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The *Arabidopsis* ATNRT2.7 Nitrate Transporter Controls Nitrate Content in Seeds [W]

Franck Chopin,[a] Mathilde Orsel,[b] Marie-France Dorbe,[a] Fabien Chardon,[a] Hoai-Nam Truong,[a] Anthony J. Miller,[c] Anne Krapp,[a] and Françoise Daniel-Vedele[a,1]

[a] Institut National de la Recherche Agronomique, Institut Jean-Pierre Bourgin, Unité de la Nutrition Azotée des Plantes, F-78000 Versailles, France

[b] Amélioration des Plantes et Biotechnologies Végétales, Unité Mixte de Recherche 118, Institut National de la Recherche Agronomique–AgroCampus Rennes, 35653 Le Rheu Cedex, France

[c] Crop Performance and Improvement Division, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, United Kingdom

In higher plants, nitrate is taken up by root cells where *Arabidopsis thaliana* NITRATE TRANSPORTER2.1 (ATNRT2.1) chiefly acts as the high-affinity nitrate uptake system. Nitrate taken up by the roots can then be translocated from the root to the leaves and the seeds. In this work, the function of the *ATNRT2.7* gene, one of the seven members of the NRT2 family in *Arabidopsis*, was investigated. High expression of the gene was detected in reproductive organs and peaked in dry seeds. β-Glucuronidase or green fluorescent protein reporter gene expression driven by the *ATNRT2.7* promoter confirmed this organ specificity. We assessed the capacity of ATNRT2.7 to transport nitrate in *Xenopus laevis* oocytes or when it is expressed ectopically in mutant plants deficient in nitrate transport. We measured the impact of an *ATNRT2.7* mutation and found no difference from the wild type during vegetative development. By contrast, seed nitrate content was affected by overexpression of *ATNRT2.7* or a mutation in the gene. Finally, we showed that this nitrate transporter protein was localized to the vacuolar membrane. Our results demonstrate that ATNRT2.7 plays a specific role in nitrate accumulation in the seed.

INTRODUCTION

Plant seeds are important for two reasons: one is to support human life as they are a vital component of man's diet, and the other is to ensure the successful propagation of the species. For the latter function, seeds must contain all the genetic material, macronutrients, and micronutrients needed to allow efficient germination and seedling establishment, even if the external environment is hostile. Seed composition and subsequent germination depend on the environmental conditions under which the seeds are produced and are controlled by developmental and hormonal signals. In addition, metabolic control can influence embryo development and seedling establishment. For example, disruption of the *ACC1* gene, which codes for acetyl-CoA carboxylase, was lethal for the embryo (Baud et al., 2003), and mutants affected in key components of the glyoxylate cycle have demonstrated the importance of fatty acid metabolism for seedling establishment under nonfavorable conditions (Eastmond et al., 2000). Much research has been focused on the metabolism of major seed compounds, such as starch in cereals (James et al., 2003) and carbon and lipid in noncereals (Baud et al., 2002; Hills, 2004). Recently, a global metabolomic analysis was performed in the model species *Arabidopsis thaliana* (Fait et al., 2006), which, together with global transcriptomic analyses in the same species (Ruuska et al., 2002; Nakabayashi et al., 2005), gives an integrated view of seed development from early embryo morphogenesis through maturation to the early steps of germination. Surprisingly, there have been far fewer investigations of seed mineral accumulation. Seeds store minerals in the form of phytate and its cations, mostly Mg^{2+}, K^{+}, and Ca^{2+} (Lott et al., 1995), in two types of vacuoles and in the endoplasmic reticulum (Otegui et al., 2002). The seed iron is stored either as plastid ferritin in legume seeds (Lobreaux and Briat, 1991) or in globoids in the protein storage vacuoles of *Arabidopsis* (Lanquar et al., 2005). By contrast, nothing is known about the localization and the molecular basis of nitrate storage in seeds. N from nitrate represents less than one thousandth of seed nitrogen (our unpublished data), but endogenous nitrate levels can vary enormously even among seed batches from one species (Derckx and Karssen, 1993). In *Sisymbrium officinale*, seed nitrate was shown to be a necessary and limiting factor for light-induced germination in water (Hilhorst, 1990) and to be present both in seed coats (including the endosperm) and in the embryo, particularly in the axes and the radicle. The important role of nitrate alone or in combination with other factors, such as light, gibberellins, or ethylene, in breaking dormancy has also been shown in different species (Saini et al., 1985; Hilhorst and Karssen, 1988). It was recently demonstrated that the effect of nitrate in breaking dormancy in *Arabidopsis* is not nutritional but rather a signaling effect, whether it is provided exogenously during imbibition or accumulated during seed development (Alboresi et al., 2005). This effect may be of importance in agriculture because seed dormancy is generally an undesirable trait for the establishment of crops. However, a complete lack of dormancy may lead to

[1] To whom correspondence should be addressed. E-mail vedele@versailles.inra.fr; fax 33-3083-3096.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Françoise Daniel-Vedele (vedele@versailles.inra.fr).

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germination of grains while still on the ear of the parent plant (preharvest sprouting), resulting in major losses to agriculture.

During plant vegetative growth, nitrate is taken up from soil solution by active transport across the plasma membrane of root cells. As a nutrient, nitrate can be reduced inside the cell by the nitrate reductase into nitrite, which is then further reduced into ammonium by nitrite reductase (Meyer and Stitt, 2001). To cope with low or high nitrate concentrations in soils, two uptake systems exist within plant roots: high-affinity nitrate transport systems (HATS) and low-affinity nitrate transport systems (LATS) (Glass and Siddiqi, 1995). During recent years, several genes involved in LATS and HATS have been isolated. On the basis of their deduced amino acid sequence, the corresponding proteins have been classified in two families: NITRATE TRANSPORTER1 (NRT1) and NRT2. The *Arabidopsis* genome contains 53 NRT1 and seven NRT2 family members (Arabidopsis Genome Initiative, 2000; Orsel et al., 2002a, 2002b).

The first gene isolated, *ATNRT1.1*, has been shown to be a dual-affinity transporter involved in both low- and high-affinity nitrate uptake (Liu et al., 1999), the molecular control of this switch being a protein phosphorylation/dephosphorylation mechanism (Liu and Tsay, 2003). Until now, the best characterized member of ATNRT2 family is the *ATNRT2.1* gene, which was isolated either by a differential display approach (Filleur and Daniel-Vedele, 1999) or using degenerate primers (Zhuo et al., 1999). Analysis of the mutant *atnrt2.1-1*, disrupted in the *ATNRT2.1* and *ATNRT2.2* genes, showed that this plant is specifically deficient in the HATS but not the LATS (Cerezo et al., 2001; Filleur et al., 2001). Recent studies showed that the ATNRT2.1 protein requires a second gene, *ATNAR2.1*, for function (Okamoto et al., 2006) and possibly forms a complex with the NAR2 protein to transport nitrate efficiently (Orsel et al., 2006). Based on the sequence homology between to the *ATNRT2.1* coding sequence, six other genes with various degrees of homology were identified (Orsel et al., 2002a). Except for the *ATNRT2.2* gene (Li et al., 2007), the function of the other *NRT2* genes has not been elucidated. Regarding the nitrate regulation of expression, some of the genes were found to be inducible (*ATNRT2.1* and *ATNRT2.2*), while another was repressible (*ATNRT2.5*) (Okamoto et al., 2003). By contrast, the expression of the *ATNRT2.3*, *ATNRT2.6*, and *ATNRT2.7* genes seemed to be insensitive to changes in nitrate supply. The *ATNRT2* genes chiefly show expression in the roots, with the exception of *ATNRT2.7*, whose expression is higher in shoots than in roots (Orsel et al., 2002a; Okamoto et al., 2003). In this study, we focus on the *ATNRT2.7* gene, and using several different approaches, we demonstrate its importance for the efficient storage of nitrate in seed vacuoles that can then influence the kinetics of seed germination.

