# Analysis of the NRT2 Nitrate Transporter Family in Arabidopsis. Structure and Gene Expression

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Nitrate is an essential element for plant growth, both as a primary nutrient in the nitrogen assimilation pathway and as an important signal for plant development. The uptake of nitrate from the soil and its translocation throughout the plant has been the subject of intensive physiological and molecular studies. Using a reverse genetic approach, the *AtNRT2.1* gene has been shown to be involved in the inducible component of the high-affinity nitrate transport system in Arabidopsis. The Arabidopsis Genome Initiative has released nearly the whole genome sequence of Arabidopsis, allowing the identification of a small NRT2 multigene family in this species. Thus, we investigated the phylogenetic relationship between NRT2 proteins belonging to several kingdoms and compared the structure of the different members of the Arabidopsis family. We analyzed, by semiquantitative reverse transcriptase-polymerase chain reaction, the expression pattern of each gene depending on plant organ and development or nutritional status, and compared the relative level of each gene by real-time polymerase chain reaction. We also evaluated the significance of each paralog on the basis of the relative levels of gene expression. The results are discussed in relation with distinct roles for the individual members of the AtNRT2 family.

Nitrate uptake by root cells is the first step of the nitrate assimilation pathway in higher plants. To cope with large variations in nitrate concentrations in cultivated soils, plants have developed two different uptake systems (for review, see Glass and Siddiqi, 1995; Forde and Clarkson, 1999). The low-affinity nitrate transport system is used preferentially when external nitrate concentration is high (above 1 mM; Siddiqi et al., 1990), whereas the high-affinity transport system (HATS) takes place at very low external concentrations (between 1 $\mu$M and 1 mM; Behl et al., 1988). After its entry in the cytoplasm of root epidermal cells, nitrate is either translocated and stored in the vacuole or excreted back into the apoplasm. It also can be reduced into nitrite in the cytoplasm by nitrate reductase. The nitrite is then translocated to the chloroplast where it is further reduced into ammonium by nitrite reductase (Faure et al., 2001). Finally, nitrate can be excreted from the root cytoplasm in the xylem vessels and unloaded in aerial organs, where it can follow the same fates as in roots. Although it is known that nitrate transfer across the root occurs by diffusion along a radial concentration gradient in the symplasm, many of these nitrate or nitrite fluxes entail the passage across the plasmalemma or the tonoplast. The passage through one or the other of these membranes requires active processes that involve transporters anchored in these structures.

In higher plants, the molecular basis of root nitrate uptake has been the matter of intensive studies during the last decade (for review, see Crawford and Glass, 1998; Daniel-Vedele et al., 1998; Forde, 2000). So far, two gene families have been identified: the NRT1 and NRT2 families involved in the low-affinity nitrate transport system and HATS, respectively. This classification appears to be an oversimplification because one so-called low-affinity transporter has also been shown to play a role in the high-affinity transport process (Liu et al., 1999; Wang et al., 1999). The complexity of nitrate/nitrite transport is enhanced by the fine regulation that occurs at the transcriptional level: Both low and high-affinity systems have constitutive and inducible components that are clearly distinct (Glass et al., 2001). External nitrate concentration, photosynthesis via sugars, nitrogen status of the plant, and possibly developmental signals act as positive or negative regulators of NRT2 gene expression (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999; Ono et al., 2000). Moreover, most genes that have been studied so far are expressed preferentially in roots and nothing is known about the elements that could ensure the long-distance transport of nitrate through the whole plant or between intracellular compartments.

In the model species, Arabidopsis, the complete sequencing of the genome reveals the importance of gene families, both in number and in size, for the organization and evolution of the genomes (Bevan et al., 2001). The duplication of an ancestral gene, followed by the divergence of both copies, leads to the construction of these families (Ohno, 1970). The general view is that such family members are both selected and preserved in evolution because they ex-

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press variable levels of proteins in different temporal and spatial patterns (Meagher et al., 1999). To understand the biological function of a gene, the analysis of the complete family is thus required. Until now, the best characterized member of the AtNRT2 family is the *AtNRT2.1* gene, which was isolated by a differential display approach (Filleur and Daniel-Vedele, 1999). We demonstrated that external nitrate, nitrogen starvation, and light regulate the *AtNRT2.1* mRNA steady-state levels in correlation with nitrate influx in roots (Lejay et al., 1999). The isolation of a T-DNA mutant, disrupted for the *AtNRT2.1* and *AtNRT2.2* genes, allowed us to demonstrate that one or two NRT2 genes are specifically involved in the nitrate-inducible component of the HATS and are not involved in low-affinity transport (Filleur et al., 2001). BLAST search using the *AtNRT2.1* coding sequence revealed the presence of six other related genes in the Arabidopsis genome. We have numbered the genes in the chronological order of their emergence by our own work on the genome sequencing project. These *NRT2* gene family members are distributed, although unequally, on three chromosomes of the genome (Orsel et al., 2002). Four of these genes form two tandems present on two bacterial artificial chromosomes (BACs), either in a tail-to-tail configuration for *AtNRT2.1* (T6D22-6) and *AtNRT2.2* (T6D22-7) on chromosome I, or in a head-to-tail configuration for *AtNRT2.3* (mae1-30) and *AtNRT2.4* (mae1-20) on chromosome V. Within each cluster, the genes are separated by 1.5 and 3.8 kb, respectively. Two other genes, *AtNRT2.5* (F13K23-15) and *AtNRT2.7* (T15N1-60), are found on isolated BACs on chromosomes I and V. The last gene of the family, *AtNRT2.6* (F14D17-130), is found isolated from the other members on chromosome III. The function of all the NRT2 proteins is far from being elucidated. Sequence comparison supports the idea that these proteins are involved in high-affinity transport. All members are characterized by a high degree of homology to *AtNRT2.1*, which is specifically involved in this high-affinity transport process.

A major challenge arises from the multiplicity of these genes. The activities of the proteins might be additive within the same cell or the high-affinity nitrate uptake process might rely on a major component. They could be strictly redundant or they could display organ-specific or developmentally regulated expression. In an attempt to start addressing this issue, we report in this paper the phylogeny of these sequences, the specificity of the different domains that make up the proteins, and the patterns of the gene expression according to the plant organ or nutritional status.

