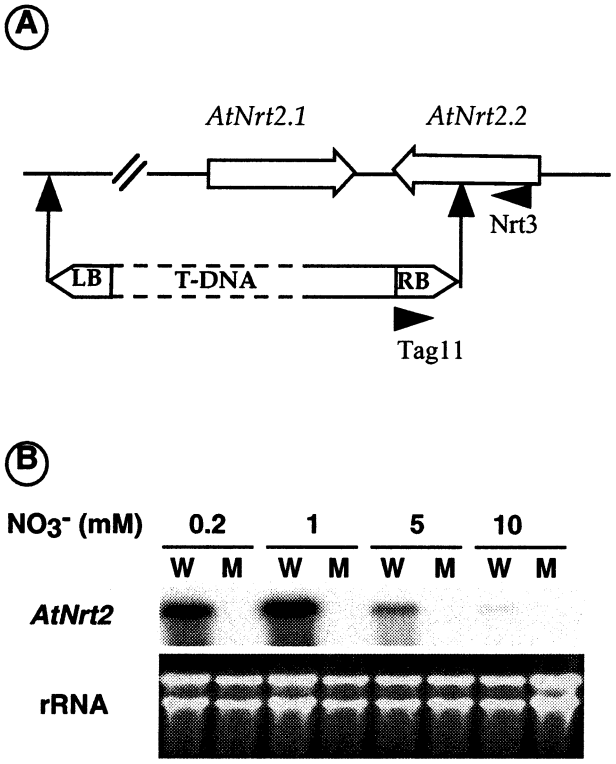

Fig. 1. Characterisation of a *Nrt2* mutant. The collection of T-DNA insertion mutants was screened by PCR, using the NRT3 and Tag11 oligonucleotides, designed, respectively, from the *AtNrt2.1*/*AtNrt2.2* genes and the T-DNA sequences. A: Schematic representation of the Nrt2::T-DNA insertion characterised in the *atnrt2-A* mutant. The structure of the *Nrt2* genes and T-DNA insertion were deduced from PCR and Southern analyses. B: Expression analyses of *Nrt2* genes: total RNAs were isolated from plants grown under various nutritive conditions ranging from 0.2 to 10 mM nitrate concentrations as indicated at the top of the figure. RNA were extracted from roots of wild-type (W) and mutant (M) plants and hybridised with the entire *AtNrt2.1* cDNA as probe. The ethidium bromide-stained rRNA bands are shown as sample loading control.