RESULTS

Expression of ATNRT2.7 in Different Organs

The AtGenExpress initiative, supported by different countries (Germany, Japan, the United States, and the UK), compiles data from >1300 microarrays and can be used as a tool to follow the expression of your favorite gene. When we analyzed these in silico data, *ATNRT2.7* appeared to be highly expressed in seeds. The expression of *ATNRT2.7* increases by a factor of 6.5 between the 7th and 8th stages and a factor of 15 between the 7th and 10th stages of seed development (https://genevestigator-1.ethz.ch/). We thus decided to validate these results by real-time PCR analysis alongside all the other members of the *NRT2* family.

Two different stages of seed development were tested together with root and shoot extracts to compare with our previous results (Orsel et al., 2002a). Results of both experiments are summarized in Table 1 for the *ATNRT2.1* and *ATNRT2.7* genes. As found previously, the *ATNRT2.1* gene is predominantly expressed in roots as compared with shoots, while the *ATNRT2.7* gene is more expressed in shoots. However, the most striking point is the huge amount of *ATNRT2.7* mRNA in seeds (nearly 200% of the EF1α control gene), especially at the end of seed maturation when the tissue is completely dry. Interestingly, the expression decreases after 6 h of imbibition. With the exception of *ATNRT2.5*, which reaches only 6% of the EF1α level in dry seeds, we could not detect significant expression of any other NRT2 family members in this organ in two independent experiments (data not shown).

To further characterize the expression of *ATNRT2.7* in seeds, we have used a fusion between a 2-kb fragment of the *ATNRT2.7* promoter and either the β-glucuronidase (GUS) or green fluorescent protein (GFP) reporter gene. These constructs were stably introduced into the *Arabidopsis* Wassilewskija (Ws) ecotype, and Figure 1 shows typical results from GUS staining of seeds harvested from one of these transformants. After 1 h of imbibition to enable better separation of embryos from seed coats, histochemical GUS staining was performed (Figures 1A and 1B). A uniform and strong GUS activity was detected in transgenic embryos, while wild-type embryos remained unstained (Figure 1A). The seed coat is characterized by a faint blue staining, which reveals a weak expression of *ATNRT2.7* in the surrounding endosperm (Figure 1B). Homozygous lines were also obtained carrying the GFP reporter gene under the control of the same 2-kb fragment of the *ATNRT2.7* promoter. In these lines, embryos were also dissected after 1 h of water imbibition and observed using confocal microscopy (Figure 1C). Membranes were stained with FM4-64 (red dye; Figure 1D), making the embryonic cells clearly visible. Strong green fluorescence was observed in all tissues, including the cotyledons and radicles (Figure 1E). Altogether, these data suggest strongly that the

Table 1. Gene Expression Level in Different Organs

	Dry Seed	Imbibed Seed	Root	Leaf
ATNRT2.1	ND	ND	62.1 ± 12.9	ND
ATNRT2.7	174.7 ± 28.1	46.5 ± 20.8	0.2 ± 0.1	4.3 ± 2.2

ATNRT2.1 and *ATNRT2.7* expression levels in seeds (dry and imbibed), roots, and shoots. Relative expression level was determined on Col-8 plants. Values are means of 30 mg of dry or imbibed seeds. Values are means ± SD of two experiments and are given as a percentage of the EF1α gene used as a constitutive reference. ND, not detectable (below 0.01% EF1α).

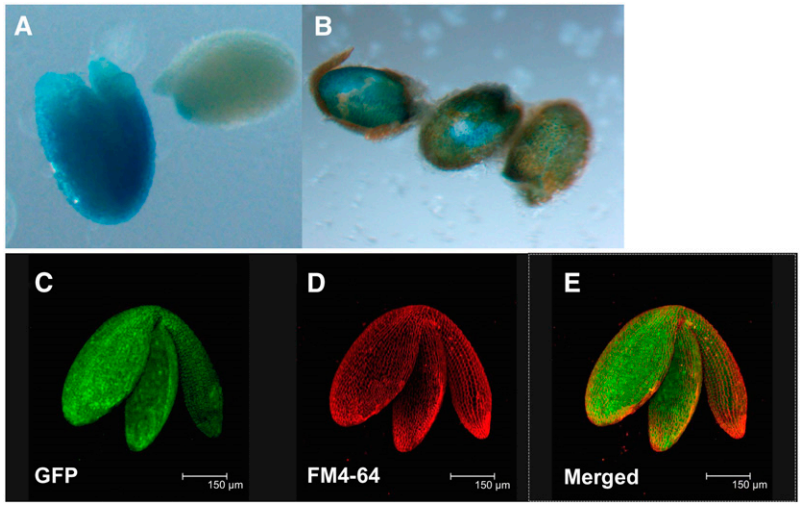

Figure 1. Expression of *ProATNRT2.7* in Mature Seed after 1 h of Imbibition.

(A) and **(B)** The *ProATNRT2.7:GUS* reporter gene.
(A) Embryo without seed coat.
(B) Seed coat of the embryo.
(C) to **(E)** The *ProATNRT2.7:GFP* reporter gene, with green indicating GFP fluorescence. Red indicates FM4-64 staining. Bars = 150 μm.
(C) GFP signal.
(D) FM4-64 dye where cytoplasmic membrane is stained in red.
(E) Merged image of GFP and FM4-64 signals.

expression of *ATNRT2.7* is developmentally regulated and that *ATNRT2.7* transcripts accumulate in seeds undergoing dessication.

ATNRT2.7 Nitrate Transport Activity

A large number of *NRT2* gene sequences are now available, and homologous sequences are found in three clades: mosses, gymnosperms, and angiosperms (5, 5, and 26 genes, respectively; see Supplemental Table 1 online). When the evolutionary relationship between these sequences and those of two *NRT2* genes from algae was investigated using a Bayesian method, the *NRT2* genes appear to be grouped into well distinct classes for algae, mosses, and seed plants (Figure 2). In the seed plant clade, the *NRT2* genes appeared to be clustered into two monophyletic groups, Group A (*NRT2.1*, *NRT2.2*, *NRT2.3*, *NRT2.4*, and *NRT2.6*) and Group B (*NRT2.5* and *NRT2.7*). This phylogenetic analysis highlights the fact that the *NRT2* genes that have been so far characterized as nitrate transporters in *Arabidopsis* (Filleur et al., 2001; Li et al., 2007), *Nicotiana plumbaginifolia* (Fraisier et al., 2000), or *Hordeum vulgare* (Tong et al., 2005) belong to Group A. The *NRT2.7* gene is classified in a distinct monophyletic group within this best-characterized family of plant nitrate transporters.

Thus, to evaluate the capacity of the ATNRT2.7 protein to transport nitrate, we introduced the *ATNRT2.7* cDNA driven by the 35S promoter into the *atnrt2.1-1* mutant background (Filleur et al., 2001). As a positive control, we used transgenic plants expressing the Np *NRT2.1* gene under the control of the RolD promoter that has been shown to restore nitrate influx in the *Arabidopsis* mutant back to 90% of that shown by the wild type (mR2 clone; Filleur et al., 2001). Homozygous transgenic plants were grown for 6 weeks in a hydroponic system on 0.2 mM NO_3^-, a N-limiting condition for the *atnrt2.1-1* mutant (Orsel et al., 2004). Root nitrate influx was measured at an external concentration of 0.2 mM using $^{15}NO_3^-$ (atom% ^{15}N: 99%). At this concentration, the difference in the HATS activity between the wild type and the *atnrt2.1-1* mutant was largest (Filleur et al., 2001). In two independent experiments, the overexpression of the two genes (*ATNRT2.7* and Np *NRT2.1*) was correlated with an increase in the root $^{15}NO_3^-$ influx (Figure 3A). This influx in the transgenic plants represents 160% of that measured for the *atnrt2.1-1* mutant for both genes, indicating a similar extent of phenotypic recovery as HATS activity was restored in both types of plants. Quantitative RT-PCR analyses confirmed that this restoration of nitrate influx in the transgenic plants was not due to altered expression or compensation by other members of the *NRT2* family (Table 2).