## RESULTS

### Intron-Exon Structure of the AtNRT2 Genes

For the report of the genomic and cDNA sequences of the *AtNRT2.1* gene (Filleur and Daniel-Vedele, 1999), a complete alignment of the seven AtNRT2 sequences, beginning and ending at the putative ATG and stop codons, was performed using the Gap program of the Genetics Computer Group (GCG; Madison, WI) package (Table I). The highest values of nucleotide sequence identity are found between the *AtNRT2.1* and *AtNRT2.2* and between the *AtNRT2.3* and *AtNRT2.6* genes. One or several introns are present in between the primer sets used in reverse transcriptase (RT)-PCR reactions. Their numbers, positions, and sizes have been determined for each gene by comparing the sequence of these RT-PCR products with the Arabidopsis genome sequence. A schematic representation of the *AtNRT2* gene structures is presented in Figure 1. According to the number of exons and relative positions of introns within the coding sequence, the *AtNRT2* genes can be classified into two different groups. Three members of the first group (*AtNRT2.1*, *AtNRT2.2*, and *AtNRT2.3*) are characterized by two conserved introns, whereas the *AtNRT2.6* gene has only the first conserved intron and the *AtNRT2.4* genes exhibits an additional intron of 236 bp in length. The second class is characterized by one intron shared by the *AtNRT2.5* and *AtNRT2.7* genes, the former gene possessing one additional large intron.

### Relationships within the Arabidopsis NRT2 Family and with Other NRT2 Proteins

Amino acid sequences of plant NRT2 proteins from monocotyledons or dicotyledons, algae, fungi, or *Escherichia coli* sequences were aligned using the ClustalW program. The best alignment on the entire sequences was then used to derive a phylogenetic

**Table I.** *Identity/similarity matrix for the NRT2 family*

| Nucleotide Identity | Amino Acid Similarity | | | | | | |
|---|---|---|---|---|---|---|---|
| | AtNRT2.1 | AtNRT2.2 | AtNRT2.3 | AtNRT2.4 | AtNRT2.5 | AtNRT2.6 | AtNRT2.7 |
| AtNRT2.1 | – | 90.4 | 76.4 | 87.7 | 67.1 | 76.4 | 55.6 |
| AtNRT2.2 | 87.98 | – | 72.6 | 83.3 | 65.7 | 73.0 | 55.7 |
| AtNRT2.3 | 67.12 | 65.94 | – | 78.9 | 64.6 | 91.5 | 55.2 |
| AtNRT2.4 | 72.86 | 70.73 | 69.2 | – | 65.9 | 78.2 | 54.8 |
| AtNRT2.5 | 60.85 | 57.83 | 55.75 | 52.26 | – | 65.5 | 60.0 |
| AtNRT2.6 | 66.95 | 66.30 | 80.67 | 68.34 | 60.40 | – | 55.3 |
| AtNRT2.7 | 50.97 | 51.50 | 56.18 | 48.68 | 57.89 | 54.09 | – |

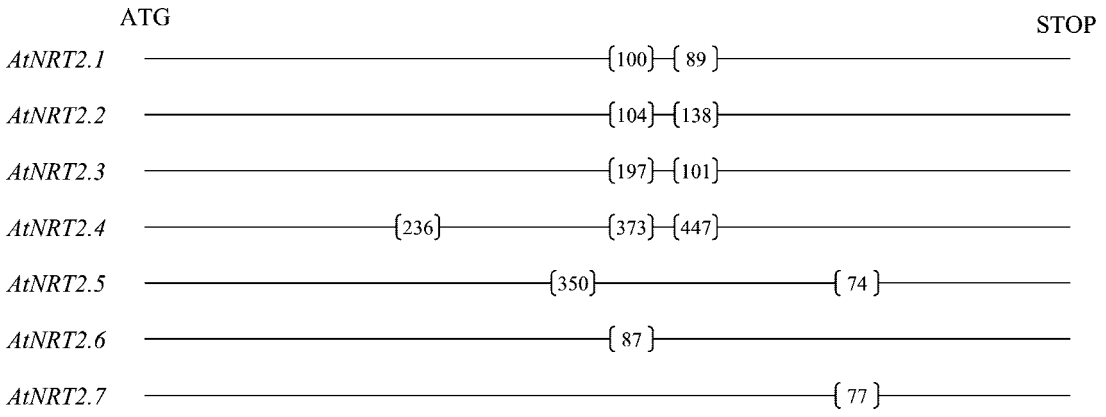


**Figure 1.** Gene structure of the AtNRT2 family. Sequences were aligned using the Gap program of the GCG package. Sizes of the introns, indicated between brackets, are given in bp.

unrooted tree using the "neighbor joining" method of the Philip package (Fig. 2). Bootstrap analysis with 1,000 replicates was performed to assess the statistical reliability of the tree topology.

The existence of two distinct classes of NRT2 proteins is suggested. The first class contains five Arabidopsis paralogs (AtNRT2.1, AtNRT2.2, AtNRT2.3, AtNRT2.4, and AtNRT2.6), and comprises also NRT2 proteins from *N. plumbaginifolia*, soybean, and *L. japonicus* (clade D in Fig. 2). The NRT2 proteins from monocotyledons (clade M: barley, rice, and wheat) are clustered in a second class. The AtNRT2.5 and AtNRT2.7 appear as isolated and individual sequences. The same is true for sequences from lower eukaryotic organisms such as algae (*C. reinhardtii*), fungi (*A. nidulans*), and yeast (*H. polymorpha*), or prokaryotic organisms like *E. coli*. Thus, the *AtNRT2.5* and *AtNRT2.7* genes seem to be less related to the first D group and might be more closely related to bacteria and fungi genes.

### Comparison of the NRT2 Proteins

Overall amino acid sequences deduced from the different AtNRT2 genes have been compared using the GCG Gap program (Table I). The highest percentage of similarity is found between the AtNRT2.3 and AtNRT2.6 proteins (91.5%), followed by the AtNRT2.1 and AtNRT2.2 pair (90.4%). The lowest score was observed with the AtNRT2.5 and AtNRT2.7 sequences when compared with all the other members of the family.

