

Expression Patterns of Nitrate Transporter Genes (*AtNRT*)  
in *Arabidopsis thaliana*.

by

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## Abstract

Higher plants possess both high- and low-affinity nitrate transporters. Since the first *Arabidopsis thaliana* low-affinity nitrate transporter gene, *AtNRT1.1(CHL1)*, was isolated (Tsay et al., 1993), three low-affinity (*NRT1*), and seven high-affinity (*NRT2*) nitrate transporter gene homologues have been identified in this species. We investigated the transcript abundances of all eleven genes both in shoot and root tissues in response to  $\text{NO}_3^-$  provision, by relative quantitative RT-PCR method. Based upon their patterns of expression following  $\text{NO}_3^-$  provision, the genes were classified into three groups: 1. nitrate-inducible, 2. nitrate-repressible, and 3. nitrate-constitutive. *AtNRT1.1*, *2.1*, and *2.2*, representatives of the first group, were strongly induced by 1 mM  $\text{NO}_3^-$ , peaking at 3 to 12 hours, and declining during 3 subsequent days. By contrast *AtNRT2.4* showed only modest induction both in shoots and roots. Expression of *AtNRT2.7*, one of the nitrate-repressible genes from the second group, was strongly suppressed by nitrate provision in both roots and shoots. The last group, characterized by a constitutive expression pattern, contains the largest membership, including *AtNRT1.2*, *1.4*, *2.3*, *2.5*, and *2.6*. Tissue-specific expression patterns of limited number of *AtNRT* genes were observed by using *GUS* reporter DNA fusion lines. In the root tip regions, *AtNRT1.1*, *2.1* and *2.4* were expressed, while in the epidermal cells of mature roots, *AtNRT2.1*, *2.2*, *2.4*, and *2.6* were expressed. Expression of *AtNRT2.1* was also found in the cortex and endodermal cells. In shoots *AtNRT1.1* showed expression in leaves and flowers, while *AtNRT2.6* was specifically expressed in pollen grains.  $^{13}\text{NO}_3^-$  influx from 100 $\mu\text{M}$  and 5mM  $[\text{NO}_3^-]$ , chosen to examine high-affinity and low-affinity transport, respectively, corresponded closely with the expression patterns of *AtNRT2.1* and

*1.1*, respectively. These results indicate that despite the close homology among members of the *NRT2* and *NRT1* families, individual members appear to be differently regulated, and may therefore perform different functions with respect to nitrate transport.



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## List of Abbreviations

C : Carbon  
cHATS : Constitutive high-affinity transport system  
CK2 : Casein kinase 2  
cLATS : Constitutive low-affinity transport system  
CRLR : Calcitonin-receptor-like-receptor  
GFP : Green fluorescent protein  
GOGAT : Glutamate synthase  
GS : Glutamine synthetase  
GUS :  $\beta$ -glucuronidase  
iHATS : Inducible high-affinity transport system  
iLATS : Inducible low-affinity transport system  
 $K^+$  : Potassium  
MFS : Major facilitator superfamily  
N : Nitrogen  
NiR : Nitrite reductase  
NNP : Nitrate/nitrite porter  
NOS : Nopaline synthase  
NR : Nitrate reductase  
ORF : Open reading frame  
PKC : Protein kinase C  
PTR : Peptide transporter  
RAMP : Receptor-activity-modifying protein  
RT-PCR : Reverse transcription-polymerase chain reaction  
TMS : Trans-membrane-spanner  
WT : Wild-type  
X-Gluc: 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucoronide cyclohexylamine

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# 1

## General Introduction

Nitrogen (N) constitutes a large proportion (~3-5%) of the dry weight of most plants and hence it is required in large quantities. By virtue of its relative scarcity in natural systems, its availability commonly limits plant growth, while among agricultural species also, nitrogen is the one of the most influential factors controlling rates of plant growth and absolute crop yield. In order to sustain current high levels of crop productivity global N fertilizer use has risen to  $\sim 10^{11}$  kg per annum, yet estimates of N use efficiency suggest values of 30 to 40% for cereals, the major crops with respect to fertilizer consumption (Raun and Johnson, 1999).

In temperate agricultural soils, under aerobic conditions nitrate ( $\text{NO}_3^-$ ) is commonly the principal form of available N, since ammonium ( $\text{NH}_4^+$ ) is quickly nitrified to  $\text{NO}_3^-$  under those conditions. Because of its chemical nature (negatively charged),  $\text{NO}_3^-$  is hardly retained on soil surfaces, which are usually negatively charged. Therefore  $\text{NO}_3^-$  in soil solution would be mobile, moving in soil according to mass flow, which is affected by

transpiration and irrigation. Excessive fertilization can cause environmental damage. Unabsorbed free  $\text{NO}_3^-$  can leach from soils and contaminate surface and/or ground water, escape to lakes, rivers, and oceans, and cause eutrophication. However, if N fertilizer were reduced by an appropriate amount, it is claimed that plant growth might be maintained with minimum N losses from the soil (Raun and Johnson, 1999; Andersson et al., 2001; McIsaac et al., 2001). The processes involved in nitrate acquisition by higher plants have therefore been of considerable interest to plant scientists and been extensively studied for several decades.

Nitrate utilization by higher plants starts at the root surface where nitrate is absorbed into root cells through the plasma membrane. In many species both roots and shoots have the capability to assimilate nitrate into organic nitrogen compounds. Thus in those species undertaking significant assimilation of nitrate ( $\text{NO}_3^-$ ) in the shoots, there is a requirement to transfer  $\text{NO}_3^-$  to the stele, and on to the leaves, either in the transpiration-driven ascent of sap, or by means of root pressure. In such cases there is a need to re-absorb  $\text{NO}_3^-$  from the leaf apoplast. Whether in roots or in shoots,  $\text{NO}_3^-$  is reduced to nitrite ( $\text{NO}_2^-$ ) by the enzyme nitrate reductase (NR) in the cytosol. Nitrite is then rapidly transported into plastids (i.e., proplastids in non-photosynthetic tissues, chloroplasts in photosynthetic tissues), and converted into ammonium ( $\text{NH}_4^+$ ) by the enzyme nitrite reductase (NiR). Ammonium is incorporated into amino acids by glutamine synthetase (GS) and glutamate synthase (GOGAT), and amino acids are the precursors of higher molecular weight organic nitrogen compounds such as proteins, nucleic acids, coenzymes, or secondary metabolites (Marschner, 1995).



Nitrate uptake has been classified according to kinetic criteria as occurring as a result of three discrete transport systems: a constitutive high-affinity transport system (cHATS), an inducible high-affinity transport system (iHATS), and a low-affinity transport system (LATS) (Glass and Siddiqi, 1995; Crawford and Glass, 1998; Forde, 2000). The cHATS and iHATS typically operate in the range of 10 to 250  $\mu\text{M}$   $\text{NO}_3^-$ , while the LATS only becomes evident above these concentrations. At such concentrations total uptake rates for  $\text{NO}_3^-$  are the sum of these three transporter activities (Siddiqi et al., 1990; Glass et al., 1992).

The first high-affinity nitrate transporter gene, *NrtA* (originally called *crnA*), was isolated from a filamentous fungus, *Aspergillus nidulans* (Unkles et al., 1991). An *A. nidulans* mutant with a defective *NrtA* absorbed  $\text{NO}_3^-$  at roughly 20% the rate of WT (Unkles et al., 2001). Although this suggests that NrtA is the principal transporter, a second transporter (NrtB) absorbs  $\text{NO}_3^-$  with a much higher affinity ( $K_m$  value of 11  $\mu\text{M}$  compared to 108  $\mu\text{M}$  for NrtA), suggesting that this transporter may be important for scavenging  $\text{NO}_3^-$  from low external concentrations. In *Chlamydomonas reinhardtii* there are also two high-affinity  $\text{NO}_3^-$  transporters and these exhibit differences in  $K_m$  values of the same magnitude as observed in *A. nidulans* (Galvan et al., 1996). Genes encoding nitrate-inducible high-affinity transporters have been cloned from various higher plants, including *Arabidopsis thaliana*, using the reported sequences of the *A. nidulans* and *C. reinhardtii* high-affinity transporter genes (Forde, 2000).

In *A. thaliana*, representatives of the high-affinity nitrate transporter (*AtNRT2*) family, *AtNRT2.1* and *AtNRT2.2*, were first isolated by use of degenerate primers based

upon the *NrtA* homologues (Zhuo et al., 1999), and by a differential display method (Zhuo et al., 1999; Filleur and Daniel-Vedele, 1999). The Arabidopsis genome project unveiled five more putative high-affinity nitrate transporters in the family (The Arabidopsis Genome Initiative, 2000). Preliminary experiments showed that all seven members were expressed in roots (Glass et al., 2001). It is well established that *AtNRT2.1* is induced by  $\text{NO}_3^-$  (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999; Zhuo et al., 1999; Gansel et al., 2001), as are other *NRT2* genes in barley (Trueman et al., 1996; Vidmar et al., 2000), *N. plumbaginifolia* (Quesada et al., 1997; Krapp et al., 1998), soybean (Amarasinghe et al., 1998), and tomato (Ono et al., 2000). The transcript abundances of *AtNRT2.1* and the patterns of high-affinity nitrate influx under different conditions of N provision showed high correlations, suggesting that *AtNRT2.1* is primarily responsible for iHATS activity (Zhuo et al., 1999; Lejay et al., 1999). This conclusion was substantiated by the recent finding that a T-DNA mutant, lacking *AtNRT2.1* and a part of 2.2, lost about 70% of high-affinity  $\text{NO}_3^-$  uptake capacity compared to the WT plants, while LATS transport was unaffected (Filleur et al., 2001). Studies of expression patterns of *AtNRT2.1* and *HtNRT2.1*, in response to treatments with amino acids and various inhibitors of  $\text{NO}_3^-$  assimilation strongly suggest that *NRT2.1* is regulated at the transcript level by glutamine (Zhuo et al., 1999; Vidmar et al., 2000). Nevertheless, there is evidence to suggest that  $\text{NO}_3^-$  influx may be also regulated at the post-translational level (Forde, 2000; Vidmar et al., 2000). Therefore correlations between transcript abundance and  $\text{NO}_3^-$  influx must be interpreted with caution. Characterizations of other members of the *NRT2* family (i.e., *AtNRT2.3-2.7*) and their functional determination await investigation.

Low-affinity  $\text{NO}_3^-$  transport is thought to be mediated by members of separate (unrelated) family, the NRT1 family, which belongs to a larger peptide transporter family (PTR, also called POP), which contains 51 or 52 members (<http://www.cbs.umn.edu/arabidopsis>). The first member of this family of putative low-affinity nitrate transporter genes, *AtNRT1.1* (originally designated *CHL1*), was isolated from an *Arabidopsis* mutant that was resistant to chlorate, a toxic  $\text{NO}_3^-$  analogue (Tsay et al., 1993). The *AtNRT1* family contains at least four members, including *AtNRT1.1(CHL1)*, *AtNRT1.2(NTL1)*, *AtNRT1.3(NTP3)*, and *AtNRT1.4(NTP2)* that were isolated by homology searches using *AtNRT1.1* against the EST database (Hatzfeld and Saito, 1999; Huang et al., 1999; Forde, 2000). The present study has focused on these four members (i.e., *AtNRT1.1*, *1.2*, *1.3*, and *1.4*). Although the *AtNRT1* family is generally designated as encoding LATS, as stated above, it may express both HATS and LATS activities (Liu et al., 1999; Wang et al., 1998). Interestingly, the latest findings suggested that *AtNRT1.1* might also be involved in organogenesis (Guo et al., 2001).