In addition, to confirm the hypothesis that ATNRT2.7 is a nitrate transporter, we used a heterologous expression system. In this experiment, *Xenopus laevis* oocytes were injected with nuclease-free water or *ATNRT2.7* mRNA. After 3 d, oocytes were incubated in a solution enriched with 5 mM $Na^{15}NO_3$ (atom% ^{15}N: 98%), and the ^{15}N enrichment of individual oocytes was measured after 16 h. These measurements showed that *ATNRT2.7* mRNA–injected oocytes took up significantly more nitrate than water-injected controls (Figure 3B).

We next investigated the effect of a mutation in the nitrate transporter *ATNRT2.7*. T-DNA mutants were isolated from two independent T-DNA libraries, in the Columbia (Col-8) and Ws backgrounds, and homozygous lines were selected, called

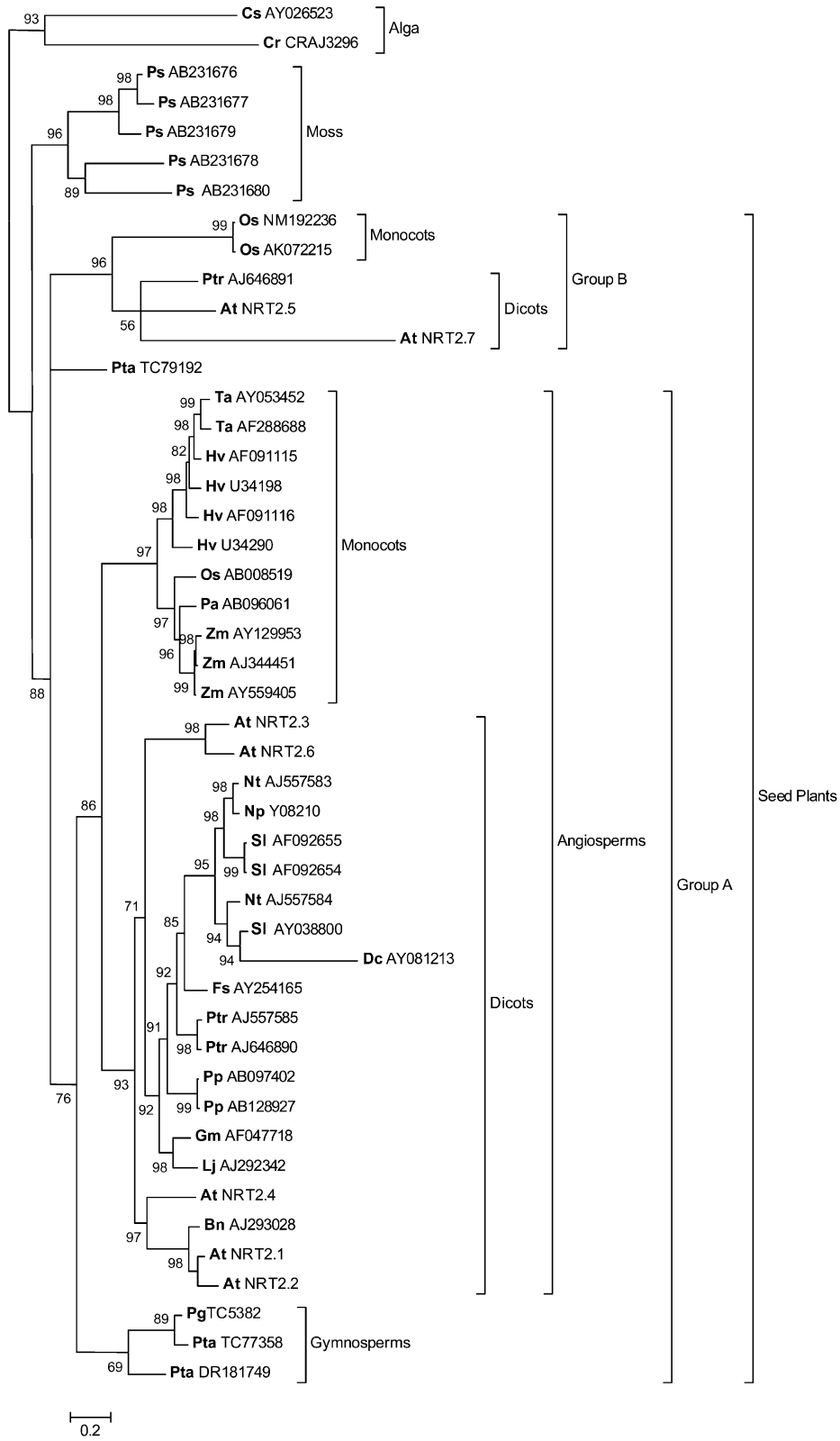

Figure 2. Rooted Phylogenetic Tree of the NRT2 Gene Family from Several Plant Species.

Support values for branches are shown and represent Bayesian posterior values. The *NRT2* genes in seed plants were classed into monophyletic Groups A and B. Species abbreviations are as follows: At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Cr, *Chlamydomonas reinhardtii*; Cs, *Chlorella sorokiniana*; Dc, *Daucus carota*; Fs, *Fagus sylvatica*; Gm, *Glycine max*; Hv, *Hordeum vulgare*; Sl, *Solanum lycopersicum*; Lj, *Lotus japonicus*; Np, *Nicotiana plumbaginifolia*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Pa, *Phragmites australis*; Pg, *Picea glauca*; Pp, *Prunus persica*; Ps, *Physcomitrella patens*; Pta, *Pinus taeda*; Ptr, *Populus tremula*; Ta, *Triticum aestivum*; Zm, *Zea mays*.

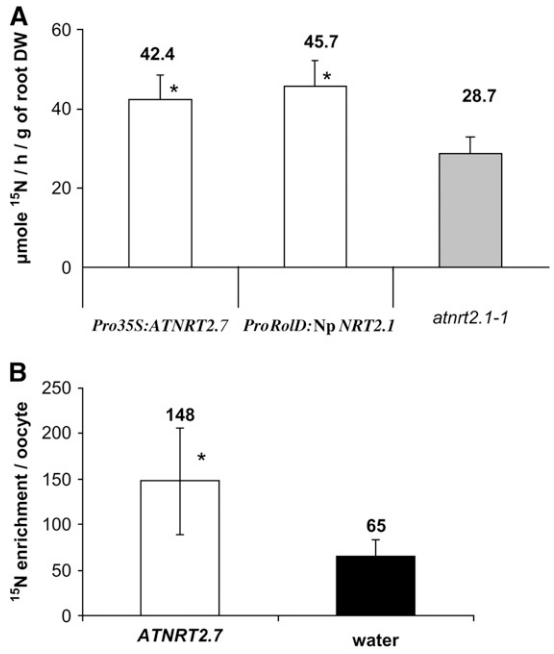

Figure 3. ATNRT2.7 Can Transport Nitrate.

(A) Root $^{15}NO_3^-$ influx in *atnrt2.1-1* and in *Pro35S:ATNRT2.7* and *ProRolD:*Np *NRT2.1* overexpressors. Plants were grown on 0.2 mM NO_3^- solution. Root $^{15}NO_3^-$ influx was measured with complete nutrient solution containing 0.2 mM $^{15}NO_3^-$. The values are means ± SD of five replicates (pooling three to five plants). Asterisks indicate statistically significant differences between overexpressors and mutant ($P < 0.0001$).
(B) Uptake of ^{15}N nitrate into oocytes injected with water or mRNA mixtures as indicated. Oocytes were incubated for 16 h in ND96 solution at pH 8.0 enriched with 5 mM $^{15}NaNO_3$. The delta ^{15}N values are means ± SD for five oocytes. The delta ^{15}N was calculated as described previously (Tong et al., 2005). The asterisk indicates a statistically significant difference between RNA and water-injected oocytes ($P < 0.05$).

atnrt2.7-1 and *atnrt2.7-2*, respectively (see Methods). These mutants are knockouts for the *NRT2.7* gene (data not shown), but no difference in root nitrate influx mediated by either the HATS or the LATS was found in both types of plant, whether grown with limiting (0.2 mM) or nonlimiting (6 mM) nitrate supply (Table 3). Nitrate and free amino acid contents in roots and shoots of both mutants were also measured and were found to be identical to the wild type under both levels of nitrate supply (Table 3). Taken together, these results suggest that the ATNRT2.7 protein is a functional nitrate transporter, but, possibly because of its expression site, it is not involved in the direct uptake of nitrate from soil by the root system or in nitrate distribution within the vegetative organs.