To highlight similarities and specific features of the NRT2 proteins, we analyzed their different domains using the ProDom 2000.1 program (Corpet et al., 2000; Fig. 3). This program allows specific domains to emerge from homologous sequences. Domain I is found in almost all the proteins that are potentially inserted in the membrane, and corresponds to the more specific domain VI in AtNRT2.5 or YNT1, a nitrate transporter from yeast. Domain II is found in all the high-affinity nitrate transporters with the exception of those from prokaryotes like NarK. This is also the case for domains III and IV, although these domains could not be considered as eukaryote specific because they are found in other proteins from *E. coli* than NarK; for example, the nitrite porter NarU. The fifth domain seems to be plant specific because it is found in five of seven proteins in Arabidopsis and is also found in four barley NRT2 proteins as well as in wheat, *Brassica napus*, tomato (*Lycopersicon esculentum*), rice, or *L. japonicus*. Surprisingly, the corresponding consensus sequence is modified and domain V does not appear in AtNRT2.5 and AtNRT2.7 proteins. Finally, domain VI is found in the yeast *H. polymorpha* and the AtNRT2.5 protein. Domains VII and VIII are specific of the prokaryotic NARK protein.

In summary, five of the seven Arabidopsis proteins (AtNRT2.1, AtNRT2.2, AtNRT2.3, AtNRT2.4, and AtNRT2.6) share a common structure, composed of five identical domains. AtNRT2.5 is structurally closely related to a yeast transporter, whereas AtNRT2.7 is similar to fungal and algal proteins.

### Expression Analysis of *AtNRT2* Genes during Plant Development

The plant NRT2 genes studied so far seem to be preferentially expressed in roots, but nothing is known about the organ specificity of the different members of a multigene family. Moreover, in some plant species, the uptake of mineral nutrients is regulated during plant development and, for example, the transition between vegetative and reproductive phases may lead to variations in nitrate uptake (Rossato et al., 2001).

Thus, we examined, in two independent experiments corresponding to two independent plant cultures, the level of expression of each member of the *AtNRT2* family in the aerial and root part of plants during the vegetative and reproductive stages of development using semiquantitative RT-PCR (see "Materials and Methods").

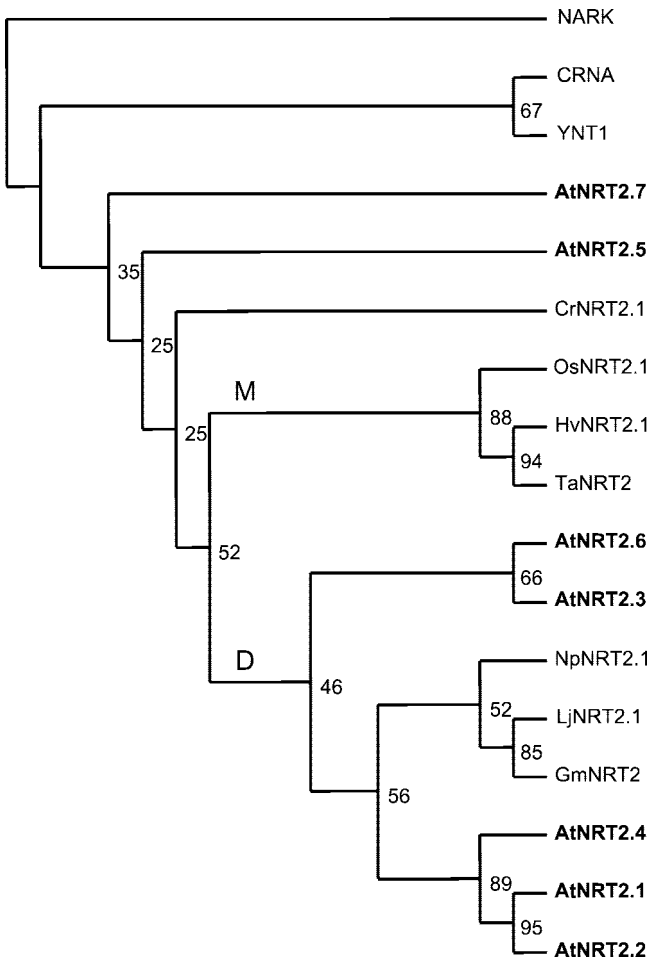


**Figure 2.** Unrooted, bootstrapped tree of NRT2 proteins. ClustalW was used to create an alignment of the entire sequence of amino acids that was then bootstrapped ($n$ = 1,000) to create the final tree using the Philip package. Values indicate the number of times (in percent) that each branch topology was found during bootstrap analysis. D, Dicotyledons; M, monocotyledons. GenBank protein ID numbers are: AtNRT2.1, AAC64170; AtNRT2.2, AAC35884; AtNRT2.3, BAB10099; AtNRT2.4, BAB10098; AtNRT2.5, AAF78499; AtNRT2.6, CAB89321; AtNRT2.7, CAB87624; *Nicotiana plumbaginifolia* NpNRT2.1, CAA69387; *Lotus japonicus* LjNRT2, CAC35729; soybean (*Glycine max*) GmNRT2, AAC09320; rice (*Oryza sativa*) OsNRT2, BAA33382; barley (*Hordeum vulgare*) HvNRT2.1, AAC49531; wheat (*Triticum aestivum*) TaNRT2.1, AAK19519; *Chlamydomonas reinhardtii* CrNRT2.1, CAA80925; *Aspergillus nidulans* CRNA, AAA62125; *Hansenula polymorpha* YNT1, CAA93631; and *E. coli* NARK, CAA34126.

Figure 4 describes the typical results of this semiquantitative RT-PCR approach using the example of the *AtNRT2.7* gene and *AtAPT1* gene as a control. The expression of this housekeeping gene has been shown to be constitutive within the different plant organs (Moffatt et al., 1994). Reverse transcription of total RNA isolated from different organs was performed using oligo(dT)$_{15}$. Standardization was based on *AtAPT1* mRNA content using the APT1 primer set (see "Materials and Methods" and Table I). We determined the specificity of the seven NRT2 primer sets by cloning and sequencing each amplified fragment. Then, the numbers of PCR cycles that corresponded to the linear range of amplification were evaluated. The results were analyzed by Southern blotting using the cloned fragments as probes, quantified by phosphor imager, and compared with results obtained similarly with the *AtAPT1* gene.