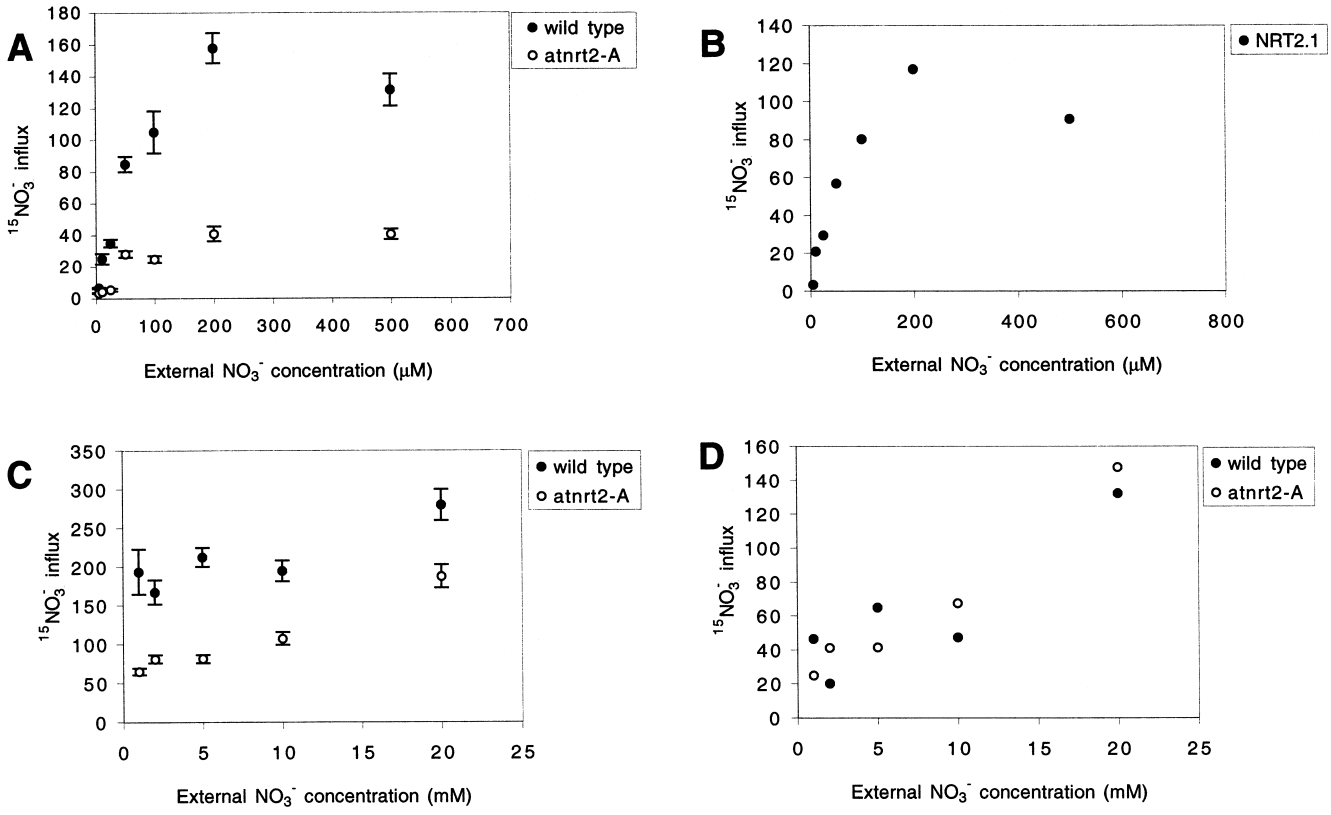

Fig. 2. Kinetic characteristics of nitrate influx in wild-type and mutant plants. Plants were grown in hydroponic conditions in the presence of 1 mM NH_4NO_3 as sole nitrogen source and then transferred to 1 mM NO_3^- for 7 days. Root influx is given in μmol $^{15}NO_3^-$ h^{-1} g^{-1} of root dry wt. Bars indicate standard error ($n=6$). A: HATS activities: root $^{15}NO_3^-$ influx was measured after 5 min labelling with a complete nutrient solution containing between 0.005 and 0.5 mM $^{15}NO_3^-$ to determine the nitrate influx mediated by the HATS system. B: Schematic representation of the influx mediated by the *NRT2* genes. The curve represents the difference of the nitrate influx between wild-type and mutant (A). C: Combined HATS and LATS activities: root $^{15}NO_3^-$ influx was measured after 5 min labelling with a complete nutrient solution containing between 1 and 20 mM $^{15}NO_3^-$ to determine the nitrate influx mediated by the HATS and the LATS system. D: LATS activities: the V_{max} of the HATS (A) were subtracted for each genotype.

used in combination with a T-DNA primer, Tag11, allowed us to identify one single positive line. Sequence analysis of the corresponding PCR fragment revealed that the T-DNA was located in the middle of the *AtNrt2.2* gene, 850 bp after the putative ATG initiation codon (Fig. 1A). After self-crossing of this positive line, we then selected a homozygous mutant by PCR, named *atnrt2-A*. The presence of a single T-DNA insertion in *atnrt2-A* was shown by Southern blot analysis of the mutant using either the right and left borders or the entire T-DNA probes (data not shown).

To evaluate the impact of the T-DNA insertion on the expression of the two *AtNrt2* genes, total RNAs were extracted from wild-type and mutant roots of plantlets cultivated with NO_3^- concentrations ranging from 0.2 to 10 mM. Fig. 1B shows the results of Northern blot analyses, using the entire *AtNrt2.1* cDNA that allows the detection of both *AtNrt.2.1* and *AtNrt2.2* mRNAs [15]. In wild-type, the steady-state *AtNrt2* mRNA level is higher on 0.2 and 1 mM NO_3^- than on 5 or 10 mM NO_3^-. The repression of *Nrt2* gene under high-nitrate nutrition has already been reported for *N. plumbaginifolia* [17] and barley [26] and probably occurs because of larger internal nitrogen pools in the 5 and 10 mM NO_3^--treated plants. In the *atnrt2-A* mutant, the *AtNrt2.1* and *AtNrt2.2* mRNAs were not detectable even after longer exposure of the autoradiograph and whatever the plant nutritional conditions. This result was surprising in the case of *AtNrt2.1*. Two hypotheses could explain this absence of signal: the insertion of the T-DNA could have either modified by an unknown mechanism the expression of the *AtNrt2.1* gene or led to a deletion of the gene. Southern blot experiments using the entire *AtNrt2.1* cDNA as a probe showed that indeed, after the T-DNA insertion event, this gene was completely deleted in the *atnrt2-A* mutant, together with the 3′ end of the *AtNrt2.2*. Using a walking PCR approach [27], we isolated the flanking region of the T-DNA left border and found that the deletion was 25 kb long. Although most insertions correspond to simple, unique inserts where both T-DNA region ends are present, more complex cases of T-DNA integration, such as deletions or duplications of T-DNA extremities have also been described [28].