Nitrate Contents in Seeds of Different ATNRT2.7 Genotypes

Due to its specific expression pattern, we asked if this gene could be involved in nitrate accumulation in seeds. In *Arabidopsis*, a recent study showed that N and C metabolites are accumulated in seeds in a specific way during seed formation and maturation (Baud et al., 2002), but to our knowledge, only a few studies have been devoted to nitrate storage in *Arabidopsis* seeds (Derckx and Karssen, 1993).

Six independently isolated plant lines per genotype were grown together in the greenhouse under long days, on the same soil type and under nonlimiting N conditions (watered with 10 mM nitrate nutrient solution). Seeds were harvested from individuals after complete maturation and senescence of the plants. The results of seed nitrate content determinations are shown in Figure 4A. The *atnrt2.7-1* seeds had only 35% less nitrate than Ws wild-type controls. This decreased seed nitrate phenotype was even greater in the Col-8 ecotype, where a statistically significant 70% reduction of nitrate content in the mutant was observed. As the seed weights are very similar in both the genotypes, these differences are observed in nitrate content whether it is expressed on a per seed or on a milligram/dry weight (DW) basis (data not shown).

We next compared seed nitrate content in over- and under-expressing *ATNRT2.7* plants. As the *Pro35S:ATNRT2.7* plants were obtained in the Ws genotype, we increased the amount of nitrate in the nutrient solution up to 50 mM after flowering to enhance the small difference already observed on 10 mM between the mutant and Ws wild type (Figure 4A). Results of a typical experiment are shown in Figure 4B. As expected, the amount of nitrate in seeds of wild-type plants supplied with 50 mM is higher than with 10 mM nitrate (82 and 58 nmol/mg DW, respectively). When comparing seed nitrate content after either 10 to 50 mM nitrate supply, the amount in the *atnrt2.7-2* mutant had increased only slightly, leading to twofold less nitrate compared with the wild type (Figure 4B), and this difference was statistically significant. These data support the idea that ATNRT2.7 plays a major role in seed nitrate accumulation and agrees with the result obtained when seed nitrate contents were compared between overexpressor and wild-type plants. Nitrate contents in *Pro35S:ATNRT2.7*-overexpressing plants reached 160 and 310% of the nitrate in Ws and *atnrt2.7-2* seeds, respectively.

Germination Kinetics of the Different Genotypes

Nitrate was demonstrated to be a signal relieving seed dormancy in *Arabidopsis* (Alboresi et al., 2005). In this study, we used the

Table 2. Relative Gene Expression Level of *ATNRT2* and Np *NRT2.1* Genes in Roots of the *atnrt2.1-1* Mutant Complemented by *Pro35S:ATNRT2.7* or *ProRolD:*Np *NRT2.1* Constructs

	Pro35S:ATNRT2.7	*ProRolD:*Np *NRT2.1*	*atnrt2.1-1*
ATNRT2.1	ND	ND	ND
ATNRT2.2	ND	ND	ND
ATNRT2.3	ND	ND	ND
ATNRT2.4	0.34 ± 0.01	0.27 ± 0.02	0.33 ± 0.04
ATNRT2.5	0.25 ± 0.06	0.16 ± 0.03	0.14 ± 0.01
ATNRT2.6	ND	ND	ND
ATNRT2.7	86.16 ± 18	0.04 ± 0.01	0.03 ± 0.01
Np *NRT2.1*	ND	20.79 ± 4	ND

Relative expression level was determined on the plants as described for Figure 3A. Values are means ± SD of three replicates (pooling three to five plants). Results are given as a percentage of the *EF1*α gene used as a constitutive reference. ND, not detectable (below 0.01% *EF1*α).

Table 3. Physiological Characterization of the *atnrt2.7* Mutants

	Col-8	*atnrt2.7-1*	Ws	*atnrt2.7-2*
Plants grown on 0.2 mM nitrate				
Nitrate influx (0.2 mM) (μmol ^{15}N/h/g DW)	32.4 ± 2.9	30.6 ± 7.0	53.1 ± 7.3	51.4 ± 13.9
Nitrate influx (6 mM) (μmol ^{15}N/h/g DW)	137.6 ± 24.4	133.3 ± 14.5	164.9 ± 41.1	122.9 ± 20.8
Root nitrate content (μmol NO_3^-/g FW)	22.6 ± 2.3	22.2 ± 1.8	22.9 ± 2.21	20.6 ± 1.7
Shoot nitrate content (μmol NO_3^-/g FW)	132.2 ± 9.3	125.0 ± 5.6	122.0 ± 9.1	141.2 ± 8.2
Root amino acid content (μmol/g FW)	3.4 ± 0.5	4.4 ± 0.6	5.0 ± 0.6	4.4 ± 0.6
Shoot amino acid content (μmol/g FW)	3.5 ± 0.5	4.1 ± 0.5	5.2 ± 0.7	5.2 ± 1.1
Plants grown on 6 mM nitrate				
Nitrate influx (0.2 mM) (μmol ^{15}N/h/g DW)	21.9 ± 3.3	18.9 ± 2.8	19.0 ± 2.7	20.9 ± 4.6
Nitrate influx (6 mM) (μmol ^{15}N/h/g DW)	167.8 ± 12.6	128.9 ± 19.7	220.0 ± 25.5	181.8 ± 13.8
Root nitrate content (μmol NO_3^-/g FW)	45.6 ± 2.6	47.9 ± 0.9	41.1 ± 2.9	45.4 ± 1.8
Shoot nitrate content (μmol NO_3^-/g FW)	121.9 ± 17.1	132.3 ± 18.8	126.1 ± 3.7	125.2 ± 8.4
Root amino acid content (μmol/g FW)	5.1 ± 0.9	9.6 ± 1.4	5.4 ± 0.6	6.1 ± 0.7
Shoot amino acid content (μmol/g FW)	9.1 ± 1.3	9.6 ± 0.8	9.8 ± 1.4	9.6 ± 1.3

Plants were grown for 42 d in hydroponics on 0.2 mM nitrate or 6 mM nitrate. Nitrate influx was measured using 0.2 mM $^{15}NO_3^-$ (HATS system) or 6 mM $^{15}NO_3^-$ (HATS + LATS systems). Results are for two independent experiments (values are means ± SD of four replicates). FW, fresh weight.

same batch of seeds as those used for nitrate determination to test the impact of endogenous nitrate content on the seed germination of our different genotypes.

Freshly harvested seeds were sown on agar medium containing only distilled water, and the percentage of germination was scored at different times after sowing. In the same experiment, the viability of seeds was compared after stratification on media containing 1 mM nitrate, and in this case, the percentage of germination of all genotypes had increased to 100%. However, just 2 d after sowing on water, both mutants showed a delay in germination compared with their respective wild-type controls (Figure 5A). The strongest phenotype was observed for the *atnrt2.7-2* mutant, which displayed significantly fewer germinated seeds throughout the 7 d of measurements compared with its Ws control. By contrast, the Col-8 wild-type background showed less dormancy, and the germination difference with the corresponding *atnrt2.7-1* mutant was only significantly different 2 d after sowing.

This correlation between nitrate content and seed dormancy was further highlighted by measuring the kinetics of germination of freshly harvested seeds from over- and underexpressing plants. The same batches of seeds that had already been tested for their nitrate content (Figure 4B) were sown on water-containing medium, and germination was scored as described above (Figure 5B). As found when plants are grown on 10 mM nitrate, the percentage of germination of seeds of the *atnrt2.7-2* mutant grown on 50 mM nitrate was always lower when compared with wild-type seeds. By contrast, seeds from the *Pro35S:ATNRT2.7* overexpressors showed a significantly higher percentage of germinated seeds only at 2 d after sowing.