The two independent experiments gave similar results and representative values for gene expression in roots and shoots of young plants are given in Figure 5A. Although several assays using different sets of primers were performed with *AtNRT2.2*, we were unable to detect any amplification under these RT-PCR conditions. For the other individual genes, the levels of transcripts in different organs were calculated relative to the *AtAPT1* gene and added. The amount of NRT2 mRNA in each organ was then calculated as a percentage of the resulting sum. Four of the analyzed genes, *AtNRT2.1*, *AtNRT2.4*, *AtNRT2.5*, and *AtNRT2.6*, showed a strongly preferential expression in roots. At least 95% of the amount of the mRNA for each of these genes was located in the root parts. On the contrary, the expression of the *AtNRT2.7* is fully specific of the aerial parts, and in these experimental conditions, no amplified fragment was detectable in RNA extracted from roots. The last gene, *AtNRT2.3*, could be considered as an intermediate between these profiles. In our experimental conditions, approximately 26% of the total amount of the corresponding mRNA in the whole plant was found in the roots, whereas 74% was located in the aerial part.

When plants were maintained under the same growth conditions, they flowered approximately 45 d after sowing. At this stage, different organs were harvested, from roots, rosette leaves, stems, cauline leaves, to flowers. Total RNA was extracted and the RT-PCR reactions were performed as described above (Fig. 5B). The patterns of expression of *AtNRT2.1*, *AtNRT2.4*, and *AtNRT2.6* were not strongly modified between young and old plants. Approximately all the corresponding mRNA was found in the roots. Likewise, *AtNRT2.7* transcripts were again only found in the aerial part of the plant. The steady-state levels of these mRNAs were higher in stems and cauline leaves than in the rosette leaves or in the flowers. The expression patterns of the two remaining genes, *AtNRT2.3* and *AtNRT2.5*, were found to be modified between the two developmental stages. Whereas the amounts of *AtNRT2.5* mRNA increased in the aerial parts of old plants as compared with young plants (from 1%–21%), the levels of *AtNRT2.3* transcripts strongly decreased from 75% to a total of 15% in the aerial parts of young and old plants, respectively.

### Influence of Nitrate Supply on NRT2 Gene Expression

A major regulation of the HATS is the feedback control by the nitrogen status of the plant. This reg-

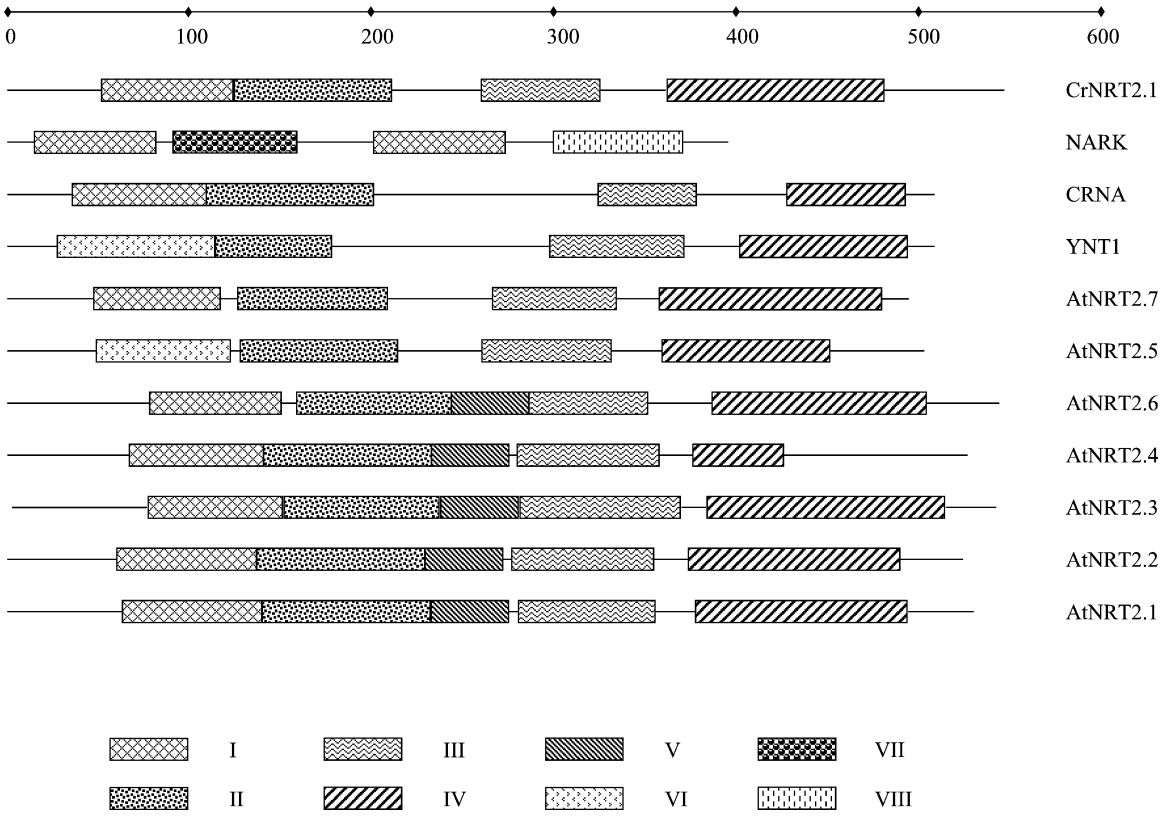


**Figure 3.** Comparison of NRT2 proteins. The amino acid sequences (identity nos. are indicated in Fig. 2) were analyzed with the ProDom 2001.2 program.

ulation is illustrated by the derepression of NRT2 gene expression during nitrogen starvation (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999), or by a differential expression according to the nitrogen availability (Fraisier et al., 2000). Plants were grown under two different nutritional conditions. The first one corresponds to non-limiting conditions where plants were supplied with high nitrate concentration (10 mM $KNO_3$). In contrast, a second set of plants was cultivated under limiting conditions and fed with 0.5 mM $KNO_3$. Plants were harvested at the rosette stage, total RNA was extracted from leaves and roots, and RT-PCR reactions were performed as previously described.

Figure 6 shows, for each of the six genes that could be tested, the variations of transcript levels between plants grown under limiting and non-limiting conditions within both organs. Three classes of genes can be distinguished. The first one, corresponding to the *AtNRT2.4* gene, seemed to be highly responsive to low external nitrate concentrations. The corresponding mRNA was even undetectable in leaves on high nitrate (Figs. 5 and 6). The level of this transcript was increased by a factor of 80 in roots of limited plants. The second class comprised the *AtNRT2.1*, *AtNRT2.3*, and *AtNRT2.5* genes that showed smaller variations, from a factor of 2.7 for expression of *AtNRT2.3* in leaves to a factor of 15 for *AtNRT2.1* expression also in leaves. In roots, the expression of the three genes increased 6 times when plants were grown on limiting conditions. The last class corresponds to the *AtNRT2.6* and *AtNRT2.7*, which both exhibited only slight variations in roots and in leaves, respectively. The expression of these latter genes appeared to be more or less constitutive with regards to N status of the plants.