In this study I have characterized 11 members of *NRT* gene families in *Arabidopsis thaliana* (i.e., 4 *AtNRT1*; 7 *AtNRT2*). First of all, gene and predicted protein structures of *AtNRT* families were analyzed. These analyses provided the fundamental information upon which subsequent genomic analysis relied. Additional bioinformatics data will be presented in order to predict gene regulation and functional aspects of gene products (Chapter 2).

Chapters 3 and 4 will focus on the expression patterns of *AtNRT* family members in total tissue extraction, and intact tissues, respectively. In Chapter 3, the transcript abundances were analyzed quantitatively by RT-PCR in both roots and shoots, in response to the provision of  $\text{NO}_3^-$  following a 7-day period of  $\text{NO}_3^-$  deprivation. Gene expression

patterns of all 11 *NRT* genes were revealed with the sensitive RT-PCR method. The patterns were grouped into three categories, namely, nitrate-inducible, nitrate-repressible, and nitrate-constitutive. These expression patterns were then used to predict possible roles in nitrate uptake of the nitrate transporters in the species.

In Chapter 4, tissue-specific expression patterns are presented using transgenic plants which carry *AtNRT* promoter regions fused with *GUS* reporter DNA. Previous *in situ* hybridization studies showed that *AtNRT1.1(CHL1)* is expressed in epidermal and endodermal cells in the root tip region, and endodermal cells in mature root region, while expression of *AtNRT1.2* is primarily in epidermal cells and root hairs regardless of the stages of root development (Huang et al., 1996). In *Nicotiana plumbaginifolia* a high-affinity nitrate transporter *NpNRT2* expression was found primarily in epidermal and endodermal cells in root tips, and in lateral root primordia in mature roots. To date, however, no information is available for the *NRT2* family of genes in Arabidopsis. Therefore this is the first report showing the localization of expression of four high-affinity nitrate transporter genes (*AtNRT2.1*, *2.2*, *2.4*, and *2.6*) in *A. thaliana*.

Molecular analysis can predict possible gene and protein structures, and expression analysis provides clues concerning the regulation of genes at the *in vitro* and *in situ* levels. Subsequent questions relate to how these genes and/or gene products function. Do they function in isolation, or do they need to be coordinated with other proteins to generate functional transporters? In Chapter 5,  $^{13}\text{NO}_3^-$  influx studies were performed in parallel to compare patterns of influx in response to provision of  $\text{NO}_3^-$  after 7 days of N-deprivation, with patterns of gene expression. The kinetic study confirmed that Arabidopsis had four nitrate transport systems (i.e., iHATS, cHATS, inducible LATS, and constitutive LATS).

Nitrate transporter genes were then assigned to these systems based upon their expression patterns. The ultimate goal of this thesis is to elucidate the function of each of the nitrate transporters by integrating molecular and physiological information.

# 2

## Gene Structure and Predicted Protein Sequence Analysis of the *AtNRT* Gene Families

### 2.1 Introduction

Since the first nitrate transporter gene, *AtNRT1.1* (previously called *CHL1*), was isolated from higher plants (Tsay et al., 1993), three more genes were identified as *AtNRT1* homologues in *A. thaliana*. Although *AtNRT1* proteins are considered to be nitrate transporters, this family also belongs to a family of peptide transporters (PTR) (Pao et al., 1998). Seven *AtNRT2* members encode high-affinity nitrate transporters, belonging to the nitrate/nitrite porter (NNP) (Pao et al., 1998) family, which is a subfamily of the major facilitator superfamily (MFS) (Pao et al., 1998). Typical MFS members possess 400-600 amino acid residues with 12-trans-membrane-spanner (TMS) which consists of two sets of 6-TMS.

As genome projects have developed, a huge genome information base has been built up from viruses and bacteria to eukaryotes including *Homo sapiens*, and higher plants (e.g., *Arabidopsis thaliana*, and *Oryza sativa*). In order to interpret and apply those enormous biological databases, biological informatics (so called bioinformatics) has been developing rapidly. The advantage of bioinformatics is that we can characterize and predict the function of genes and gene products from various perspectives prior to initiating experiments. This is helpful for proteins such as membrane proteins, which are difficult to work with because of their hydrophobicity.

In this chapter, gene structures and predicted protein sequences of all 11 members of *AtNRT* families will be presented. As well, further bioinformatic searches including *cis*-elements, signature motifs, transmembrane topology, and phylogenetic analyses were carried out based on the gene and protein sequences using a computational approach.

## **2.2 Methods**

### **Bioinformatics**

DNA and predicted amino acid sequences were obtained from previous reports or from the online databases (accession numbers are provided in Table 2-1). *AtNRT2.1* and 2.2 were isolated by our group (Zhuo et al., 1999), and Filleur and D-Vedele (1999), and the sequence of *AtNRT2.1* was used for a homology search, which was carried out on the BLAST server (<http://www.ncbi.nlm.nih.gov/blast>). Nucleotide motifs of *cis*-acting regulatory DNA elements were searched with the PLACE database (Higo et al., 1999; <http://www.dna.affrc.go.jp/htdocs/PLACE>). CLUSTAL W or X was used for an initial

Table 2-1. NRT(nitrate transporter) families in *Arabidopsis*

Gene (Alternative name)	Chromosome Location	Amino acids	Identity <sup>a</sup> (%)	Accession number		References
				DNA	Protein ID	
NRT2 family						
<i>AtNRT2.1 (ACH1)</i>	1	530	100	AF019748	AAC35883	Filleur et al., 1999; Zhuo et al., 1999
<i>AtNRT2.2 (ACH2)</i>	1	522	87	AF019749	AAC35884	Zhuo et al., 1999
<i>AtNRT2.3</i>	5	539	68	AB015472	BAB10099	
<i>AtNRT2.4</i>	5	527	82	AB015472	BAB10098	
<i>AtNRT2.5</i>	5	493	44	AL163792	CAB87624	
<i>AtNRT2.6</i>	3	542	68	AL353992	CAB89321	
<i>AtNRT2.7</i>	1	502	56	AC012187	AAF78499	
NRT1 family						
<i>AtNRT1.1 (CHL1)</i>	1	590	100	Q05085	AAA32770	Tsay et al., 1993
<i>AtNRT1.2 (NTL1)</i>	1	585	34	AF073361	AAC28086	Huang et al., 1999
<i>AtNRT1.3 (NTP3)</i>	3	590	47	AB019232	BAB02362	Hatzfeld et al., 1999
<i>AtNRT1.4 (NTP2)</i>	2	586	51	AC003105	AAB95302	Hatzfeld et al., 1999

<sup>a</sup> Percentage identity at the amino acid level.



alignment analysis (Thompson et al., 1994). Transmembrane prediction was performed by HMMTOP (Tusnady and Simon, 1998), MEMSTAT (Jones et al., 1994), SOSUI (Hirokawa et al., 1998), TMAP (Persson and Argos, 1994), TMHMM (Sonnhammer et al., 1998), Tmpred (Hofmann and Stoffel, 1992), and Toppred2 (Claros and von Heijne, 1994). Other bioinformatic programs including Bioedit (Hall, 1999), GeneDoc (Nicholas and Nicholas, 1997), Sequence Assistant (<http://www2s.biglobe.ne.jp/~haruta/>), AnnHyb (<http://annhyb.free.fr>), and Altemis (<http://www.sanger.ac.uk/Software/altemis>) were also employed to support the analysis.

## **2.3 Results and Discussion**

### **Gene Structure of the AtNRT2 Family**

Besides the first two *AtNRT2* genes (*AtNRT2.1*, and 2.2) cloned experimentally (Filleur and Daniel-Vedele, 1999; Zhuo et al., 1999), five more homologues were retrieved by a homology search against the database generated by the Arabidopsis genome project (Table 2-1). Three genes (i.e., *AtNRT2.1*, 2.2, and 2.7) are located on chromosome 1, where *AtNRT2.1* and 2.2 are closely located in a tail-to-tail configuration (Zhuo et al., 1999). *AtNRT2.3*, 2.4, and 2.5, were found on chromosome 5, with *AtNRT2.3* and 2.4 in tandem 3.8 kb apart (Forde, 2000) (Figure 2-1).

The open reading frames (ORF) of *AtNRT2.1*, 2.2 and 2.3, which were interrupted by two introns in the same positions, encode 530 (57.7 kD), 522 (56.7 kD), and 539 (58.2 kD) amino acid residues, respectively (Figures 2-2, 2-3, and 2-4). The *AtNRT2.4* gene has