Subcellular Localization of the ATNRT2.7 Protein

To localize ATNRT2.7 at the subcellular level, we used a chimeric protein where GFP6 was fused to the N-terminal part of ATNRT2.7. The capacity of the GFP-tagged protein to transport nitrate was first tested. We compared the *Pro35S:ATNRT2.7*-with the *Pro35S:GFP-ATNRT2.7*-overexpressing lines in the *atnrt21.1-1* mutant background and found similar effects on the root nitrate influx of $^{15}NO_3^-$ and on the seed nitrate contents (Table 4). In addition, the dormancy of the overexpressors was tested, and GFP-tagged plants were less dormant than the wild type, like the *Pro35S:ATNRT2.7* lines (data not shown). Altogether, these results demonstrate that the ATNRT2.7 protein was still active when it was fused to GFP.

Confocal microscopy revealed an ATNRT2.7-dependent green fluorescent labeling that coincided with the tonoplast in the mature part of the root (Figure 6A). This tonoplast localization was even more evident in the root tip (Figure 6D), where this subcellular compartment was present as little vacuoles that fused to give the large central vacuole. This localization was further confirmed by staining lipid membranes using the red fluorescent probe, FM4-64 (Figures 6B and 6E). By merging the two pictures, GFP and FM4-64, the tonoplast was clearly

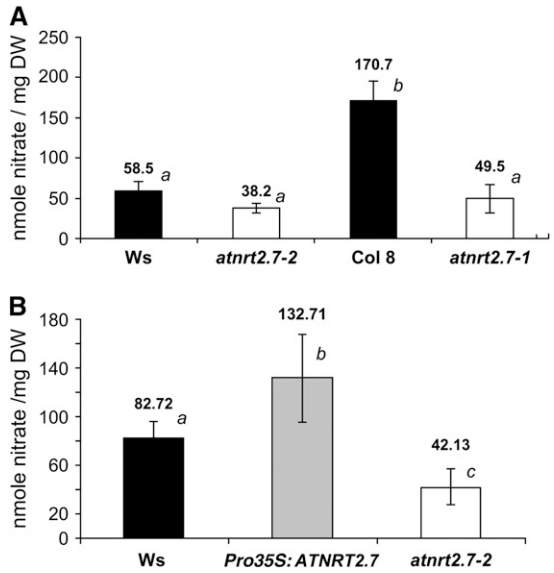

Figure 4. Nitrate Content in Seeds.

(A) Seed nitrate content of *atnrt2.7-1* and *atnrt2.7-2* mutants and their wild-type controls. Mother plants were grown on 10 mM NO_3^- solution (values are means ± SD of four replicates). Letters indicate the statistically significant different classes ($P < 0.05$).

(B) Nitrate content of seeds of Ws, *Pro35S:ATNRT2.7* overexpressor in Ws background, and *atnrt2.7-2* mutants. Mother plants were grown on 10 and 50 mM NO_3^- solution 2 weeks after bolting (values are means ± SD of four replicates). Letters indicate the statistically significant different classes ($P < 0.05$).

distinguishable in the root meristem (Figure 6F) and in the mature part of the root where formation of the main central vacuole occurs (Figure 6C). In embryos (Figure 6G), although GFP imaging in dry seeds led to a diffuse labeling (data not shown), 12 h of water imbibition allowed us to clearly distinguish two different intracellular patterns: a tonoplast localization surrounding the vacuoles that corresponded to seed protein bodies and another more punctuated localization (Figure 6H).

DISCUSSION

The sequence of the *Arabidopsis* genome identified >600 predicted open reading frames that encode potential membrane-bound transporters (Arabidopsis Genome Initiative, 2000). These proteins were classified into different families of transporters each playing specific roles in uptake or the control of nutrient and metabolite distribution. The ATNRT2.7 protein is the NRT2 family member that shows the lowest degree of homology with the other NRT2 proteins (55% similarity with ATNRT2.1) (Orsel et al., 2002a; this work). The gene organ expression pattern and its absence of regulation by nitrate suggest that *ATNRT2.7* is a specialized member of this family and implies that it has a very precise role in nitrate distribution within the plant.

We first demonstrated that *ATNRT2.7* exhibits a strong seed-specific pattern of expression. In dry seeds, only two members of the *ATNRT2* family are significantly expressed, *ATNRT2.5* and *ATNRT2.7*, the latter gene being expressed 20 times more strongly than the former. During seed imbibition, the expression of both of these NRT2 genes decreases and becomes hardly detectable for *ATNRT2.5*. Dry mature seeds contain a large number of mRNA species. Stored RNAs were first described in cotton (*Gossypium hirsutum*) but are universal in plant species. Recently, a genome-wide profiling of stored RNAs in dry seeds and imbibed germinating seeds was performed in *Arabidopsis* (Nakabayashi et al., 2005). Expression analyses during late embryogenesis and subsequent germination have shown that these developmental phases occur gradually (Nambara et al., 2000); therefore, stored RNAs contain transcripts necessary for both phases (Hughes and Galau, 1991). Data from the AtGenExpress initiative (https://genevestigator-1.ethz.ch/) show that *ATNRT2.7* expression is dramatically enhanced between stages 7 and 8, and this increase is maintained until seed maturation, leading to

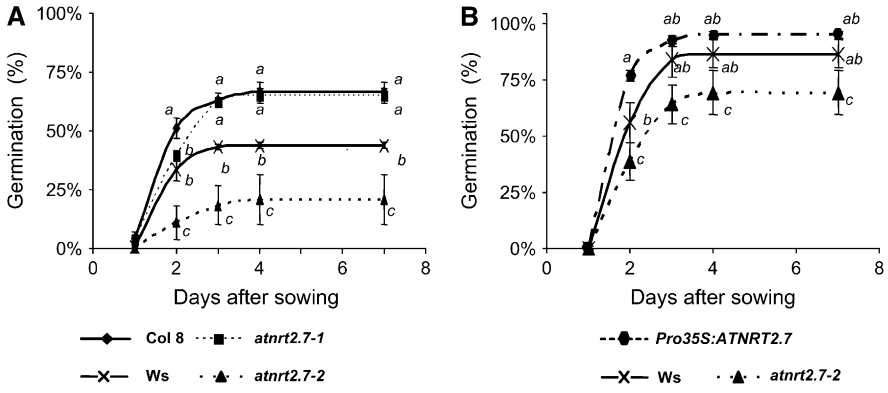

Figure 5. Kinetics of Seed Germination during 7 d.

(A) Kinetics of germination of *atnrt2.7* mutants during 7 d. Mother plants were grown on 10 mM NO_3^- solution. Values are means ± SD of four replicates, and letters indicate the statistically significant different classes ($P < 0.05$).

(B) Kinetics of germination of Ws, *Pro35S:ATNRT2.7* overexpressor in Ws background, and *atnrt2.7-2* during 7 d. Mother plants were grown on 50 mM NO_3^- solution (values are means ± SD of four replicates). Letters indicate the statistically significant different classes ($P < 0.05$).

Table 4. Nitrate Transport Activity of the *Pro35S:GFP-ATNRT2.7* Fusion

	NRT2 Constructs		
	Pro35S:ATNRT2.7	*Pro35S:GFP-ATNRT2.7*	*atnrt2.1-1*
Seed nitrate content (nmol NO_3^-/g DW)	31.0 ± 4.4	16.1 ± 2.8	8.6 ± 2.5
Nitrate influx (0.2 mM) (μmol ^{15}N/h/g DW)	5.7 ± 1.1	7.3 ± 2.3	3.3 ± 1.2

Nitrate accumulation was measured in seeds of plants overexpressing *ATNRT2.7* constructs in the *atnrt2.1-1* mutant background. Mother plants were grown in a greenhouse with 10 mM nitrate solution. The values are means ± SD of six replicates. Root nitrate influx was measured on in vitro–grown plants. The values are means ± SD of five replicates (pooling three to five plants).

a huge accumulation of transcripts for the gene in dry seeds (Table 1). The *ATNRT2.7* transcript abundance was found to decline during seed imbibition (Table 1), which may suggest a potential role of the protein during late embryogenesis rather than during subsequent germination. Genome-wide profiling has identified a G-box–like CACGTG sequence as the most prominent abscisic acid–responsive element in the dry seed transcriptome (Nakabayashi et al., 2005). However, no abscisic acid–responsive element element was found in the *ATNRT2.7* promoter.