### Comparison of the Expression of AtNRT2 Genes with One Another

Using the semiquantitative RT-PCR technique, we can compare the expression of a given gene within different organs or different nutrition conditions. However, it is very difficult to compare quantitatively the expression of different genes by this technique. The efficiency of the primer sets during amplification, on which the yield of PCR relies, is evaluated by measuring the end point amounts of amplified products by Southern blot (see "Materials and Methods"). This quantification, in turn, relies on the efficiency of probe labeling and hybridization process. Thus, we decided to use the real-time PCR, a technique that allows by fluorescence detection the measurements of PCR products after each cycle of the reaction (Schmittgen et al., 2000). To reach the highest levels of gene expression, this quantitative

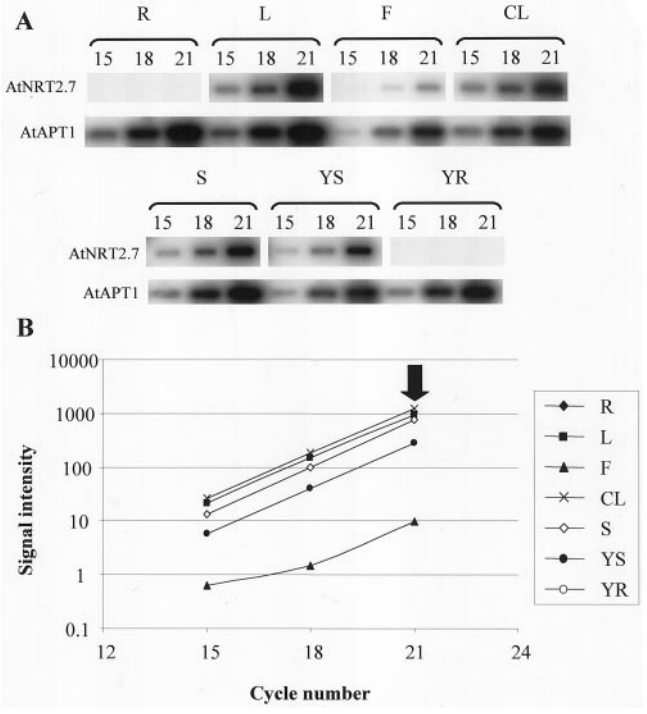


**Figure 4.** Quantification of *AtNRT2.7* expression in the linear range using the semiquantitative PCR method. Total RNA from roots (R), leaves (L), flowers (F), cauline leaves (CL), stem (S), young shoots (YS), and young roots (YR) of plants grown on 10 mM nitrate were isolated. A, mRNA was reverse transcribed and PCR amplified at different cycles using an *AtNRT2.7*- or *AtAPT1*-specific primer set. PCR products were blotted and specifically probed for quantification on a phosphor imager. B, Signal intensity was plotted versus cycle number of PCR reactions. The arrow indicates the cycle number within the linear phase that was chosen to standardize the treatments.

comparison was performed on leaves and roots of adult, flowering Arabidopsis plants grown on limiting nitrate. At this developmental stage, plants growing on 1 mM nitrate behaved very similar to plants grown on 0.5 mM nitrate concerning the expression of *AtNRT2.1*, but were bigger in size and allowed us to harvest more material. Figure 7 shows the expression levels of all the *NRT2* genes, relative to the expression of the *APT1* gene, in roots and leaves of adult Arabidopsis plants grown on 1 mM nitrate.

In roots, *AtNRT2.1* was expressed at the highest level; *AtNRT2.4*, *AtNRT2.5*, and *AtNRT2.6* were expressed at 4 to 6 times lower levels; and *AtNRT2.3* was expressed at the lowest level (40 times less than *AtNRT2.1*). The expression of *AtNRT2.2* and *AtNRT2.7* genes is barely detectable; thus, no valid quantitative comparison with the other genes can be done in roots. In leaves of adult plants, *AtNRT2.4* transcripts amounts reached the highest levels, followed by *AtNRT2.5* (6-fold less). The *AtNRT2.1*, *AtNRT2.6*, and *AtNRT2.7* were expressed at very low levels (32-, 38-, and 50-fold less than *AtNRT2.4*, respectively). In contrast, the amounts of *AtNRT2.2* and *AtNRT2.3* mRNA were too low to be correctly quantified.

In summary, the most abundant NRT2 transcript in roots originates from the *AtNRT2.1* gene, whereas in leaves, the *AtNRT2.4* mRNA is predominant.

## DISCUSSION

The NRT2 family in Arabidopsis comprises seven different genes, one of them being located alone on chromosome III. On chromosomes I and V, there are a cluster of two genes and one isolated gene.

Comparison of intron-exon structures and protein domains, as well as phylogenetic studies, support the hypothesis that the NRT2 members are distributed in two groups. The AtNRT2.1, AtNRT2.2, AtNRT2.3, AtNRT2.4, and AtNRT2.6 proteins share similar domains both in their positions and their features and belong to the first group, corresponding to the dicotyledons in the phylogenetic tree. The second group contains the AtNRT2.5 protein, which shares a common domain with yeast YNT1, whereas the AtNRT2.7 structure is identical to CRNA, the first of the two high-affinity nitrate transporters that have been isolated in *A. nidulans* (Unkles et al., 2001). Functional high-affinity nitrate transport has been achieved after expression of *CRNA* in oocytes without the assistance of a second gene (Zhou et al., 2000b), in contrast to the *C. reinhardtii CrNRT2.1* transporter, which requires the co-injection of *Nar-2* mRNA (Zhou et al., 2000a). It would be interesting to test if, according to the very close structure of the AtNRT2.7 protein to CRNA, the *AtNRT2.7* gene could by itself lead to a functional expression in oocytes.