3.2. atnrt2 mutant is altered in root NO_3^- influx

To investigate the role of *AtNrt2* genes in NO_3^- uptake, the kinetics of root $^{15}NO_3^-$ influx were compared between the wild-type and the mutant. After culture on NH_4NO_3 solution, the plants were transferred for 7 days to a solution containing 1 mM NO_3^- as sole N source. This treatment was expected to relieve *AtNrt2.1* expression from repression by exogenous NH_4^+ in the wild-type [16,18]. In both genotypes, the kinetics of root $^{15}NO_3^-$ influx displayed a classical biphasic pattern

[1,4], with a saturable Michaelis–Menten component corresponding to the HATS in the low concentration range (5–500 μM, Fig. 2A), and a linear component due to the additive activities of both HATS and LATS in the high concentration range (1–20 mM, Fig. 2C). The activity of the HATS was strongly reduced in the mutant as compared to the wild-type (Fig. 2A), with a calculated V_{max} in the mutant representing only 27% of that in the wild-type (39.7 as compared to 147 μmol h^{-1} g^{-1} root dry wt, Table 1). However, the apparent K_m was not markedly different between the two genotypes (Table 1), and agrees well with that previously reported for *Arabidopsis* [29]. The difference between root $^{15}NO_3^-$ influx in the wild-type and that in the mutant was taken as an estimate of the transport activities of AtNRT2.1 and/or AtNRT2.2 proteins. The values for this difference also follow a saturable Michaelis–Menten kinetics (Fig. 2B), with calculated V_{max} and K_m at 101 μmol h^{-1} g^{-1} root dry wt and 41 μM, respectively. In the high concentration range, root $^{15}NO_3^-$ influx, due to combined HATS and LATS activities, always remained lower in the mutant than in the wild-type (Fig. 2C). However, once the respective values for V_{max} of the HATS were substracted for each genotype, the resulting $^{15}NO_3^-$ influx, attributed to the LATS only, did not differ between the mutant and wild-type (Fig. 2D). This demonstrates that the LATS for NO_3^- is not affected by the absence of both *AtNrt2.1* and *AtNrt2.2* genes, reflecting that the N-assimilation systems which are known to disturb LATS- and HATS-mediated $^{15}NO_3^-$ influx [19] are not de-regulated in the mutant. Collectively, our data show that a main component of the HATS has been suppressed in the mutant. Classically, the HATS for NO_3^- is believed to be constituted of at least two separate transport systems, a cHATS and a NO_3^--inducible iHATS [1,3,4]. The iHATS is generally reported to be much more active than the cHATS in NO_3^--induced plants [30,31]. Thus, it is tempting to postulate that the large difference in V_{max} of the HATS between the wild-type and the mutant is due to the absence of the iHATS in the latter genotype. This is in agreement with the fact that expression of *AtNrt2.1* is inducible by NO_3^- [15,16,32], and is correlated with the activity of the HATS in NO_3^--induced plants [16,19]. The basal HATS activity should correspond to cHATS and could correspond to the activity of either ATNRT2.3 or ATNRT2.4 proteins. The *chl8* mutant seems to be genetically affected in this cHATS [33], but the molecular basis of this system has not yet been identified.

3.3. HATS activity is restored in atnrt2-A mutant complemented with a N. plumbaginifolia Nrt2 gene

In order to assign more precisely a function to the plant NRT2 family in the nitrate transport process, we attempted to complement the *atnrt2-A* mutant with a cDNA coding for *N. plumbaginifolia* NpNRT2.1 protein, driven by the constitutive 35*S* promoter or the more root-specific *rolD* promoter from *A. rhizogenes*. These constructs were shown to be functional in transgenic *N. plumbaginifolia* plants [17]. The *atnrt2-A* mutant was transformed as already described [24], and transgenic plants were selected on the basis of their resistance to hygromycin. Three homozygous lines which showed the highest expression in roots were studied further: mR2 and mR4 (*rolD* promoter), and mS5 (35*S* promoter). Fig. 3A shows the results of Northern blot analyses performed on roots from the wild-type, the mutant and these three lines, grown continuously on ammonium/nitrate for a 5 weeks period and then transferred for 7 days to 1 mM NO_3^-. Using the *AtNrt2.1* cDNA as probe, a high hybridisation signal was found in wild-type plants while as expected, no signal was detected in the mutant background. *NpNrt2.1* mRNAs are undetectable in the wild-type and mutant plants, but accumulated in mR2-transformed plants, and to a lesser extent in mR4 and mS5, the latter transformant showing the lowest level of expression.