If *ATNRT2.7* is important during late embryogenesis, is its function still related to nitrate transport? To address this question, we used a heterologous system and we took advantage of the *atnrt2.1-1* mutant phenotype. *Xenopus* oocytes have already been used to measure the nitrate uptake capacity of several *NRT1* and some *NRT2* genes. In the latter case, the coexpression of two components, one *NRT2* gene and one *NAR2* gene was found to be necessary to obtain an efficient nitrate uptake for *Chamydomonas reinhardtii* (Zhou et al., 2000), barley (Tong et al., 2005), and *Arabidopsis* (Orsel et al., 2006). Our experiments with *ATNRT2.7* mRNA-injected oocytes show that this protein alone is able to take up nitrate from the external solution. This result is in agreement with the expression pattern of the two *NAR2* genes in *Arabidopsis*. In complete contrast with *ATNAR2.1* and *ATNRT2.1*, which show identical tissue expression patterns (Okamoto et al., 2006), *ATNRT2.7* has a shoot- and seed-specific pattern that is very different from that shown by *ATNAR2.1*. We also confirmed the capacity of the ATNRT2.7 protein to transport nitrate in planta by *atnrt2.1-1* mutant complementation. This mutant is affected in the *ATNRT2.1* and *ATNRT2.2* genes and, as a consequence, exhibits only 20% of the wild-type nitrate uptake capacity (Cerezo et al., 2001; Filleur et al., 2001). We showed that the ATNRT2.7 protein, when expressed ectopically under the control of a strong promoter, is able to increase by a factor of 2 the nitrate uptake mediated by the HATS system. This increase is not due to compensation by enhanced expression of other *NRT2* genes still present in the mutant, as was found to be the case for ammonium transporters (Kaiser et al., 2002). However, this result is quite surprising because of the tonoplastic localization of the ATNRT2.7 protein. The overexpression of the *GFP-ATNRT2.7* fusion protein may lead to some targeting of the protein to the plasma membrane. Similarly, strong expression in *Xenopus* oocytes may result in default targeting to the plasma membrane. On the other hand, we cannot rule out the possibility that an enhanced flux of nitrate through the tonoplast of overexpressors could direct an increase in root influx mediated by other unidentified channels or transporters. These coordinated fluxes would participate in the homeostasis of cytosolic nitrate (der Leij et al., 1998). Although the ATNRT2.7 may participate in high-affinity nitrate transport (Figure 3A), the concentration of nitrate in xylem (Andrews, 1986) and cytoplasm (Cookson et al., 2006) is likely to be in the low mM range, and the seed dessication process will further increase the local concentration. The oocyte experiments used a 5 mM concentration to demonstrate nitrate transport (Figure 3B), but the affinity range of ATNRT2.7 has yet to be determined.

If the ATNRT2.7 protein is able to transport nitrate and this activity mainly takes place during late embryogenesis, then the

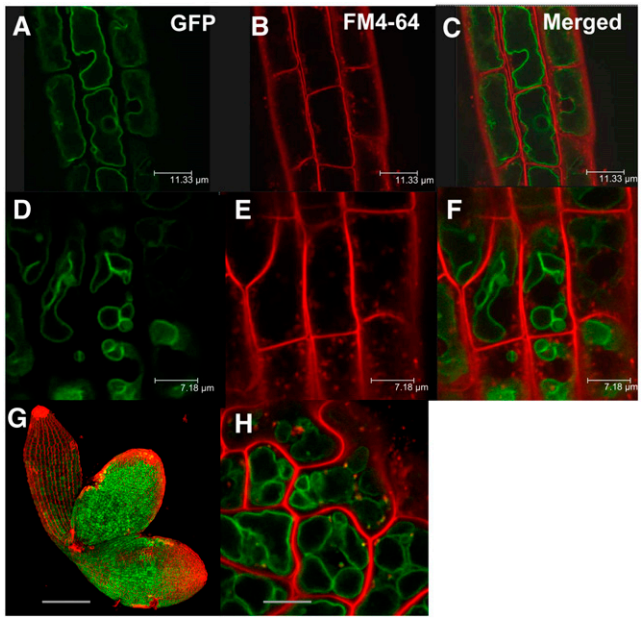

Figure 6. Expression of Translational Fusion between *ATNRT2.7* and the *GFP* Reporter Gene.

Green indicates GFP fluorescence, and red indicates FM4-64 staining.
(A) to **(C)** Epidermal cells in the mature root of 4-d-old *Pro35S:GFP-ATNRT2.7* plantlets. The plasma membrane is stained red. Bars = 11.33 μm.
(D) to **(F)** Epidermal cells near the root tip of young plantlets. Bars = 7.18 μm.
(G) Overview of mature seeds after 12 h of imbibition. Bar = 142.82 μm.
(H) Cotyledon cells of an embryo after 30 min of FM4-64 staining. Bar = 6.9 μm.

impact of a modification of the expression of *ATNRT2.7* should lead to a variation in seed nitrate content. To assess this possible role, we studied two allelic *atnrt2.7-1* and *atnrt2.7-2* mutants in Col-8 and Ws backgrounds, respectively. Both mutants shared the same phenotype, namely, normal growth under greenhouse conditions and no difference from wild-type nitrogen metabolism during the vegetative phase, but both had a lower nitrate content in their mature seeds. Moreover, an increase in seed nitrate content was observed in *ATNRT2.7*-overexpressing plants. As we demonstrated that the ATNRT2.7 protein was able to take up external nitrate when it was expressed ectopically in roots, we cannot rule out that this could simply explain the increase in seed nitrate content. We observed a strong interaction between the genetic background and the effect of a modification of *ATNRT2.7* expression and the seed nitrate content. The Col-8 genotype was more sensitive than Ws, and it was easier to show more significant results in the former than the latter ecotype (Figures 4A and 4B). Whether or not these results reflect the presence of unknown transporters, more active in Ws than Col-8 in seed nitrate accumulation, remains to be demonstrated. Alternatively, this may reflect natural variability between these ecotype backgrounds, the ATNRT2.7 protein itself could be less efficient in Ws than in Col-8, leading to a less dramatic phenotype in a null mutation. In *Arabidopsis*, seed development can be divided in three stages. After an early period of morphogenesis, the maturation phase includes both the synthesis of carbon (lipids) and nitrogen storage compounds as seed storage proteins (Heath et al., 1986). Recently, an integrated overview of seed development revealed two peaks of amino acid accumulation during the maturation and late maturation phases, explained by enrichment in Ser and Gly (Baud et al., 2002). Nothing is known, however, about the time course of nitrate storage in seeds. Nitrate content in leaves is higher during the vegetative rather than the reproductive stages, but in *Arabidopsis*, contrary to cereals, this storage pool does not significantly contribute to the bulk flow of resources to the seeds (Schulze et al., 1994). Nitrate in seeds seems to come directly from that taken up by roots, and this result is in agreement with the observed net increase in seed nitrate content when wild-type plants are transferred from 10 to 50 mM nitrate after bolting (Figure 4B).