Sequence divergence is frequently associated with functional divergence and the catalytic plasticity of enzymes within a multigene family has been shown to contribute to the evolution of metabolic diversity in plants (Richmond and Somerville, 2000). The members of the NRT2 family have undergone various degree of diversification in their amino acid sequences (Table I; Orsel et al., 2002). The high similarity between AtNRT2.1 and AtNRT2.2 sequences is not surprising because the genes are located in tandem on chromosome I, suggesting that they have derived from a duplication event. In contrast, the even higher level of conservation between AtNRT2.3 and AtNRT2.6 proteins is quite unexpected because the corresponding genes are located on chromosomes III and V, respectively. Recent studies, however, revealed that large-scale duplications occurred in a large part of the Arabidopsis genome (Mayer et al., 1999; Terryn et al., 1999). Such a duplication/translocation event has been actually shown to occur between chromosomes V and III, covering a region between BACs F18N11 and F26O13 and F2O15 and K9I9, respectively (Blanc et al., 2000). The *AtNRT2.6* gene is located on BAC F14D17, which is close to the

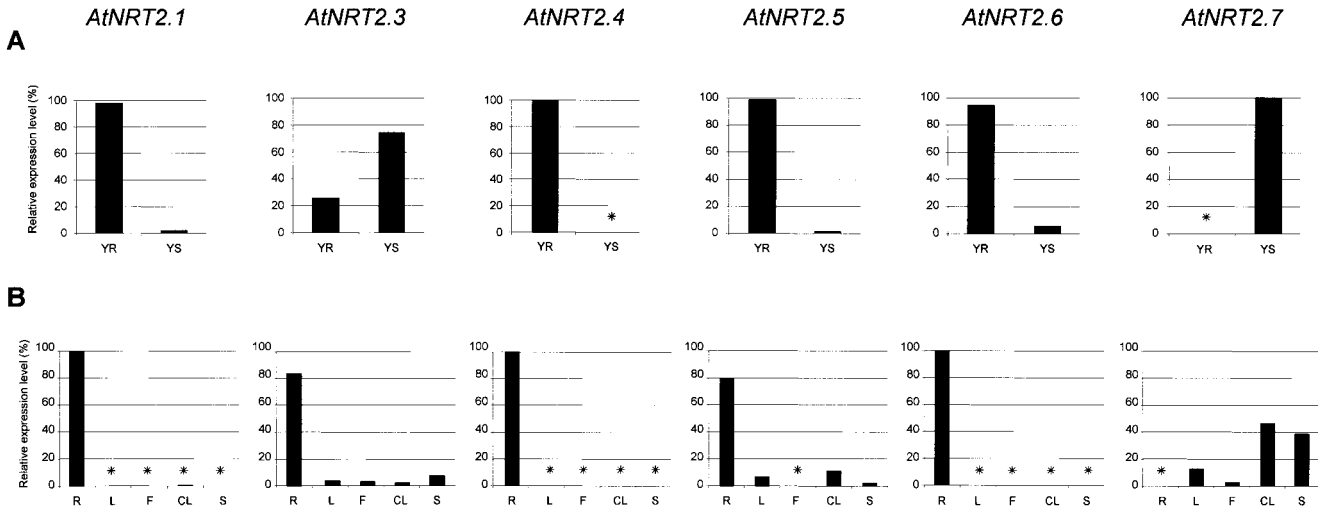


**Figure 5.** Expression profiles of individual *AtNRT2* genes (semiquantitative RT-PCR). For each gene, relative amounts of mRNA in different organs were added and then expressed as a percentage of the sum, in plants at the vegetative (A) or reproductive (B) stages of development. *, Below detection limit in the linear range of amplification.

F18N11 and could represent the border of the duplication. These sequential rearrangements might explain the high degree of similarity between two genes, *AtNRT2.3* and *AtNRT2.6*, despite their location on different chromosomes.

In higher plants, the organ specificity of NRT2 gene expression might depend on the species. For example, *NRT2* mRNA has not been detected in aerial parts of tomatoes (Ono et al., 2000), whereas in *N. plumbaginifolia*, these transcripts are present in leaves, petioles, buds, flowers, and seeds (Quesada et al., 1997). However, all these experiments were performed using the northern technique, which, due to the high degree of homology between the paralogs, does not allow a specific detection of each gene transcript and is not, moreover, sensitive enough to unravel a gene expressed at very low level. The semiquantitative RT-PCR, by carefully choosing the cycle number, was found to be highly sensitive and reproducible. For example, the results of two separated RT-PCR experiments, performed on the same RNA samples, are shown in Figures 5A and 6 (black bars). Although some variability is observed particularly when the genes are expressed at very low levels (*AtNRT2.5* and *AtNRT2.6* in shoots), all the genes exhibit the same expression pattern in both experiments. Nevertheless, a quantitative comparison of the expression levels between different genes is difficult. By using real-time PCR, this comparison becomes possible. Our results indicate that five of the seven NRT2 genes are preferentially expressed in roots of young plants.

The *AtNRT2.7* is the unique member of the gene family that exhibits a strong leaf specific pattern of expression, whereas *AtNRT2.3* shows only a preference for leaves at this stage of development. In this way, the AtNRT2.7 protein could not be involved in the direct uptake of nitrate from the soil. Specific transport systems might be responsible for efflux of ions from cells to xylem parenchyma. The potassium release into xylem vessels of Arabidopsis is mediated by SKOR, a potassium-selective voltage-regulated outward-rectifying channel (Gaymard et al., 1998). Recently, Takahashi and coworkers (Takahashi et al., 2000) made the hypothesis that the *SULTR1:2* gene might play a similar role, ensuring that any excess sulfate is transported back into xylem parenchyma cells for efflux into xylem vessels. The *AtNRT2.7* gene could

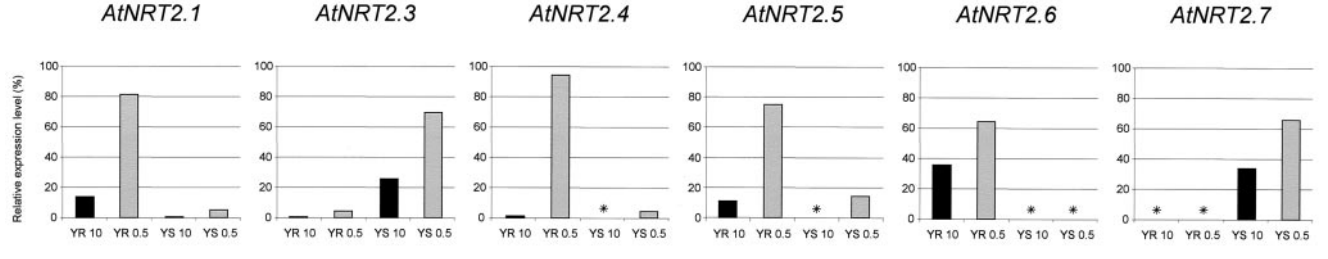


**Figure 6.** Effect of limiting nitrate conditions on *AtNRT2* gene expression (semiquantitative method). Gene expression was analyzed on plants cultivated on non-limiting (10 mM $KNO_3$, black bars) and limiting (0.5 mM $KNO_3$, gray bars) conditions. Semiquantitative RT-PCR was performed on young roots (YR) and young shoots (YS) as described in Figure 4. *, Below detection limit in the linear range of amplification.