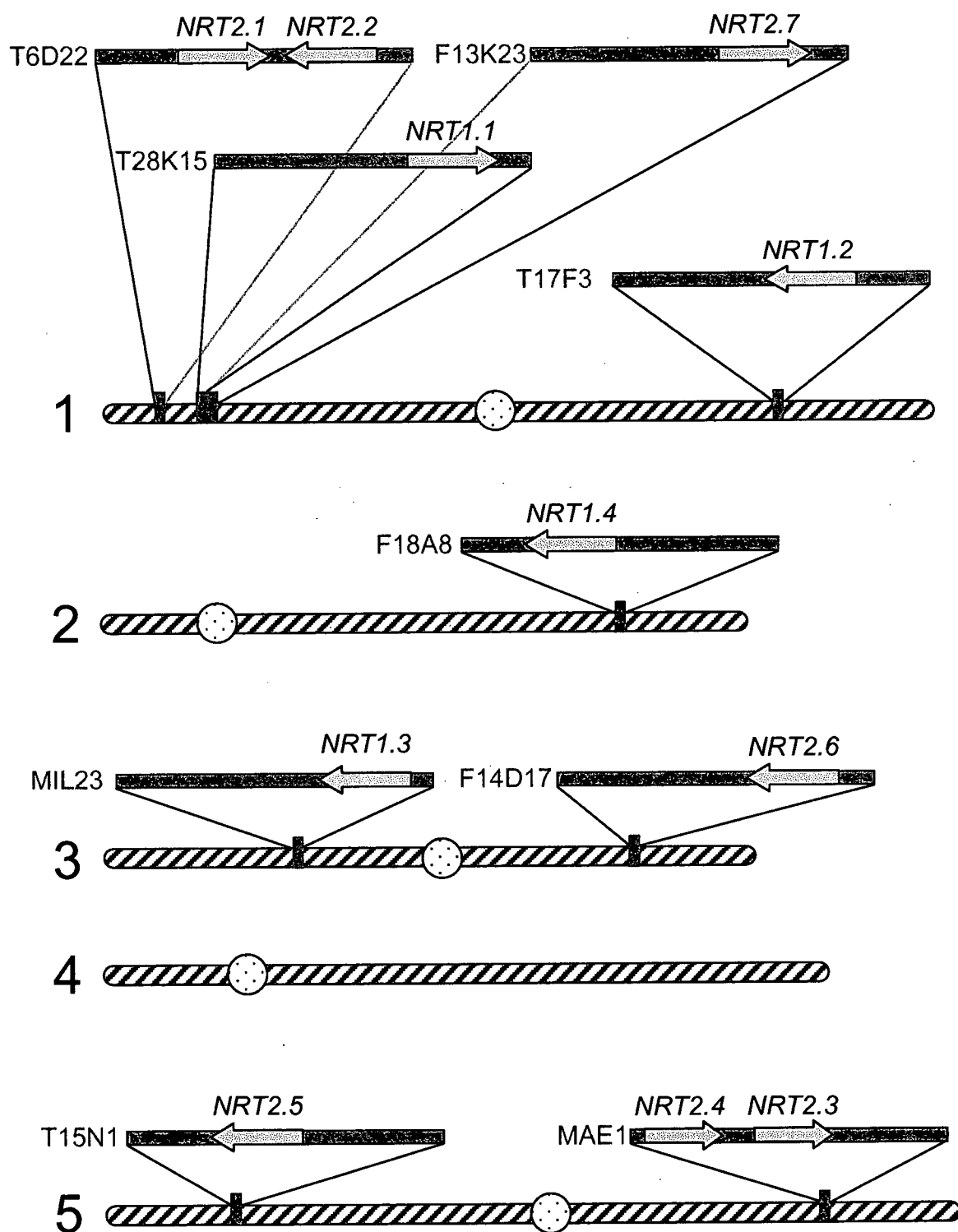


Figure 2-1. Physical map of *AtNRT1* and *AtNRT2* gene family members. Chromosomes and centromeres are indicated in stripes and dots, respectively. BAC clones are indicated in arrows.



-1326 TCAAACCCCAACAGGCCTCAACAGAGGGAACACCGGCCACGTACCAAGATTGATGTATATACGCTAGTGGCACGGAATGG  
 -1244 TACGGCATAAAATATACGAATACAACACTCTTTCTTGTATATTTAAGACGTATCTACAACCTACATTGTAATATATTTTG  
 -1162 GGTATATAATTTAGAACAAAGACGTATAGCTCTACCTATCAATTCGAGGAGTCGATTTAATACCTACCTATTTCTTTATGTAA  
 -1080 CTATTCTTATAAACACGTGATTAGGAAGTTTCTTGTTCATGTATGATGTTTATGTCACATATTTATATTTATGATTTTTTTG  
 -998 GTCTGAGTTGTTTAAATCAATTTCCAAAGCAGCAACCATTTTCCAGCAACTGAAACCATTTAAACAAAATTTACCCAAAA  
 -916 AATTAAGAAGATTTTGAATTTTGAATTCGAAATCAGACCACCTCAAAGTCAAATCTTATATATTTTATGTTTATATCCAAAGCATAAT  
 -834 GCATATATTTTCATAGTTTGTGGGAATCCAAGTTTCTAAATTTATTCAAATTAAGTCATTAATTAATCCATGTTTAAAT  
 -752 AATCAAAGGACATGTGAAATTCGTAATGCCAGTCAAATTACATTACAACCAACTAAAATATCGAACAAATTAAGTTATGTCT  
 -670 ATATGTGCTATACAAAATTAATCTAATTTTACAGTGTATATATGGAATAATATGATAAGTTTGTGAAGTAATAAGCTTA  
 -588 TTGATTTTTTAGAGACAACATAATGTGCAGCTAAGGCCACAACAAATTAATCGTCCAGGAGACAGCAGTTTATCCACCACAA  
 -506 TCATTCCTATTTCTGATACGATGTGATATAGAATTACATTGATACCCCTTAAGAAATATGAAGAATATATGGTCTAAAAAGAG  
 -424 AATCACAATGACATCGCTTAAAAATTAATTTAAATATGGATTTTAATGAGTTCACGATGTGGTGCTTGATCTCTGTATAAGAT  
 -342 AATATGTATTACAGACATGTAAAAAATAATCCACTTCTGAAACACAAAAGGAGAAAAGGATACAGTTGTCACCTTGTCTATCT  
 -260 GAGAGGTGAATATCGGTAACCTTTGGGGATTAGCGAGAGAGTGGGAAAGATGCTTGCGGCGAATATGGATTCTCCGAAAAA  
 -178 ATGTATTTTAAATATGTTTGTAGTTAGCCGTTGAGAACTCCAAAGAATACTTTTCTTTTCTTTTGTGAATTTGCAAAAGCT  
 -96 ATACGAATCCTATATAATCCCTCGTGTGACTAATATCTAGTACATCAATCATATAAACTTGAATTTTCTCAAAGGAACCTTGA  
 -14 TACGTTTAAATATACATGGGTTCTACTGATGAGCCCGGAATTCCTATGATGAGATTACCGGTAGAGAACAGAGCTATGCTTT  
     M G S T D E P G S S M H G V T G R E Y A F 23  
 69 CTCGGTAGATGGTAGTGAGCCGACCAACACAAGAAAAAGTACAATCTGCCGGTGGACGCGGAGGATAAGGCAACGGTTTTTC  
     S V D G S E P T N T K K K Y N L P V D A E D K A T V F 50  
 151 AAGCTCTTTCCTTCGCCAAACCTCACATGAGAAGTTCCACCTCTCGTGGATCTCTTTCTCCACATGTTTGTTCGACGT  
     K L F S F A K P H M R T F H L S W I S F S T C F V S T F 78  
 233 TCGCAGCTGCACCACTTATCCCGATCATCAGGGAGAATCTTAACCTCACCAACATGACATTGGAACGCTGGAGTTGCCTC  
     A A A P L I P I I R E N L N L T K H D I G N A G V A S 105  
 315 CGTCTCGGGGAGTATCTTCTTAGGCTCGTGATGGGAGCCGTGTGTGATCTTTGGGTCTCTGTTACGGTTGTGCCTTCTCTT  
     V S G S I F S R L V M G A V C D L L G P R Y G C A F L 132  
 397 GTGATGTTGTCTGCCCAACGGTGTCTCCATGAGCTTCGTGAGTGACGCGAGGCTTCATAACGGTGAGGTTTCATGATTG  
     V M L S A P T V F S M S F V S D A A G F I T V R F M I G 160  
 479 GTTTTTCCTGGCGACGTTTGTGTCTTGTCAATACTGGATGAGCACTATGTTCAACAGTCAGATCATCGGCTGGTGAACGG  
     F C L A T T F V S C Q Y W M S T M F N S Q I I G L V N G 187  
 561 GACAGCAGCGGATGGGAAACATGGGTGGCGGATAACGCAGTTGCTCATGCCATTGTGTATGAAATCATTAGCGCGCTGC  
     T A A A G C W G N M G G G I T Q Q L L M P I V Y E I I R R C 214  
 643 GGATCAACAGCGTTTACGGCTGGAGGATCGCCTTCTTQCTCCCGGTTGTTGCACATCATCATGTTGGAATCTTGGTGTCA  
     G S T A F T A W R I A F F V P G W L H I I M G I L V L T 242  
 725 CGTAGGTCAAGATCTGCCAGGTGGAACAGAGCTGCCATTGGAGAAAGCGGAGAGTTGCCAAAGACAAATTCGGAAGgt  
     L G Q D L P G N R A A M E K A G E V A K D K F G K 268  
 807 atatctctatctacatgaatatgtcaaaacaagtttaggctaactatgtcatagatggatcgatataataggctaactcgtg  
     intron I  
 889 atatcgataaatcggttagATTCTATGGTACGCCGTTTACAAATACAGGACTTGGATTTCGTTCTTCTGTATGGATATTC  
     I L W Y A V T N Y R T W I F V L L Y G Y S 289  
 971 CATGGGAGTTGAGTTAAGCACAGACAATGTTATCGCCGAGTACTTCTTTGATAGgtttgtttctgtctcgattggaaattg  
     M G V E L S T D N V I A E Y F D R 308  
 1053 acaacttcacatatattcaggaataacataagagtaaaatttatcccttttattttttttgtcaatcggtccctttt  
     intron II  
 1135 attttaaataaaaaaatggtatgttcagGTTTCACTTGAAGCTTACACAGCGGGGATTATAGCAGCATGTTTCGGAATGGC  
     F H L K L H T A G I I A A C F G M A 325  
 1217 CAATTTCTTTGCTCGTCCAGCAGGAGGCTGGGATCTGACATTCAGCCCAAGCGCTTCGGAATGCGAGGAGGTTGTGACT  
     N F F A R P A G G W A S D I A A K R F G M R G R L W T 352  
 1299 TTGTGGATTTTACAGCGTCCGGTGGTCTCTTTTGTGTGGTCTGGGACGTCGCCAACACCTCGTCACTGCCGTTGTATCTA  
     L W I I Q T S G Q L F C V G L G R A N T L V T A V V S M 380  
 1381 TGGTCTCTTCTCTTTAGGAGCACAAGCCGCTTGGGAGCCACCTTTGCTATCGTGCCTTTTGTCTCCCGGCGAGCTCTAGG  
     V L F S L G A Q A A C G A T F A I V P F V S R R A L G 407  
 1463 CATTATCTCGGTTTAAACGGGGCTGGAGGGAACTTGGGTGAGGACTCACACAGCTCGTCTTTTCTCGACTTCGCGCTTC  
     I I S L G A T G A G G N F G S G L M T Q L V F F S T S R F 434  
 1545 ACACTGAAGAAGGCTAACGTGGATGGGAGTGATGATGTTGCTGACGTTGCTGTACCTTAATCCACTTTTCTCAGT  
     T A E G L T W M G V M I V A C T L P V T L I H F P Q W 462  
 1627 GGGGAAGCATGTTCTTCCCTCCTTCCAACGATTCCGTCGACGCTACGGAGCACTATTATGTTGGCGAATATAGTAAGGAGGA  
     G S M F F P P S N D S V D A T E H Y Y V G E Y S K E E 489  
 1709 GCAGCAGATTGGCATTTTAAAGCAAACCTGTTTGTGATGGAGCCAGACCGAGGAGGCAGCAGCTCCACAAGGG  
     Q Q I G M H L K S K L F A D G A K T E G G S V H K G 516  
 1791 AACGCAACCAACATGCTTGTATGATGATCAAGAAATTAATAATTTCACTTATGTGAAATGGACATAAACTGT  
     N A T N N A \* 522  
 1873 TGGAAATAAAGAACCATTTCTTTTCATCATTTGCTTTTA

Figure 2-3. DNA and deduced amino acid sequences of the *AtNRT2.2* gene. The number on the left starts at the proposed start codon, and the numbers on the right refer to amino acid residues. Intron sequences are shown in lower cases. GATA boxes are underlined. TATA box is indicated with thick underline. Nitrate-dependent transcription motifs are double underlined.