The physiological role of nitrate in seeds could be as a nutrient, either to participate to reserve synthesis during the maturation phase or to sustain growth of young seedlings after germination. The high levels of mRNAs coding for genes involved in amino acid biosynthesis during seed maturation and during the dessication period suggest that amino acid biosynthesis takes place during these developmental stages (Fait et al., 2006). However, even if photosynthesis efficiency is maintained until the dessication period (Fait et al., 2006), providing the reducing power necessary for nitrate reduction, the mRNAs of the two key enzymes of the pathway, nitrate and nitrite reductase, are hardly detectable during these two phases (https://genevestigator-1.ethz.ch). On the other hand, an increase in the levels of many metabolites is observed very early in postimbibition germination. This reactivation of metabolic pathways must require the availability of key internal precursors. Among the genes involved in nitrogen assimilation and metabolism, the mRNA level of the nitrite reductase is multiplied by a factor of 4 after 3 h of imbibition (Fait et al., 2006). It is thus possible that the nitrate storage pool is used during the very early hours after imbibition to sustain the metabolic boost that precedes the mobilization of storage compounds. The role of ATNRT2.7 may be to secure a supply of nitrate in the seed, ensuring cytosolic homeostasis during very early growth, until the young root can access external sources.

However, the total amount of nitrate in the seeds is negligible compared with organic N (<1 in 1000). This prompts us to propose that, rather than a nutritional function in seed maturation, this nitrate plays either a signaling or osmotic role during the first steps of imbibition. The role of nitrate in the osmotic control of plant development has already been suggested (McIntyre, 1997). In barley seeds, exogenous nitrate increased water uptake by the seedling (Lieffering et al., 1996). The seed nitrate storage pool, mediated by ATNRT2.7 during seed development, may act as an osmoticum to drive water uptake during imbibition and germination and so may have an osmotic, rather than a nutritional, function. Nitrate could also act as a signal to turn on or off the expression of the numerous nitrate-regulated genes (Wang et al., 2003, 2004) or, as it has been demonstrated recently, to release seed dormancy (Alboresi et al., 2005). Bethke et al. (2006) proposed a simple four-component model that explains how nitric oxide (NO) derived from nitrogen-containing compounds can reduce seed dormancy. Nitrate reductase was shown to catalyze the reduction of nitrite to NO under anaerobic conditions or darkness (Meyer et al., 2005; Crawford, 2006). Possibly, during the very early hours of imbibition, the level of NO could increase due to both the presence of both nitrate in the seed vacuole and increasing expression of the *nia1* gene coding for one of the two nitrate reductase isoforms (https://genevestigator-1.ethz.ch). Here, 2 d after sowing the genotypes lacking *ATNRT2.7* expression were more dormant than their wild-type controls and the reverse was found for *ATNRT2.7* overexpressors (Figure 5). We can thus probably link the relationship between *ATNRT2.7* expression and seed dormancy through seed nitrate content.

GFP-tagged ATNRT2.7 proteins seem to be located in the vacuolar membrane (Figure 6). In 5-d-old plantlets, ATNRT2.7 was localized in the tonoplast of the main central vacuole, while when dry seeds are imbibed during 12 h, it is present on small vacuole bodies or as punctuate structures (Figures 6C and 6H). Both lytic vacuoles and protein storage vacuoles have been postulated to coexist in some plant embryos (Jiang et al., 2001), but the situation is less clear in *Arabidopsis*. Results of Otegui et al. (2006) using different markers suggest that only protein storage vacuoles occur at the late bent-cotyledon and mature embryo stages. However, the localization analysis of the iron transporters At NRAMP3 and At NRAMP4 has led to the hypothesis that seed storage vacuoles have within them a membrane-bound compartment that surrounds globoids and is a marker for the lytic vacuole (Lanquar et al., 2005). The authors proposed that during germination, the membrane surrounding storage vacuoles, where the ATNRT2.7 protein could mediate the accumulation of nitrate, rapidly acquires the properties of a lytic vacuole.

Many transporters have been found to be targeted to the tonoplast, such as ammonium transporters (Loque et al., 2005), sulfate transporters (Kataoka et al., 2004), and sucrose transporter (Endler et al., 2006). Depending on their substrate and

mechanism, these transport proteins can be involved in influx or efflux. The SULTR4-type transporters actively mediate the efflux of sulfate from the vacuole lumen into the cytoplasm (Kataoka et al., 2004), and At NRAMP3 and At NRAMP4 are essential for mobilization of vacuolar iron during seed germination on low iron (Lanquar et al., 2005). By contrast, a nitrate/proton antiporter, At CLCa, was recently found to mediate nitrate accumulation in *Arabidopsis* vacuoles, showing for the first time how these anions channels acted as ion exchangers (De Angeli et al., 2006). However, this gene is not expressed in seeds (https://genevestigator-1.ethz.ch). We demonstrated in this work that *ATNRT2.7* expression increases during seed maturation and subsequently decreases during germination and *atnrt2.7* mutants show lower seed nitrate content; therefore, we can postulate that the ATNRT2.7 transporter acts in a similar manner, mediating nitrate loading in the seed vacuole.

With these findings we provide additional information on the complementary roles of two members of the NRT2 family for the distribution of nitrate within the plant. One gene, *ATNRT2.1*, is involved in root uptake and entry of nitrate into the plant at the beginning, while the other gene, *ATNRT2.7*, is important at the final destination for nitrate loading into the vacuole during seed maturation.

METHODS

Plant Material and Growth Conditions

The SALK_073582 line (*atnrt2.7-1*) was obtained from the ABRC and was derived from a T-DNA–mutagenized population of the Col-8 ecotype (Alonso et al., 2003), while the *atnrt2.7-2* line was derived from a T-DNA–mutagenized population of the Ws ecotype (Bechtold et al., 1993; Bouchez et al., 1993). Homozygous mutant plants were isolated by PCR with the primers 2.7A (5′-CGGTATCTCTCAGCTCCTTATGCCTCTC-3′) and 2.7B (5′-GATGACGAAGGCCCACAACACATTCCAC-3′). Both of the T-DNA insertions were located in the first exon of *ATNRT2.7*. The absence of transcript downstream from the insertion sites was checked by quantitative RT-PCR with the primers 7E (5′-CCTTCATCCTCGTCCGTTTC-3′) and 7F (5′-AATTCGGCTATGGTGGAGTA-3′). Wild-type *Arabidopsis thaliana* ecotypes Col-8 and Ws as well as mutants or transgenic plants were grown either in the greenhouse or under hydroponic conditions in a Sanyo growth chamber with an 8-h-light/16-h-dark cycle at 21°C/17°C, respectively, 80% relative humidity, and 150 μmol m^{-2} s^{-1} irradiation. In greenhouse conditions, seeds were stratified for 48 h at 4°C in the dark in a 0.1% agar solution and then sown and cultivated as already described (Loudet et al., 2003). The nutrient solutions contained 10 mM nitrate, and in some cases, 2 weeks after bolting (flower initiation), the nitrate regime of plants was changed to 50 mM nitrate.

Phylogenetic Analysis of the NRT2 Gene Family

Nucleotide sequences were manually aligned by first translating into proteins and then using the sequence alignment editor software BioEdit. Only regions where the assessment of primary homology appeared reasonable were kept, generating a 1517-nucleotide position matrix. A phylogenetic tree was reconstructed using MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003) after back-translating to nucleotide sequences. The model of evolution used is the general time reversible model estimating separate rates of change for each type of nucleotide substitution with gamma distribution shape parameter estimated to account for rate heterogeneity. Analysis was run with the following parameters: number of generations = 500,000; sampling frequency = 10 generations; number of chains = 4. Posterior probabilities were computed after discarding 200,000 generations (burn-in period) and computing a consensus of trees saved in the remaining generations. The tree was visualized using MEGA3.1 (Kumar et al., 2004).

Quantitative RT-PCR

RNA was extracted with the Gen Elute Mammalian Total RNA kit from Sigma-Aldrich, modified by adding a DNase step, which was performed with the Qiagen RNase-free DNase kit. First strands were synthesized according to Daniel-Vedele and Caboche (1993) using M-MLV reverse transcriptase and oligo(dT) 15 primers (Promega). The PCR was performed on a LightCycler instrument (Roche) with the LightCycler-FastStart DNA Master SYBR Green I kit for PCR (Roche) according to the manufacturer's protocol. Each reaction was performed on a 1:20 dilution of the first cDNA strands, synthesized as described above, in a total reaction of 20 μL. With this dilution, the SYBR green signal was linear. Specific primer sets were used for the seven *ATNRT2* genes, as previously described (Orsel et al., 2002a). The experiment was performed twice on independent biological samples.