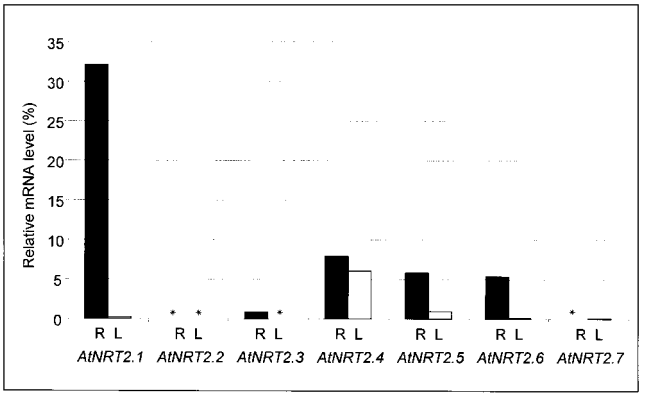


**Figure 7.** Relative expression of the 7 *AtNRT2* genes in roots (R) and leaves (L; real-time PCR). Expression was studied by real-time RT-PCR in roots and leaves of flowering plants cultivated on 1 mM $KNO_3$. mRNA levels of individual genes are given as percentage of the constitutive *AtAPT1* gene expression level. *, Below 0.01% of the *AtAPT1* expression level.

be involved in this type of nitrate flux and participate in the balance in leaves between the amount of nitrate used for assimilation and that re-absorbed for further transport. It might be expected that this balance would be particularly important during periods of plethoric nutrition or, in contrast, under starvation. This interpretation must be taken with precaution because of the high level of variations in the relative intensity of the transcription of each gene. The *AtNRT2.7* is expressed strictly in leaves, but it is not the only gene that is expressed in this organ (Fig. 5). Moreover, because it is one of the genes that is expressed at a very low level, there are more *AtNRT2.4* mRNAs than *AtNRT2.7* mRNAs at the same time within the same organ (Fig. 7). In addition, it will be of high interest to use reporter genes such as β-glucuronidase or green fluorescent protein to follow the fine distribution of each NRT2 gene, both in subparts of shoots or roots and within the cell.

As opposed to the xyloglucanendotransglucosylase/hydrolase family (Yokoyama and Nishitani, 2001), there is obviously no correlation in the level of expression of duplicated genes. The difference is particularly striking in the case of the *AtNRT2.1* and *AtNRT2.2* genes. The first accounts for 60% of total *NRT2* mRNA in roots, whereas the mRNA of *AtNRT2.2* has been hardly detectable overall in our experimental conditions. However, it is not a pseudogene because it was already shown to be expressed in the earlier stages of development (Zhuo et al., 1999).

Constitutive high-affinity nitrate transporters (CHATS) are thought to operate at low capacity and with high affinity to facilitate the entry of nitrate into roots, allowing the induction of high-capacity transporters (Kronzucker et al., 1995). Evidence of the constitutive component was obtained genetically with the isolation of an Arabidopsis mutant affected in CHATS activity (Wang and Crawford, 1996), but the corresponding gene has not yet been identified. External nitrate is not the only metabolite regulating the HATS. Numerous studies have reported a down-regulation of nitrate influx by downstream metabolites of nitrate assimilation such as Gln or Asn. As a consequence, negative regulation of *NRT2* gene expression has already been observed when plants are grown under non-limiting nitrogen conditions (Fraisier et al., 2000). Our results show that, in addition to the already studied *AtNRT2.1*, the *AtNRT2.3*, *AtNRT2.4*, and *AtNRT2.5* genes show significant responses to these nutritional conditions (Fig. 6). On the contrary, *AtNRT2.6* and *AtNRT2.7* appear to be constitutive regarding the nutritional status of the plant.

From this preliminary study, it is difficult to speculate on the role of each of these genes in the nitrate uptake process. However, it is tempting to associate, as for the *AtNRT2.7* (see above), a particular pattern of expression and a putative function. What is certain is that there is no pseudogene in this gene family. Sets of genes found in duplicated segments could have redundant/duplicated gene function. For example, for the mutant phenotypes of genes encoding the SHATTERPROOF or SPETALLATA families of MADS-box transcription factors to become visible, all

**Table II.** *Primer sets for PCR amplification*

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| Semiquantitative PCR | | |
| *AtNRT2.1* | 5′-GAA ACG CCG GAG TTG CCT CTG TCT CTG GG | 5′-CAC CAT AGC CAC AAC GGC AGT TAC AAG GG |
| *AtNRT2.2* | 5′-GAG CCG ACC AAC ACA AAG AAA AAG TAC | 5′-TCT GCT GCT CCT CCT TAC TAT ATT CGC C |
| *AtNRT2.3* | 5′-AGG ACT AGT GAA CGG CTG TGC AGG CGG G | 5′-AGA GAG CGG CGC GAG ACA AAA GGT GCA A |
| *AtNRT2.4* | 5′-ATG GTA ATA GAA GCA CAC TCG AGA AGA A | 5′-TAA GGA GCG GCG TGA GAT GAA AGG TAT G |
| *AtNRT2.5* | 5′-CTC TCC ACC GCT CCC GCC GTC TAT TTC ACC | 5′-CCT CCC GCA CCA GTC ATT CCC GAT ACC ACC |
| *AtNRT2.6* | 5′-AAC CGC CTT CTC ACT TAT GCT TAC AGC TCC | 5′-GAG AAC GGC GGG AAA CAA AAG GAG CAA CAC |
| *AtNRT2.7* | 5′-CGG TAT CTC TCA GCT CCT TAT GCC TCT C | 5′-GAT GAC GAA GGC CCA CAA CAC ATT CCA C |
| *AtApt1* | 5′-TCC CAG AAT CGC TAA GAT TGC C | 5′-CCT TTC CCT TAA GCT CTG |
| Real-time PCR | | |
| *AtNRT2.2* | 5′-GCT ATG CTT TCT CGG TAG ATG GTA G | 5′-AAT GTC ATG TTT GGT GAG GTT AAG A |
| *AtNRT2.5* | 5′-CTC TGC TTT CGC CGT TCT CTT GTT C | 5′-CGC TGC TAT AAT CCC TGC TGT CTG G |
| *AtNRT2.6* | 5′-TTG TTT CCC GCC GTT CTC TTG | 5′-TCT GTC CAT TCC GCT CCA TAG |
| *AtNRT2.7* | 5′-CCT TCA TCC TCG TCC GTT TC | 5′-AAT TCG GCT ATG GTG GAG TA |