-1462 TATATATATAGAACTAAAACCATACAAAAAGAAAAGATTTCGCCCTTTTAAATGATGGATAATTCTAAAGCCTATAAGACTT  
 -1380 AAACCTTTTATTTAAATCTACAGTTTGTGCCCCAAATAAAATGTGATAAGTATAGAGATAACAAAATATAAAATTATTTCTT  
 -1298 ACCAAAAAATATATATATATATATAAGACTATTTTACCGTAATTTATTTGATGATTGCAGACAATACAAGATATCTCT  
 -1216 AGTTTACAAAAATATCATCAACAATATTCGAGAGAATAGTTCCTTTTGAGTATTTGCGGATGGCCGGAACAACAACACTTAG  
 -1134 AAATCATATTTAGTAAAGAATTATGTTTAGAGTTTAGAGTTTATAGCTTATATATTATTTAGATGATGATGATTATTAAG  
 -1052 GAAAAACATAAGGAAGTCAACTTATCATAACGCTTCATTGTAAGGATATGCGAATATCAAAGCGGAAAGATTTAGAATAAG  
 -970 TGAATAACCCATAATTTTCATAACGTTAGAGCAATTTTTCAGGAAATTTTAAAGATAGCAATTTATTCATTGGGTGAGTC  
 -888 GACCACATGGAATCCTCAAGAGACAAATGAAAAATAATTAGACTTTTTCGACGATTTTTCGCTTCATTGATACGTACGCGCTC  
 -806 TTAGGCTTTTTCAGTATTTCCAATGCCAAGTCAACATTCGTATCTCTTTCGCCATTTTAGTGAATTTCCACAAAGTTCATT  
 -724 ATCTTTGATGATATAACTTGCCAGGCTGCTACTATACCAATAGATTTATAAATAATTATTTTTCAGTTTCTTTCCATACT  
 -642 TTTTAATTTCTCAACGAACTTTATGCAAGTTGAAATAAAGTTTCTCTGCAATTTCACTTAAATTTATAATTTGTCAAAC  
 -560 AGAAGTGCAACATTTGATTTTCTGACTGAAATTTTCAACCTTACAAAAGTTGCTTGAACCTTTGTTAGAAAAAGAAAAAT  
 -478 ACTAATACCTTGAATAACCTCAGGTTCCCTATTTTAAACCAAAACCTTCAAAATCAAAGACTCAAGAATCAATTCATAATG  
 -396 TGTGAGACAAAACCTCATAACCCAAATATGGTACACGTACGTATGAAACGTACTTAAATGCTACAAAAGAATAATATGTTTAG  
 -314 TTCTCAAAATTTAAATAATTTTTCCTTCTATCATTAATCAAAATTTACAACCTATATCTATACTAAATGCTATATCAGC  
 -232 TCAACATAATAAAATTTTCAAAATTTGGTAAAGTCTTTTAACTTTTACTTATCTTATTTCAAAATCTATTATTTGGACCCAC  
 -150 TAACCCCTACCCACCATATGATATTTGGTATCCCTCTGGCCTCTATATAAACTTTGCAATTTCTCTAATCTCAACTTACACC  
 -68 AACAAAACACAACAAAGACCATTAGCCTTCTCTCCACTCAGAGTCTTTTCATCTCACTCTCGCCTTAAATGACTCACAACCA  
 M T H N H 5  
 15 TTCTAATGAAGAAGGCTCCATTGGAACCTCCTTGCAATGGAGTTACAGCAAGAGAACAAGTCTTCTCTTCCGTCGATGCT  
 S N E E G S I G T S L H G V T A R E Q V F S F S V D A 32  
 97 TCGTCTCAACAGTCCAATCAGACGATCCAACAGCTAAATTCGCCCTTCCGGTTGATTCCGAACATCGAGCCAAAGTGTTC  
 S S Q T V Q S D D P T A K F A L P V D S E H R A K V F N 60  
 179 ACCCATCTCTTTTGCTAAACCTCAGATGAGAGCCTTCCACTTAGGATGGCTCTCATTCTTCACATGCTTCATCTCCACCTT  
 P L S F A K P H M R A F H L G W L S F F T C F I S T F 87  
 261 CGCGCAGCACCATTAGTCCCATCATCCGCGACAACCTCGACCTCACTAAAACCGACATTGGAACGCGCGAGTCGCATCC  
 A A A P L V P I I R D N L D L T K T D I G N A G V A S 114  
 343 GTCTCTGGTGCCATTTTCTCAAGGTTAGCCATGGGAGCGGTTGTGATCTCTCGGTGCACGATATGGGACTGCCTTCTCCC  
 V S G L A F S R L A M G A V T D L L G A R Y G L V N G 142  
 425 TCATGCTAACCGCCCCAACCGTCTTCTCAATGTCGTTTGTGGGTGGCCCTAGCGGATACTTAGGCGTCCGGTTTCATGATCGG  
 M L T A P T V F S M S F V G G P S' G Y L G V R F M I G 169  
 507 ATTCTGTCTCGCCACGTTTGTATCATGCCAGTATTGGACCAAGTGTATGTTCAACGGTAAGATCATAGGACTAGTGAACGGC  
 F C L A T F V S C Q Y W T S V M F N G K I I G L V N G 196  
 589 TGTGACGGCGGGTGGGTGATATGGGCGGTGGAGTGAACCTCAATGCCGATGGTCTTCCACGTCATCAAACTTGCCG  
 C A G G W G D M G G G V T Q L L M P M V F H V I K L A G 224  
 671 GAGCCACTCCGTTTCATGGCTGGCGGATAGCTTTCTCTCGTTCCCGGATTTCTTCAAGTTGTTATGGGCAATCTCTGCTCCTCAG  
 A T P F M A W R I A F F V P C G F L Q V V M G I L V S 251  
 753 TCTCGGCCAAGATCTCCCTGACGGTAACCTAAGTACCCTTCAAGAGTGGTCAAGTCTCTAAAGACAAATCTCCAAGta  
 L G Q D L P D G N L S T L Q K S G Q V S K D K F S K 277  
 835 cttttctatatatatatttctgtctcatcttcaaatatatacaaaatccagaccaagaagaatatggctctcattaaaaag  
 intron I  
 917 aatcttgcaaacattaaacatactcatgaagttttgcaataaattcggtgaagaactatttcggtttatattagttaaataaatt  
 999 tctagtgtctaaaggtttttctgttatagGTTTCTGGTTTGTGTGAAGAATACAGAACATGGATTTTATTCTGTTCTTT  
 V F W F A V K N Y R T W I L F V L Y 295  
 1081 ATGGATCTTCCATGGGAATTGAATTAACCTATCAACAACGTTATCTCCGGATATTTTACGACAGgttcgatttcttattcgt  
 G S S M G I E L T I N N V I S G Y F Y D R 316  
 1163 taaaacccaactgatgaacttacttacttctgtcacaaggtaataacctaatttggttttctgttctctacgtatttatca  
 intron II  
 1245 gGTTTAAACCTTAAGCTTCAACAGCTGGTATAGTAGCAGCCAGCTTTGGAATGGCTAACTTCATCGCCCGTCCCTTCGGTGG  
 F N L K L Q T A G I V A A S F G M A N F I A R P F G G 343  
 1327 TTACGCTTCTGATGTAGCGGCTCGGTTTTCGGCATGAGAGGCCGGTTATGGACCTTATGGATCTTTCAACCGTAGGAGCT  
 Y A S D V A A R V F G M R G R L W T L W I F Q T V G A 370  
 1409 CTTTCTGTATCTGGCTAGGTCGAGTACGTTTCACTCCCATAGCAATCCTAGCAATGATGCTCTTCTCAATCGGTACACAAG  
 L F C I W L G R A S S L P I A I L A M M L F S I G T Q A 398  
 1491 CAGCTTGGGAGCCCTCTTCGGAGTTGCACCTTTTGTCTCGCGCGCTCTCTAGGCTCATATCGGGACTAACCGCGCAGG  
 A C G A L F G V A P F V S R R S L G L I S G L T G A G 425  
 1573 AGGAACTTCGGGTCGGTTTGACTCAACTGCTTTTCTCTCATCAGCAGGTTTATGACAGTGAAGGACTCTCATTGATG  
 G N F G S Q E E Y Y Y G S E W T E N E K Q Q G L H E G 452  
 1655 GCGTATGGCGGTTTGTGCACACTCCAGTTGCGTTTATACATTTTCCGCAATGGGAAGCATGTTTAAAGACCGTCA  
 G V M A V L C T L P V A F I H F P Q W G S M F L R P S 480  
 1737 CCGATGGAGAAAGATCACAGGAGGAATATTATACGGTTCTGAGTGGACGGAGAATGAGAAACAAGGATTGCACGAAG  
 D G E R S Q E E Y Y Y G S E W T E N E K Q Q G L H E G 507  
 1819 AAGCATCAAATTTGCAGAGAATAGTAGGTCAGAGAGAGGCCGGAAGTAGCTTTGGCTAACATTTCAACGCCGAGAACGGA  
 S I K F A E N S R S E R G R K V A L A N I P T P E N G 534  
 1901 ACTCCAAGTCATGTTGAAGACAAACTTACAAGAGATTTTCCTTTTACATTAAATCCCCTTTTAAATAGTTTCATACTCCAC  
 T P S H V \* 539  
 1983 TCATATATGTACCGAGTAAGACCTTTTCTGTGATAGGTAAGGATCGATGAGCTTGGTACTTTAGTATAAATTATGTAATGT  
 2065 CATCAGAATGTTCAACTACTCTTTTCTGCAACAAAGCTTTATGTTCAATCAGATTTACATGTCAAGTGAAAAAACAA  
 2147 AAAAGTCTCTGTTTACGGTGTGTGAATGCTAAGAGAGTCCACATGAAGAAAACCTTTATGGACAAGGACCA

Figure 2-4. DNA and deduced amino acid sequences of the *AtNRT2.3* gene. The number on the left starts at the proposed start codon, and the numbers on the right refer to amino acid residues. Intron sequences are shown in lower cases. GATA boxes are underlined. TATA box is indicated with thick underline. Nitrate-dependent transcription motifs are double underlined.

3 introns and 4 exons, containing introns 2 and 3 in the same position as those of *AtNRT2.1-2.3* (Figure 2-5). *AtNRT2.5* and *2.6* have one short intron (Figures 2-6 and 2-7). The ORF of *AtNRT2.5* encodes 493 amino acids (52.7 kD) which is the smallest protein among the NRT families, whereas *AtNRT2.6* encodes the largest transporter (58.6 kD) in the NRT2 family. *AtNRT2.6* shares the same intron position as the first intron of *AtNRT2.1-2.3*. Two introns were found in the *AtNRT2.7* gene, the second intron having the same position as that of *AtNRT2.5*, while the first one is unique (Figure 2-8).