Complementation of the *atnrt2.1-1* Mutant

In this experiment, the full-length *ATNRT2.7* cDNA was amplified by RT-PCR from total RNAs extracted from Ws leaves using the start primer (5′-CGAGAGTAATGGAGCCATCTCAACG-3′), the stop primer (5′-CGCACATCAACAAACGGGACGTAGA-3′), and the high-fidelity Taq enzyme (Roche) and cloned into pGEM-T Easy vector (Promega). The nucleotide sequence of the insert was checked before transferring the cDNA first in the pRT103 vector (Töpfer et al., 1987) downstream from the 35S promoter, and this whole chimeric gene was then transferred into a pGREEN vector (Hellens et al., 2000) already containing the 35S terminator. Binary vectors were introduced into *Agrobacterium tumefaciens* strain C58C1 (pMP90). The *atnrt2.1-1* mutant was transformed by the in planta method using the surfactant Silwet L-77, and transformants were selected on 20 μg/mL of hygromycin B.

Root ^{15}N Influx

Influx of $^{15}NO_3^-$ was assayed as previously described (Orsel et al., 2004). The plants were transferred first to 0.1 mM $CaSO_4$ for 1 min and then to complete nutrient solution containing 0.2 mM $^{15}NO_3^-$ (atom% ^{15}N: 99%) for 5 min and finally to 0.1 mM $CaSO_4$ for 1 min (300 mL for plants grown in hydroponics and 20 mL for plants grown in vitro). After homogenization, an aliquot of the frozen powder was dried overnight at 80°C and analyzed using the ANCA-MS system (PDZ Europa). Influx of $^{15}NO_3^-$ was calculated from the total N and ^{15}N content of the roots (1 mg DW).

Xenopus laevis Oocyte Expression System

The pGEM-T Easy vector containing the full-length *AtNRT2.7* cDNA was fully digested with *Not*I. The cDNA fragment was blunted using the klenow enzyme and subcloned into the *Eco*RV site of the pT7TS expression vector containing the 5′- and 3′-untranslated regions of the *Xenopus* β-globin gene (Cleaver et al., 1996). For in vitro synthesis of mRNA, pT7TS clones were linearized by digestion with *Bam*HI. Synthesis of capped full-length mRNAs and *Xenopus* oocyte preparation were performed as previously described (Orsel et al., 2006). Healthy oocytes at stage V or VI were injected with 50 nL of water (nuclease free) or *ATNRT2.7* mRNAs at 1 $\mu g \cdot \mu L^{-1}$. After 3 d incubation at 18°C, 5 to 10 oocytes were incubated in 3 mL of ND96 solution enriched with 5 mM $Na^{15}NO_3$ (atom% ^{15}N: 98%) during 16 h at 18°C. The oocytes were then thoroughly washed four times with ice-cooled 5 mM $NaNO_3$ ND96 solution and dried at 60°C. The

$^{15}N/^{14}N$ ratio of the single dried oocyte was measured as previously described (Orsel et al., 2006). The values are means ± SD of five replicates; results from a representative experiment are shown.

Nitrate Content Measurements

Nitrate contents of seeds were determined as described previously (Alboresi et al., 2005). For the extraction, 50 seeds were homogenized in 500 μL 80% (v/v) ethanol at 4°C and extracted for 120 min. Nitrate content of seeds was analyzed by high-performance liquid chromatography on a DX-120 analyzer (Dionex). Nitrate and free amino acid contents in leaves or roots were measured as previously described (Orsel et al., 2004).

Germination Test

For each experiment, all genotypes were harvested on the same day. Four independent batches of 50 to 80 mature seeds were sown on 0.5% agarose plates (Litex agarose; FCM A/C) sometimes containing an additional 1 mM potassium nitrate. The plates were incubated in a growth chamber (Percival; Cu-36L6) at 25°C, with 16 h of light (100 μmol m^{-2} s^{-1}), 8 h of dark, and 60% relative humidity. Germination was scored as positive when the radicle protruded from the seed. A cold treatment at 4°C for 3 d was also performed to test the viability of mature seeds.

GUS Staining

Histochemical GUS staining was performed according to the method described by Jefferson (1987), with some modifications. Dry seeds were first sown on imbibed filter paper during 1 h, and the seed coat was removed before staining embryos. Embryos were vacuum-infiltrated for 1 h in a 50 mM potassium phosphate buffer, pH 7.2 (1% Triton, 0.5 to 5 mM ferro/ferricyanide, and 2 mM X-glucuronide). Subsequently, samples were incubated overnight in the dark at 37°C. Stained embryos was cleared by incubation in a chloralhydrate solution (8:2:1 [w/v/v] of chloralhydrate, water, and glycerol, respectively) and then observed under a light microscope (Axioplan 2; Zeiss).

Construction of GUS and GFP Fusions

Binary vectors containing GUS or GFP fusions with the *ATNRT2.7* promoter or coding sequences were obtained using Gateway technology (Curtis and Grossniklaus, 2003). A genomic *Arabidopsis* ATNRT2.7 region, starting from position –2,000 bp upstream of the translation initiation site and terminating before the ATG codon, was amplified from ecotype Ws by PCR with primers NRT2.7 PGW5′ (5′-AAAAAAGCAGGCCTCAGGTTGACTTCATCATTGG-3′) and NRT2.7PGW3′ (5′-AAGAAAGCTGGGTACGACTCTTACTTACACGAC-3′). Amplification was performed using the Expand high-fidelity PCR system (Roche), and the amplified fragment was cloned in front of the GFP and GUS coding sequence in the pBI101 derived gateway vector (Divol et al., 2006). The binary plasmids were transferred to *A. tumefaciens* strain C58C1 (pMP90) by triparental mating.

First primers AttB1-ATNRT2.7 start (AttB1, 5′-GAGCCATCTCAACGCAAC-3′) and AttB2-ATNRT2.7 End-Stop (AttB2, 5′-AACAAACGGGACGTAGACTACC-3′) were used to amplify a complete *ATNRT2.7* cDNA from our previously isolated clone (see above). PCR products were obtained with the Expand high-fidelity PCR system and amplified with the universal U3-endstop (5′-AGATTGGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACCTCCGGATC-3′) and U5 primers (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATG-3′) to create the recombinant site AttB. The product of recombination reactions (BP reactions) was used to transform competent *Escherichia coli*, strain TOP10 (Invitrogen), by heat shock. LR clonase reactions to T-DNA fragments from the entry clone to the destination binary vector pMDC 43 (Curtis and Grossniklaus, 2003) were performed. The vectors pMDC43/ATNRT2.7 were generated with GFP6 in N-terminal fusion, and the binary vectors, containing the *Pro35S:GFP-ATNRT2.7* construct, were sequenced before transformation of *A. tumefaciens*.

Arabidopsis plants, the wild type or the *atnrt2.1-1* mutant, were transformed according to the in planta method using the surfactant Silwet L-77 (Clough and Bent, 1998). Transgenic plants were selected on Estelle and Sommerville media (Estelle and Sommerville, 1987) containing 50 μg L^{-1} of kanamycin.

Confocal Microscopy Analyses

Seven-day-old transformed plantlets grown on Estelle and Sommerville media were observed with the TCSNT confocal microscope (Leica) equipped with an argon/krypton laser (Omnichrome). Cells in living roots and embryo were stained with 0.1 μg/mL of FM4-64 (Molecular Probes). The different fluorochromes were detected using laser lines 488 nm (Alexa 488, GFP, and FM4-64) and 543 nm (Alexa 568). The images were coded green (fluorescent isothiocyannate and GFP) and red (Alexa 568 and FM4-64), giving yellow colocalization in merged images. The samples were washed twice after staining before observation with the confocal microscope. Each image shown represents a single focal plane.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers listed in Supplemental Table 1 online.

Supplemental Data

The following material is available in the online version of this article.

Supplemental Table 1. The NRT2 Gene Family in Plants and Algae.

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