In the same experiments, root influxes of $^{15}NO_3^-$ were measured at 0.2 mM external concentration, where the difference in HATS activity between wild-type and mutant plants was maximum (Fig. 2A). Again, a 2-fold difference was observed between the two genotypes. Interestingly, the wild-type influx was nearly fully restored in the mR2 plants while this level

Table 1
Kinetic parameters of nitrate influx in wild-type and *atnrt2-A* mutant plants

	V_{max} (μmol h^{-1} g^{-1} root dry wt)	K_m (μM)
HATS WS	147	52.2
HATS *atnrt2-A*	39.7	61
NRT2	101	41.4

V_{max} and K_m were calculated for each genotype from $^{15}NO_3^-$ influx measurements. The values corresponding to NRT2 were obtained by difference between wild-type and mutant.

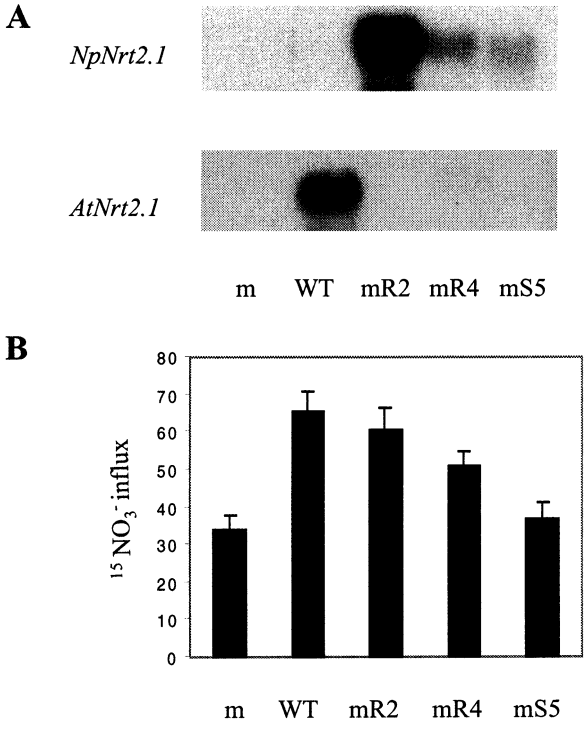

Fig. 3. Characterisation of mutants complemented by *N. plumbaginifolia Nrt2* gene. The *atnrt2-A* mutant (m) was transformed with a *NpNrt2.1* cDNA put under the control of the *rolD* (mR2 and mR4) or the 35*S* (mS5) promoters. A: Expression analyses of the exogenous *NpNrt2.1* and the endogenous *AtNrt2.1* genes: root total RNAs were extracted from plants grown in hydroponic conditions with 1 mM NH_4NO_3 as nitrogen source and then transferred for 7 days on 1 mM NO_3^-. B: Root $^{15}NO_3^-$ influx in wild-type and transgenic lines: root $^{15}NO_3^-$ influx, given in μmol $^{15}NO_3^-$ h^{-1} g^{-1} of root dry wt, was measured after 5 min labelling with a complete nutrient solution containing 0.2 mM $^{15}NO_3^-$; bars indicate standard error ($n=6$).

was lower in mR4 and mS5 plants (Fig. 3B). Statistically, the effect of the genotype on nitrate influx was shown to be highly significant (Fisher value = 12.54).

These results show a good correlation between the expression of the *NpNrt2.1* transgene and the restored root $^{15}NO_3^-$ influx in complemented mutants. Whether or not the NRT2 protein is directly involved in the transport process remains to be established. One could imagine that the expression of *NRT2* genes regulates, by a cascade mechanism, the expression of another unknown gene or that the NRT2 proteins participate in the stability/activity of the transporter itself. To investigate the possibility that, as in Chlamydomonas, another protein may be involved in this process [14], studies of the expression of plant NRT2 in oocytes would be necessary.

Nonetheless, taken together, our results demonstrate that an *Arabidopsis* mutant where the two NRT2 genes located on chromosome I are totally inactivated is impaired in a component of the HATS system. This new mutant constitutes a very valuable tool for improving our knowledge on the complex process of nitrate uptake. For example, it will be of great interest to cross this mutant with *chl1*, affected in LATS and HATS [9,10], or *chl8*, affected in the constitutive component of the HATS [33]. This defect is not compensated by an enhancement of the activities of the other *Arabidopsis Nrt2* genes but we have been able to restore this defect by a deregulated expression of an exogenous *Nrt2* gene. It is thus attractive to postulate that no structural element, possibly involved in the nitrate transport process, is encoded by the open reading frames located in the deleted region in the mutant. Nevertheless, that regulatory elements could exist in this region cannot be excluded. It will be interesting to complement our mutant with genomic *AtNrt2.1* or *AtNrt2.2* sequences and to study the kinetics of nitrate uptake in order to determine which of these two genes code for the nitrate-inducible component of the HATS.

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