Putative TATA and GATA boxes were found in the promoter regions of all members of the *NRT2* genes. Some GATA boxes were separated by 2-22 bp except in *AtNRT2.7*. Rastogi et al. (1997) found that the important region of spinach NiR promoter contained GATA consensus sequences located 24 bp apart, harboring a potential binding site for a NIT2-like transcriptional factor. Nitrate-dependent transcription motifs (an AT-rich region followed by [AG]-[CG]-T-C-A) (Hwang et al., 1997) were observed in all *AtNRT2* family members at -500 to -1000 bp from the start codon. However, those positions might be too far upstream. In the cases of nitrate reductase (NR) and nitrite reductase (NiR), the motifs were found at -140 to -250 bp upstream in Arabidopsis (Hwang et al., 1997).

The seven *AtNRT2* members encode predicted proteins consisting of 493-539 amino acids, which are typical membrane proteins. Membrane spanning regions and topologies were analyzed with prediction programs including HMMTOP (Tusnady and Simon, 1998), MEMSTAT (Jones et al., 1994), TMHMM (Sonnhammer et al., 1998), TMPred (Hofmann and Stoffel, 1992), and Toppred2 (Claros and Von Heijne, 1994). Although it is generally



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2435 AATGGGGAAGCATGTTTTTGCCTTCCACGGAAGATGAAGTGAAGTCTACGGAGGAGTATTATTACATGAAAGAGTGGACAGA
      W G S M F L P S T E D E V K S T E E Y Y Y M K E W T E 490
2517 GACCGAGAAGCGAAAGGGTATGCATGAAGGGAGTTTGAAGTTCGCCGTGAATAGTAGATCGGAGCGTGGACGGCGCGTGGCT
      T E K R K G M H E G S L K F A V N S R S E R G R R V A 517
2599 TCTGCACCGTCTCCTCCGCCGAACACGTTTAAGAGTTTTTAAATGTACATGTTGTATCAGTTTCTATATATTTCTCCAAAT
      S A P S P P P E H V * 527
2681 GTGTAATTAGTTGATCGAATTGCATGCTTTAATTTCTTTTAAAGGATATGCTACTCTTCATGTAATTCGTAACAATTATAT
2763 TGTTTTTTTAAATTCGTA

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Figure 2-5. (continued).

The number on the left starts at the proposed start codon, and the numbers on the right refer to amino acid residues. Intron sequences are shown in lower cases. GATA boxes are underlined. TATA box is indicated with thick underline. Nitrate-dependent transcription motifs are double underlined.



-1426 CTAGATTATCAGTTCCTGCAGTTGTCTATCCGTACTGCTGTGGAGTGTAATGAGGCAGAGAAATCTTCCCCTGTGAACGACAA  
 -1344 CGACAAACCAGACGCGCTGCTACATCAAAGCCAGACCGGAAACCCAAATCCACAGCCTCTAAACTGATTGCGACACAGAAG  
 -1262 GAAGAGGAGGCACTCGAATCCATTGCTCCGAGAAGAACTTCAGCTGAATGTGGTGAGTACTGAAGCAGGACGGGAAGCTAA  
 -1180 AATCCGTTTCTCCAAAAACAATAGCACTGCTTCTAACCTAGTATCATTTTCAAAGCTAAGAAATCAACTATGAAGGAGAA  
 -1098 TCTATCAGAGAACAAGGCTGAAGAGAGTGCATCGGTTAGTACCAGGAAACTGAAAATAGGAACGAGATGACAGCCACGGTC  
 -1016 GACCATGTTCGAGCCCTTGGATTGGTTCTTGATTAGGTGGTGAAATCCGCGGTATGTACATATCCAGGTATGTTTCAGTA  
 -934 AATTCTCCTTTGACTTGTCTCCCATTTTCTTGAGTTAACTTTTGAGGCGATATCTTAACTCTTGCTTTGTATTGATATTG  
 -852 TGTACTATGAATGAGTACAGGGAGATAAGTTCAAAAAGGAGACACATTGCGAGTGAATGTACAAGTTTAAACAC  
 -770 CAAAGGAGTCCCTGTGATGGCTTTGGTTGACGAAGAGGAGAGAAGATAGTGGTAAACTCTCTTTCTTTCTACAGGTTT  
 -688 TCAACCCAAGGGATCATGTGGAGTCGATCATATGGATCATACGAGAGTTTGTCTTGAATGTTTTATCATACGGATCGAAT  
 -606 CAAGAGATGCTCGTTAGCTGCTTTTGGTGAGCAATCTTGGTAGATGGATAGATTTCATTGGCGTCACAACCTTGAGGAAAGCT  
 -524 AATGAACTCGGTTAGGTAAGTCTTTTACTTTTGGCCTTGTGATGTGCCATTTTGTCTCTTTTATTGGGGGGGTTTGT  
 -442 GGAACCGAAAGTGGATTGTATTGTATTAGTGTCTTGTAGTAACTGACGCTCTGAAAGGTGCGTAACGCAAGTTTCATCATTTTGT  
 -360 CCAATTTTACAAAAATACAAAAGCACAAAACCAAGTCGGCAGCTAAAGATAGAACTACGCATCCCAATGGCATTCCTT  
 -278 GATAGATATTATTTGTCGGAATATATTTCTTCTAAATTACAAATTCATTTCACAATTGACGTCAAAAATTAATTTAAGCC  
 -196 AAGAGACGAGGAACATAAATTTGAATTTACATTTTGATTGAGCATTTGGGTTTGAATTAAGATTACTCGAAAGTTGCCAAAA  
 -114 GTTTGCCCTCGAGTCATTTTATTGTTCATGAAAGATTAAATTCAAAGATAATGTTTCATTGATTGGGTGGTCTGTAAGTAA  
 -32 GAGTCGTACGCGAGTTTCTTCTCCGAGAGTAATGGAGCCATCTCAACGCAACACCAACCGCGCTCGTTTTCAGATTCCAC  
 M E P S Q R N T K P P S F S D S T 17  
 51 TATCCCCTGTTGATTCCGATGGTTCGAGCCACCGTCTTCCGACCATCTCTCTCTCTCCGACACTCACGAGCCTTTCACCTA  
 I P V D S D G R A T V F R P F S L S S P H S R F H L 44  
 133 GCTTGGCTCTCACTCTTCTCATGCTTCTTCTCCACCTTCTCCATCCCTCTCTGGTCCCCGTATCTCTCCGACCTCAACC  
 A W L S L F S C F F S T F S I P P L V P V I S S D L N L 72  
 215 TCTCTGCCTCCACCGTATCCGCGCGCGAATCGCTTCCCTTCGCTGGCTCCATCTTCTCTCGCCTCGCTATGGGACCACTG  
 S A S T V S A S I A S F A G S I F S R L A M G P L C 99  
 297 TGATCTCATCGGACCAGTACTTCTCTCAGCGATTCTCTCTTCTCACCGCTCCTGTAATCCTCTCCGCTCACTCGTCTCC  
 D L I G P R T S S A I L S F L T A P V I L S A S L V S 126  
 379 TCTCCGAGCTCTTCATCTCTCGTCCGTTTCTTCTCGTGGCTTCTCGCTCGCTAATTTTCGTAGCCAACTAATCTGATGTCCT  
 L M S P I L V R F F V G F S L A N F V A N Q Y W M S S 154  
 461 CCATGTTCTCCGGTAACGTCATTGGTCTCGCTAACGGTGTCTCAGCGGTTGGGCTAACGTCGGCGCGGTATCTCTCAGCT  
 M F S G N V I G L A N G V S A G W A N V G A G I S Q L 181  
 543 CCTTATGCTCTCATATACTCCACCATAGCCGAATTCCTTCCACGCGCGCTCGCTGGCGCGTGTCTCTGATTTTCCCGCC  
 L M P L I Y S T I A E F L P R A V A W R V S F V F P A 208  
 625 ATTTTTCAGGTTACACGCGCGTCTCGTTTCTCTACGCGCAAGATATCCCCACGGTAACAGAAAAAATCGAACCAGA  
 I F Q V T T A V L V L L Y G Q D T P H G N R K N S N Q N 236  
 707 ACAAACTCACAATTCCTGAAGAAGAAGTACTAGTAGTTGAAGAAGACGAACGTTCCAGTTTCGTGAGATCCTTAATCGG  
 K L T I P E E E E V L V F E E D E R S S F V E I L I G 263  
 789 CGGACTTGGAAATTACAGAGCGTGGATCTTAGCGCTGCTCTACGGATACTCGTACGCGCTGAGCTAACGACGGACACGCTG  
 G L G N Y R A W I L A L L Y G Y S Y G V E L T T D N V 290  
 871 ATCGCCGATATTTCTACGAGAGATTGGAGTGAATCTGGAGGCGGCGGACGATCGCGCGAGTTTCGGGATATCGAACA  
 I A G Y F Y E R F G V N L E A A G T I A A S F G I S N I 318  
 953 TTGCGTCTCGGACCGCGGGAGGGATGATATCGGATCGCTGGGGAAGAGATTCCGTATGAGAGGGAGGCTGTGGGGCTATG  
 A S R P A G G M I S D A L G K R F G M R G R L W G L W 345  
 1035 GATCGTGCAATCGGTGGCTGGGTGTTGTGTCGTGTTACTCGGACGAGTCAACTCGCTCTGGGATCAATCCTCGTCAATGCG  
 I V Q T S V A G L L C V L F V R V N S L W G S I L V M W 372  
 1117 GTCTTCTCTGTTTTCGTTCAAGCTGCTTCTGGCCTTGTATTTGGCGTGGTCCCTTTCGTCTCCACGCGgttagttaaagtc  
 V F S V F V Q A A S G L V F G V V P F V S T R 395  
 1199 taccaatccggtttttgctaataatttcggtttggttttaattggtttgtttataatgacagATCGTTAGGAGTGGTGGC  
 intron S L G V V A 401  
 1281 GGAATTACGGGAAGCGGCGGTACGGTTGGTGGCGGTGGTACGCGAGTTTCTGTTGTTTCCGGTGATGATGTTCGAAACAG  
 G I T G S G G T V G A V V T Q F L L F S G D D V R K Q 428  
 1363 AGAAGCATTTCACTTATGGGTTTGATGACTTTTGTGTTTGTCTTTCTGTTACATCAATTTACTTTCCACAATGGGGTGAA  
 R S I S L M G L M T F V F A L S V T S I Y F P Q W G G M 456  
 1445 TGTGTTGTGGGCTTCGTCATCTTCCGAAGAAGAAGATATTTCTCGGGGACTCCTTGTAGAAGACGAAGATGAAGAAGTAA  
 C C G P S S S S E E E D I S R G L L V E D E D E E G K 483  
 1527 AGTGGTTAGTGGTAGTCTACGTCGCCGTTTGTGTAGTTAGTTTAAAGTCTACCAATCCGGTTTGTCTAATAATTTCCGTTTG  
 V V S G S L R P V C \* 493  
 1609 GTTTTAAATTTGGTTTGTATTATAATGACAGATCGTTAGGAGTGGTGGCGGGAATTACGGGAAGCGGCGGTACGGTTGGTGGC  
 1691 GTGGTGACGCACTTTCTGTTGTTTCCGGTGATGATGTTTCAAAAACAGAGAAGCATTTCACTTATGGGTTTGTGACTTTTG  
 1773 TGTTTGTCTTTCTGTTACATCAATTTACTTTCCACAATGGGGTGAATGTGTTGTGGGCTTCGTCATCTTCCGAAGAAGA  
 1855 AGATATTTCTCGGGGACTCCTTGTAGAAGACGAAGATGAAGAAGTAAAGTGTTAGTGGTAGTCTACGTCGCCGTTTGTGTA  
 1937 TGTGCACTTTGAACTGCTGGCCCA

Figure 2-6. DNA and deduced amino acid sequences of the *AtNRT2.5* gene. The number on the left starts at the proposed start codon, and the numbers on the right refer to amino acid residues. Intron sequences are shown in lower cases. GATA boxes are underlined. TATA box is indicated with thick underline. Nitrate-dependent transcription motifs are double underlined.