the genes within the relevant family must be defective (Liljegren et al., 2000; Pelaz et al., 2000). Some redundancy, if any, could occur between *AtNRT2.4* and *AtNRT2.5* and between *AtNRT2.3* and *AtNRT2.6* genes because of their similar expression patterns or their very high amino acid sequence homology, respectively. We know already that a null mutant for both *AtNRT2.1* and *AtNRT2.2* genes is affected in the inducible component of the HATS system (Cerezo et al., 2001). However, a very high-affinity component is still active in the mutant and could correspond to the *AtNRT2.4*, which is the most inducible gene by limiting nitrate conditions. Work is in progress to isolate mutants affected in the members of the family. Physiological characterization of such simple/double or triple mutants will help us to assign a precise function to each AtNRT2 gene.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Seed stocks of Arabidopsis from Wassilewskija or Columbia ecotypes were used for all experiments.

For expression studies, two independent experiments were performed. The plants were stratified at 4°C for 2 d before sowing on sand, and grown at 25°C and 70% relative humidity for 29 d (young plants) or 45 d (adult plants) under 8 h of light/16 h of dark. Cultures were supplied with a complete solution described by Lejay et al. (1999), containing 0.5 or 1 mM $NO_3^-$ for nitrogen-limiting and 10 mM $NO_3^-$ for non-limiting conditions.

### Semiquantitative RT-PCR

*RNA Extraction and Reverse Transcription*

Total RNA from roots, leaves, flowers, stem, young shoots, and young roots of plants grown on 10 mM nitrate was isolated with the guanidium chloride method (Lobreaux et al., 1992). RNA was treated by RNase-free DNase I (Boehringer Mannheim/Roche, Basel) before first-strand synthesis according to Daniel-Vedele and Caboche (1993) using Moloney murine leukemia virus reverse transcriptase (Life Technologies/Gibco-BRL, Cleveland) and oligo$(dT)_{15}$ primers (Promega, Madison, WI).

*Semiquantitative PCR and Radioactive Detection*

For each treatment, PCR amplifications were performed on first cDNA strand corresponding to 90 ng of total RNA, using a specific primer set (Table II), and stopped at three different cycle numbers. The amplified fragments were cloned in the pGEM T Easy vector and sequenced. PCR products were blotted and specifically probed using the corresponding cloned fragment and quantified by phosphor imager. Signal intensity was plotted versus cycle number of PCR reaction to retain the values that were in the linear range and to allow the comparison between the different treatments. The constitutive gene *AtAPT1* was used as a standard reference. For Southern blotting, 1.25% (w/v) agarose gels containing the PCR products were incubated 30 min in 0.4 N NaOH and transferred to Hybond $N^+$ (Amersham, Buckinghamshire, UK) overnight. The probes were $^{32}P$ labeled by random priming with a kit (Amersham-Pharmacia Biotech, Uppsala) using $^{32}P$-dCTP. The blots were pre-incubated for 2 h at 65°C in Church buffer (7% [w/v] SDS; 0.25 M $Na_2PO_4$, pH 7.4; 2 mM EDTA; 0.02 mg $mL^{-1}$ heparine; and 0.1 mg $mL^{-1}$ DNA calf thymus), and then incubated overnight at 65°C with the probe. Membranes were washed for 30 min two times in Lav I (2× SSC, 0.5% [w/v] lauryl sarkosyl, and 2% [w/v] sodium pyrophosphate) and then at least 30 min in Lav II (0.2× SSC, 0.5% [w/v] lauryl sarkosyl, and 1% [w/v] sodium pyrophosphate).

Two independent plant cultures were performed and all samples were analyzed twice by this technique.

### Real-Time RT-PCR

*RNA Extraction and Reverse Transcription*

Total RNA from roots and leaves from plants grown on 1 mM nitrate was extracted using the same guanidium chloride method (see above). First strands were synthesized with the same method except the DNAse step, which was performed with the Rnase-free DNase kit (Qiagen USA, Valencia, CA).

*Real-Time PCR and SYBR Green Detection*

The PCR was performed on the LightCycler Instrument (Boehringer Mannheim/Roche) with the LightCycler-FastStart DNA Master SYBR Green I kit for PCR (Boehringer Mannheim/Roche) according to the manufacturer's protocol. Each reaction was performed on 5 μL of 1:100 (w/v) dilution of the first cDNA strands, synthesized as described above, in a total reaction of 20 μL. New specific primer sets were designed for the *AtNRT2.2*, *AtNRT2.5*, *AtNRT2.6*, and *AtNRT2.7* genes (Table II) to obtain optimal primer sets in these conditions for the seven AtNRT2 genes. The reactions were incubated at 95°C for 8 min to activate the hot-start recombinant *Taq* DNA polymerase, followed by 55 cycles of 10 s at 95°C, 6 s at the melting temperature of the primer set, 20 s at 72°C, and 6 s at 82°C to avoid dimer formation and to measure the fluorescence signal. The specificity of the PCR amplification procedures was checked with a heat dissociation protocol (from 65°C–95°C) after the final cycle of the PCR. The efficiency of the primer sets was evaluated by performing real-time PCR on several dilutions of a mix of the different first strands. The results obtained on the different treatments were standardized to the constitutive *AtAPT1* gene expression level.

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