-1426 TTTCATATCTTATGGTATTATTGGGTAATTGAAAGCAAAAAAAAAAATGAATTTTGAATAAAGTTGATACAACACTATTTTC  
 -1344 CAAAAGTAAAAATTAAATTGATGAGTCGACCACATGGAAATACACAAAAGACAACTGCAATTATTTTGAAGAATGAGCTTT  
 -1262 TTACAATGAACCGTACGATCTCATAGTCTCAITTGACCTTTGGTGTGATGTCAAATGTCAAACATTTGTCTCTCCATAAAGATA  
 -1180 TTCTTGTACTTAGATGACTCTATAACAAAAAATAAAGAAATTAAGAGATATTCTCGTAAATTAACAATATTATATATAA  
 -1098 TGTATAAGTATAACCATTAACAAAAAGATCAACAATATTTTCAAATATTATAGTAAGCTGGAAACATCAATGCATTAGGT  
 -1016 AAGACACATGCATGTTGTACATTAGCTACGGATGCGCAAAATGGACGCTAAGCCGGCGGTGAAAGAGACACGTTGTTCTTCT  
 -934 GCATTTTATTCCATTTCGTACGTAATAGTTAATCTATAAAGTTATATAATGTTTCGACTTGATAGAGATATGATACAAGTAG  
 -852 TCATGAAACGTATTTCTAATTAAGTTAAGTTATAAACATTTTAATAGTAAATGGAGCACGTAATTTGCATTATGCAAAAA  
 -770 CACACATATGCTTAGTTCTAATAGGATATTTCTCTTTTCCGAATGAACATTTTGGAGCGGTGGACTTTTTTTTTTTATTAA  
 -688 CCGATATTAATTAGATAAAATTTAGGAGAGCCATTCATAATAATGTTATTTATATATGTTATATGATGCAGTTCCGGTATAGA  
 -606 TTTTTCGTGTCAAATTGTCGGTTTGTATTTGCACTACGAAAGATTAAGATAAAGAAAAGCTAGTGAACCTTCTCTTATC  
 -524 GTTTCGTAATTTTCTTCTGATCATTCGGTTATACGTTAACTTTCTCTTATCCTTCGTAATTTTCTCTGATAATTCCGGTT  
 -442 AGAGAAAATATATACTATCTTCGACCTCAACACAGTTTATTAACAAAATATATAAATAAATCAGGACTTTTTTTTTTTTT  
 -360 TGCTATTGAAACGTTTGCAAAACCTACAAAGAGACTACATCTAAAATGTACAAGAGACTTTACATATTGATTGTTTCTA  
 -278 CCAATAAATTTCTGAATACTATTTACCAAAAAAATAAAGCAAAAGCCATCGCTCATTTTCATTTCACGTAGTATTAT  
 -196 TCAACTCTTATTATGGATCCACCTAACGTTACCCCAAAAAATCTCTATAAATACTATGCAACTCATGCAACTTCTTCACTTAC  
 -114 ACCAAAAACAGAAAAACAAGGCCACAAAGAAGAAAGAAACCTTCTTCAACGTTTAAAGTCTCTCTCTCTCTCTCTT  
 -32 AACTTCTCCCATTTTCTTTGAACATAAGATCAATGGCTACAACCATCTAATGAAGACGGCTCTATTGGAACCTCCTTGCA  
 M A H N H S N E D G S I G T S L H 17  
 51 TGGAGTCACGGCAAGGGAGCAAGTCTTCTCCTCCGTCNCAAGAGATGTCCTTCACTCAAGCGTCCGACCAACAGAT  
 G V T A R E Q V F S F S V Q E D V P S S Q A V R T N D 44  
 133 CCAACGGCTAAGTTTGCCTTACCAGTGGACTCCGAACATAGGGCAAAAGTGTCAAACCACTATCATTGCTAAACACATA  
 P T A K F A L P V D S E H R A K V F K P L S F A K P H M 72  
 215 TGAGAGCTTCCACTTAGGATGGATCTCTTCTTCACTTGCTTCCACCTTCGACGCGGACCTTAGTCCCGTCAT  
 R A F H L G W I S F F T C F I S T F A A A P L V P V I 99  
 297 TCGGACAACTTCGACCTGACCAAAACGACATCGGAATGCTGGAGTTGCATCAGTTTCCGGCGCCATTTCTCAGACTC  
 R D N L D L T K T D I G N A G V A S V S G A I F S R L 126  
 379 GCTATGGGTGCTGTATGACCTTCTAGGGGACGTTATGGAACCGCTTCTCACTTATGCTTACAGCTCCAGACTTTTCT  
 A M G A V C D L L G A R Y G T A F S L M L T A P A V F S 154  
 461 CCATGTCGTCGTAGCTGACGCGGAAGCTACTTAGCCGTAAGGTTTCATGATCGGTTTTCGCTTAGCAACGTTTCGTATCATG  
 M S F V A D A G S Y L A V R F M I G F C L A T F V S C 181  
 543 TCAGTACTGGACGAGTGTATGTTCACTGGAAAGATTAACGCGATGTCGTCGGAGGGTGGGAGATATGGGA  
 Q Y W T S V M F T G K I I G L V N G C A G G W G D M G 208  
 625 GGAGGAGTGAAGTACTAATGCCAATGGTCTTCCACGTCATCAAACTCACGGAGCCACTCCCTTACGGCTTGGAGGT  
 G G V T Q L L M P M V F H V I K L T G A T P F T A W R F 236  
 707 TCGCCTTCTTATCCCGCATCTTTCAGATAGTTATGTTGATTTCTCGTTCTCACTCTCGGCCAAGATCTTCCGATGGTAA  
 A F F I P G I L Q I V M G I L V L T L G Q D L P D G N 263  
 789 CCTCAGTACTCTCAAAAGAGTGGTCAAGTTTCTAAAGACAAATCTCCAAGGtaatatattaacatgatcaatattttta  
 L S T L Q K S G Q V S K D K F S K 280  
 871 ggacaataactagcaataatgcatgtggttctaattacaattttgttttaatatagGCTTTTGGTTCGCTGTGAAAAACT  
 intron V F W F A V K N Y 289  
 953 ATAGAACATGGATCTTATTCATGCTCTATGGATTTTCTATGGGAGTTGAATTAACGATCAACAACGTTATATCTGGACTT  
 R T W I L F M L Y G F S M G V E L T I N N V I S G Y F 316  
 1035 CTACGATGTTTAACTTACGCTTACACAGCTGGTATTTATAGCAGCCAGCTTTGGTATGGGCAAACTTCTTGGCCGCT  
 Y D R F N L T L H T A G I I A A S F G M A N F F A R P 343  
 1117 TTTGGTGGCTACGCTTACAGTGTAGCTGCACGGCTCTTCCGGTATGAGGGGACGGTTATGGATCTTGTGGATCTTACAACTG  
 F G G Y A S D V A A R L F G M R G R L W I L W I L Q T V 371  
 1199 TTGGAGTCTCTTTTGCATCTGGCTTGGTCTGCTAGTTCACTACCTATAGCTATCTTAGCCATAGCTTTTTCATGGG  
 G A L F C I W L G R A S S L P I A I L A M M L F S M G 398  
 1281 CACACAAGCTGCTTGTGGAGCTCTCTTGGTGTGCTCTTTTGTTCCTCCGCGCTTCTTGGACTTATCTCGGATTAAT  
 T Q A A C G A L F G V A P F V S R R S L G L I S G L T 425  
 1363 GGTGCTGGTGGAAATTTGGGTGGGAGTTACTCACTTCTTTCTCTCTCTCGAGGTTTAGTACGGCGGAAGGACTAT  
 G A G G N F G S G V T Q L L F F S S S R F S T A E G L S 453  
 1445 CGTTGATGGCGGTTATGGCTGTTGTGTGCTCTTCCGGTTCGCTTTATACATTTCCGCGAGTGGGAAGCATGTTCTTGAG  
 L M G V M A V V C S L P V A F I H F P Q W G S M F L R 480  
 1527 GCCATCACAGAGAGAAATCAAAGGAAGAGCATTACTATGGAGCGGAATGGACAGAGGAAGAGAGCTTAGGACTA  
 P S Q D G E K S K E E H Y Y G A E W T E E E K S L G L 507  
 1609 CACGAAGGAAGCATTAATTTGCTGAAAACAGCCGTCAGAGAGAGGCCCAAGCGATGTTGGCTGATATCCAACGCCG  
 H E G S I K F A E N S R S E R G R K A M L A D I P T P E 535  
 1691 AAACCGGATCTCCGGCTCATGTCTAGTGAAGGATAGTCACATTACATCTTTTCCACATATAAATAACCTCAAACCTTAGCT  
 T G S P A H V \* 542  
 1773 TTATGTTTGCATGTTTATGTTCTCTTATGAGAGATGTACCGAGTAAGATCCTGACTTGATAAGTGAGGTTTCAGTGAGCTC  
 1855 GGTGCTCATGATGTACAATGTCGTTTGATATAAGACTTATGCATCAGTTTCTCTCTGTTTATCAACAAGCCGTGCTTTTA  
 1937 AAAGTCTTCCAAGATCATATTATTGATTTAACAAAATCGATCAAACTGAAAATGAGAAGAGTGTGTTGAGGCAACACTAG  
 2019 TCATACCTTAAGAAGAAGCAATTGACTTTAGTAATCTTTACGTCAGAAACAGATCACATTAAGAATCTGGAAAGAGTAA  
 2101 GAAGCAACTTAACCTAG

Figure 2-7. DNA and deduced amino acid sequences of the *AtNRT2.6* gene. The number on the left starts at the proposed start codon, and the numbers on the right refer to amino acid residues. Intron sequences are shown in lower cases. GATA boxes are underlined. TATA box is indicated with thick underline. Nitrate-dependent transcription motifs are double underlined.

-1426 ACATATATATATATATATATATATATATATATGTAAGCATATTAATTGATATGTAATAACTATTATAAGATTTTAAAGAGTAAAT  
 -1344 AAATAGTATGCCAAAACAATAATTGAATCTTATACTACTATTTTGAGTTTGTGATTTTCAATTAACAATTCATAGACAAA  
 -1262 AACAAAAAGGGAACCTAATTGAATTTATGATCTTATGCATATATATATTTTCATAGATCCAACATTTAAATAAACAACCTAGT  
 -1180 TATTTTAGACCATTTGTTTCTTTTAAATAAATGTTGCTTTTGGCCACAGAAATTAATAATGTTGTTTGTAGGATTTTAGT  
 -1098 GTAAATCTGTATAAATAATGCTTAAACTTTCTCTATATAAATTAATGAAATATGAAATAAATTTTCTTCTTAATTCGCT  
 -1016 TGAATAAATAAATAAATGACATGAATATGGAACACATCATTTGTAATTTTGTAGTTAGTCAGTTAAAGATAAGATTAT  
 -934 GGAGTTTGTGTTTGGTAACTTACAAAAACAATATTAATTTGTAACAAAAATGATTTCAAACCTCTCTCTCTTCTCATTT  
 -852 ATATACTTTATTTTTCACAAATCACAATGCACAACCTCAAAAAAATAGAAATGTTTAAAGTAGATAATGCAACAAAAAAT  
 -770 CTCAAATCGATTGTATTAATTAATGAATCTCTTGATCTTCAAATGAGTTACAATCTAACCTTTTCTATTAGATAAA  
 -688 TGTGAAAATTTATTTTAGAACTTATACTGAACACAGAGACGTAACGAAGAGAAACAGAAAACTAAAAAATATGTTGTAATA  
 -606 CGACAAAAGACTATAAAGTTAATGACATTCACGAAATTTACACGTTCTAATGGTTCGATGACACAAGGAACTTTCAATGTT  
 -524 GCCACAAGTGAATTGAGATCTCAAGTCAGCCTCCAATACCTTCTGAAAGAGCGGTGCTAACCCCTCTTAATACAAAAATTTATAT  
 -442 ATCTAAACAAAAACCCGTAATAACCAACAACTCTTAGATTCAATCGCGGTGCTTACAATCTAAGTTAGTTATAACAAAAAT  
 -360 TATATATCTAAACGAAAACTAAACACCACTTAACTCAACCTCACTTATAGATTCAATCAAAACCAAGATTTAATATATTTTAT  
 -278 TTTTCATCTAGAAAGAAAAACAGAAAACTTTATGTAATGATTCAAGTTTCTTTTATACCATCACACTAAAAATAAAAAG  
 -196 ATAGCATTTCAAAATCAAAATAAATAAATATTAATGATTACATCAACGCTTCAACAAGACATAAATCCATGAAAATGTC  
 -114 ACAGCTTTAACACTCTCTCTTATAAATACTCATAACTCAAACTGAGATTCTCTCATCTTAAACCCCTAAACCTCCA  
 -32 AAATCTGCTGAACCTTCACAAAGATCACAAAAATGGAGGTGGAAGGCAAGGAGGAGAAGCTGGAACCAACCAACCAACCGC  
 M E V E G K G G E A G T T T T A 17  
 51 ACCTCGGAGTTTCGCACTTCCGGTGGACGCGGAGAACAAGCAACAACCTTCCGACTATTTCTCAGTCGCTAACCTCACATG  
 P R R F A L P V D A E N K A C T T F R L F S V A K P H M 44  
 133 AGAGCTTTCCATCTCTCATGGTTTCAATTCTTTTGCTGCTTCGCTCCACTTTCGCGAGTCCGCTCTCTCTCTCTGTTATCC  
 R A F H L S W F Q F F C C F V S T F A A P P L L P V I R 72  
 215 GTGAAAATCTTAACCTCACCGCCACCGACATCGGAACCGGGAATAGCCTCTGTCTCCGCGCTGTTTTCGCTCGTATCTGT  
 E N L N L T A N T D I G N A G I A S V S G A V F A R I V 99  
 297 CATGGGACGGCATGTGATCTTTTCGGTCCACGCTTAGCCTCCGCGCTTTGACGCTCTCCACGCTCCCGCGCTCTATTTC  
 M G T A C D L F G P R L A S A A L T L S T A P A V Y F 126  
 379 ACCGCGGGATAAAGTCTCCGATCGGGTTATCATGTGAGATTCTTCGCGGATTTCTCTCGCCACTTTCGCTCGACTC  
 T A G I K S P I G F I M V R F F A G F S L A T F V S T Q 154  
 461 AGTTCTGGATGAGCTCCATGTTCTCTGGACCGCTCGTGGGTTCCGGTAACGGAATCGCCGCGGGTGGGTAACCTCGGAGG  
 F W M S S M F S G P V V G S A N G I A G W N L G G 181  
 543 AGCGCGGACGAGCTGATCATCGCCCTGTTCTCGTTGATTTCGTAATATGGGAGCCACCAAGTTTACCGCGTGGAGGATC  
 G A T Q L I M P I V F S L I R N M G A T K F T A W R I 208  
 625 GCTTTTTTCAATCCCGCTCTCTTTCAGACTCTCTCTGCTTTCGCGCTTCTCTTGTTCGGTCAAGtaacaaccaatcacact  
 A F F I P G L F Q T L S A F T A V L L F G Q 229  
 707 caccggttcagttatttttggttttttcaactaatccggttttgagaatttatcaccaagttattttttggttttggttaatt  
 789 tactttctttgtatagtcagtttatcctgttctatataaatgattaaaccgattcgttttagtgcaaacccggtttattgat  
 intron I  
 871 accggttttggttcgtctagtagagtcctttagtcggttatcatggttcgagtttcagtggtatggacaataactgtttaact  
 953 atctaagttgtgtctattacaaggaatagtaaaaggatacatttggtttgtcttatggtttgctatatggttttgaaatgag  
 1035 tagGATCTTCTGATGGAGATTATTTGGCGATGCATAAATCTGGAGAGGAGGAGAAAGATGATGTTGGGAAAGTGATATCTA  
 D L P D G D Y W A M H K S G E R E K D D V I S N 256  
 1117 ATGGAATCAAAAACCTATAGGGGATGGATAACAGCATTAGCATATGGCTATTTGTTTGGAGTAGAGCTTACCATTGACAACAT  
 G I K N Y R G W I T A L A Y G Y C F G V E L T I D N I 283  
 1199 CATCGCAGAATATTTCTTCGATAGATTCCATTTAAAGCTCCAGACAGCAGGATTATAGCAGCGAGTTTGGACTAGCCAAT  
 I A E Y F F D R F H L K L Q T A G I I A A S F G L A N 310  
 1281 TTTTTCGCTAGACCTGGAGGAGGAATTTTCTCTGATTATATGTCGAGACGGTTTGGGATGAGAGGAAGTTGTGGGCTTGGT  
 F F A R P G G G I F S D F M S R R F G M R G R L W A W 338  
 1363 GGATTGTGCAACATCAGGAGGTGATTATCGCATGCTTTGGCCAGATTCTCTCTTGACAGTGTCTATAATGTTATGCT  
 I V Q T S G V L C A C L G Q I S S L T V S I I V M L 365  
 1445 TGTCTTCTCTGTATTTCGTCCAAGCCGCTTGTGGACTTACCTTTGGCGTTGTTCCCTTTATTTCTAGAAGtaacatacattt  
 V F S V F V Q A A C G L T F G V V P F I S R R 388  
 1527 tggatttgagttgcttgattggttattcaacatttttgcttacaagaagtttggtttatgcagatCTCTTGGGGTGGTATCGGG  
 intronII S L G V V S G 395  
 1609 AATGACTGGTGCAGGAGCAATGTAGGCGCGGTCTTAACACAGTTGATATTCTTCAAAGGATCGACATACAGAGAGACG  
 M T G A G G N V G A V L T Q L I F F K G S T Y T R E T 422  
 1691 GGTATAACTCTAATGGGGTAATGTCAATCGCATGTTCAATATGCTTGAATTTACTTTCCGAATGGGGAGGTATGT  
 G I T L M G V M S I A C S L P I C L I Y F P Q W G G M F 450  
 1773 TTTTGGGACCTCTTCAAAAAAGTAACCTGAAGAAGACTATTATCTCGCCGAATGGAACGATGAAGAGAAAGAAAGAACTT  
 C G P S S K K V T E E D Y Y L A E W N D E K E K L 477  
 1855 ACATATCGGAAGCCAAAAATTTGCGGAAACAGCATTAGCGAAAGAGGTGAGCCACAACGACTCATCCCCAACTTGAAT  
 H I G S Q K F A E T S I S E R G R A T T T H P Q T \* 502  
 1937 ATATAAATCCAAAAGTTGATATCACCGTTTCAAAAAATAAACAAGCAAAAAGACAGATTTACGTGATAAATCTTCATAA  
 2019 TAGGATGCGAGATGTTAGTTTCAAAGTTATAGATCAAACTCAGTGAAATGAGATTTTGGTAAGTGTTATATAGTCTCTTAAA  
 2101 AATCTGGAATCCCGTATCATCTTTTCTTATATAGTTCTTACATCTTAACATATATATTTGTTATTCTATATTTGGTGGTTTT  
 2183 ATATTTGCTTGAGTTTCAAACTTCTTGATAAGATTCAAAACCGTCTTTGAAAACAAACCATCAAAATGGTCCCTCTCTT  
 2265 GTTCTTTGAAAAGATCATTTTCATACAAGCGATATAGACAAAGCATTTAAGAAACAAAGTTGAGAAGTTTGTAAACCGT  
 2347 ACGTTGTTGTAACAAAAAAGAGTAAGACTTTGCCAAAGTTTTCGAAGACTTTACCTTTGCCATGGTTAA

Figure 2-8. DNA and deduced amino acid sequences of the *AtNRT2.7* gene. The number on the left starts at the proposed start codon, and the numbers on the right refer to amino acid residues. Intron sequences are shown in lower cases. GATA boxes are underlined. TATA box is indicated with thick underline. Nitrate-dependent transcription motifs are double underlined.

accepted that the MFS has 12 TMS and a cytoplasmic N-terminus, predicted TMS numbers varied from 9 to 12 (Table 2-2). The average numbers settled between 11 and 12.

*AtNRT2.1*, *2.2* and *2.4* are highly homologous at the protein level, sharing 89% and 82% identity between *AtNRT2.1* and *2.2*, or *2.1* and *2.4*, respectively (Table 1). *AtNRT2.3* and *2.6* proteins also show high homology, with values of 89% identity and 93% similarity between the two. The middle regions of the proteins have even higher degrees of similarities, whereas N- and C-termini, which mostly consist of hydrophilic residues, share less homology within the family (Figure 2-9). In TMS5 all *AtNRT2* family members have a sequence, [AG]-G-W-[GA]-[ND]-x-G, which is highly related to a signature motif found in the NNP family (Pao et al., 1998; Trueman et al., 1996). Another conserved sequence, R-[PA]-x-G-G-x-x-[SA]-D, was identified between TMS8 and TMS9. This is not only found within plant members of the NRT2 family but it is also well conserved in all known NNP family members including *NrtA* (formerly called *crnA*) in *Aspergillus nidulans* (Unkles et al., 1991), *YNT1* in *Hansenula polymorpha* (Perez et al., 1997), and *narK* from *E.coli* (Rowe et al., 1994). Half of this sequence (G-x-x-[SA]-D) is closely related to a part of the MSF specific sequence motif, G-[RKPTY]-L-[GAS]-[DN]-[RK]-[FY]-G-R-[RK]-[RKP]-[LIVGST]-[LIM], which is located between TMS2 and TMS3 (Pao et al., 1998). It is notable that the longest conserved sequence, F-G-M-R-G-R-L-W, was found at the beginning of TMS9 in all *AtNRT2* members (Figure 2-9). This sequence is also well conserved in other photosynthetic species including *Chlamydomonas reinhardtii*, and *Chlorella sorokiniana* (data not shown), suggesting that the sequence could be applied to clone the homologues from other species. *AtNRT2.5* protein, the least homologous among the seven *AtNRT2* members, has the longest hydrophilic loop predicted between TMS6 and

Table 2-2 Prediction of membrane topology of the NRT members by seven methods

Protein	HMMTOP	MEMSTAT	SOSUI	TMAP	TMHMM	TMpred*	Toppred
AtNRT2.1	12 out	12 out	8 n/a	10 n/a	11 in	12 out	12 in
AtNRT2.2	10 out	12 out	8 n/a	10 n/a	11 in	12 out	12 in
AtNRT2.3	12 in	11 out	10 n/a	10 n/a	11 in	11 in	12 in
AtNRT2.4	12 in	12 out	9 n/a	10 n/a	12 in	12 in	12 in
AtNRT2.5	12 in	12 out	10 n/a	10 n/a	10 in	12 out	12 in
AtNRT2.6	12 in	11 out	11 n/a	10 n/a	11 in	12 out	12 in
AtNRT2.7	12 out	11 out	8 n/a	10 n/a	11 out	11 in	12 in
AtNRT1.1	12 in	11 out	12 n/a	12 n/a	11 out	12 in	12 in
AtNRT1.2	12 in	9 out	13 n/a	12 n/a	12 in	12 in	12 in
AtNRT1.3	12 in	10 out	12 n/a	12 n/a	11 out	12 out	12 in
AtNRT1.4	12 in	11 out	12 n/a	12 n/a	12 in	12 out	12 in

Predicted number of transmembrane helixes and the location of N-terminus (in=cytoplasmic, out=exoplasmic).

\* With minimum length of 17, maximum length of 29, other programs were used with default setting.



Figure 2-9. (continued).

Amino acid sequence alignments of AtNRT2 family. Sequences were initially aligned with CLUSTAL W and finely adjusted and output with GeneDoc (Nicholas and Nicholas, 1997). Amino acid residues in black background indicate identities more than 50 % (i.e., 4 of 7 sequences). 12 predicted TMS regions of AtNRT2.1, determined by HMMTOP program (Tusnady and Simon, 1998), were indicated by numbered lines above the sequences. Other members' TMS locations were close to those of AtNRT2.1. Potential protein kinase C and casein kinase 2 phosphorylation, and N-glycosylation sites, indicated with (+), (♦), and (♣), respectively, were searched with the ProSite database (<http://www.expasy.ch/prosite/>). The threonine/serine residues, indicated with asterisk, are recognition sites for both PKC and CK2. Conserved sequence regions, MFS, NNP, C1 and C2 are indicated in thick lines under the sequences.

TMS7 (Figure 2-9). This long loop is more evident in NrtA (Unkles et al., 1991), and YNT1 (Perez et al., 1997).

Some protein kinase C (PKC) phosphorylation sites (motif: [ST]-x-[RK]), and casein kinase 2 (CK2) phosphorylation sites (motif: [ST]-x-x-[DE]), were found in the cytosolic domains (Figure 2-9). The PKC sites before TMS11 and at the N- and C-termini were well conserved, whereas most of the CK2 sites were restricted to both termini with less conservation. It is intriguing that the conserved threonine residue in the N-terminus (i.e., Thr-16 in AtNRT2.1) is a phosphorylation site of both PKC and CK2. N-glycosylation sites, where the consensus pattern is (N-{P}-[ST]-{P}), were found among TMS1 and TMS2 in five of the proteins (Figure 2-9). Neither PSORT (Nakai and Kanehisa, 1992) nor SignalP (Nielsen et al., 1999) were able to find N-terminal signal sequences within the AtNRT2 members.

### **Gene Structure of the AtNRT1 Family**

The gene structures of *AtNRT1.1*(*CHL1*) and *1.2*(*NTL1*) had been characterized previously (Tsay et al., 1993; Huang et al., 1999), and a brief characterization of *AtNRT1.3*(*NTP3*) and *1.4*(*NTP2*) was also reported earlier (Hatzfeld and Saito, 1999). Briefly, *AtNRT1.1* and *1.2* are located on chromosome 1, whereas *AtNRT1.3* and *1.4* are on chromosome 3 and 2, respectively (Figure 2-1).

The *AtNRT1.1* gene possesses four introns in which the first three are 94 to 185 bp in length, whereas the fourth is 1650 bp (Figure 2-10). The nitrate-dependent transcription motif was found at 237 and 246 bp upstream from the start codon. The promoter region



-1754 GGGACTCTTCCATTGTGACTTTTGTGTTCTCGCTCTTCCACAATCTTTGGATGTGTACAAAATAATCGATTGTTGGGGATATT  
-1672 ATAGTAAATTTAGCAATATGAACTAGCAACGAAGAAGTGTTTATTGATGGGTTCAAATTTTCATCGATCTGCATATCAAT  
-1590 AACTCGGAAAGAGATGGATCATGACACATAGTATACAAATTTTATAAATTTTCAAATTAACATATTGAGGATAGAAAA  
-1508 TCATCAATTAATTAATAAATAAAAAAGATTCATTAATGTTTTACTTTCTGTAAACATACAATTTCTTGCTTTTCTT  
-1426 GAAAAATTGACTCGTTTTTTATTTGATATTTTACATAGACTTTGAGACTTATTAATAAACTAACAACCATCTCTTACAA  
-1344 ATTGTTGGAATAAATTTATTTTCTGTTGGCTCTATAAAATGAAATGACCTTTGTTCTCTCTTTTATACTCCTAT  
-1262 ATATGTTTTCTCCAGAGAGAAAGAACCATTTGAAGTTGCGAGACTTCCAATTATATATATAGTTTCATGAAAGTCCTGTGAC  
-1180 CATGCTCTTTTATTTTATTCTACTATACGTTAAATAAATGGAAGTAGGTAATATCATATTGTCAGATCATCAATTT  
-1098 TAGTGGCGTCTAAGAAGCATTCATTTTGAAGCCATTAATCTCAAGGTAGACTTTCCCATTAAACACAAAAGAAGAAAAATG  
-1016 GTACGTAATCATACCAACATCTACAAATCTTTGGTTTCAATTTTGTAAACAAAGACCTAGATTGTTGACAAACAAAAGCAAAA  
-934 TACCTAAAAAGGACTTTTATGACTTCTATAAAGATGTTTTGATGAAATTTTCATATAATTTAATAGTTTCTCCAACTATAT  
-852 GCAATAAAATATAATTTGATAATCATGTGAAGAAACATGACTTCCAATTAATATGAGTCGATTATCAATAAAATTT  
-770 TGTGATATGGCTGTGCTGCAGAACAACGAATGGGCCTAGAAGTAAAGTTGATATTGAGCAACAAAAACGAAATAGATTT  
-688 TTTTTTTCTTTTGGGCAAACTCAAAAACGAAATAGATATGCTCGACAATCTACAAAATCTCAAAATGAACAATATTGATG  
-606 AAAATGCGTTTGTAAACAGAAATCAATTTTGAATTTTTCATATATAGTACAGACGTAAAAAACATATGATTAT  
-524 CTTTCATGGAAGTAAATAATTTTCCAGAAATAGAAACAAATGTAAACAGGTGGTGGCTAAAACTGGTCTTTTAAATGGCT  
-442 AATGCCATTGAATCACTGACAAAATGACCGATGACTTCAACTTAAAGTTCAAGCCACAGTTCCAAAGGTAGAGGAAGTGG  
-360 TAAAAATTAGGTATGGTCTCTTCTCTCTCTCAATTTTCTCTTTCTACAAATATATACTACAGTTACAGTAGTATTATT  
-278 ATTTATTAATTCTCTCCCCAATCAABAACACTCATCAGAGTCACAGTGTGTCACTTTTTTTCTCAATATTTAGTTTT  
-196 ATGAATAAAAATCAAAATAAAACAATAAAACTTTTTTAACTTTGGCTAGTTTCCAGTATATAATAGATGTATCAACGAACA  
-114 CATTTAAGACCACAAAAGAAACCAAGAGCTCTCAACAAAACAGCCTTTTACATAAGAACAAACACCCCAAAACTGCAAA  
-32 AAAAAGAGAGAGATCATTAATCCATCTTCAAGATGCTCTCTCTGAAACTAAATCTGATGATATCTCTTGTATGCTTGGGA

M S L P E T K S D I L A W D

51 CTTCACAGGCCGTCCCGCCGATCGCTCAAAAACCGCGCGTGGGCCAGCGCGCCATGATTCTTTgtaagtataatcacata 17  
F Q G R P A D R S K T G G W A S A M I L C 39

133 ataaaagttcttagagaaagctaccaacaatatcatgtcgaagacaagaattgaaactttttaaggcatttactaagttta  
intron I

215 taagtttcttatggcctatcattcttattcttttatgtattctcgatgtatccaactttatcattaacaaaaattttatttgtgt  
297 gaagGTATTGAGGCCGTGGAGAGGCTGACGACGTTAGGTATCGGAGTTAATCTGGTGACGTATTGACGGGAACATTGCATT  
I E A V E R L T L G I G V N L V T Y L T G T M H L 65

379 TAGGCAATGCACTGCGGCTAACACCGTTTACCAATTTCTCGGAACCTTCTTCTGCTCTGTCTCGTTCGGTGGCTTCATCGC  
G N A T A A N T V T N F L G T S F M L C L L G G F I A 92

461 CGATACCTTTCTCGGCAGggtttgtctataaatatatttacttaactaaatcattcagattctttttaaatatta  
D T F L G R  
intron II 98

543 atttgagtttcattctttttgtatagGTACCTAACGATTGCTATATTCGCCGAATCCAAGCCACGgtaaggacctca  
Y L T I A I F A A I Q A T 111

625 aaagtcctcttctccttacccaaacttatcccgaagaaacgaagaaaataaaaaatttgtctagtcataaacacaaatcgaa  
707 atagtaaaccagcataagtcgttattttgttatagttttccgaaaaataatatccgtggtatgaactatgatgcgtatcagGG  
intron III G 112

789 TGTTTTCAATCTTAACTCTATCAACAATCATACCGGGACTTCGACCACCAAGATGCAATCCAACAACGTCGTCTACTGCGAA  
V S I L T L S T I I P G L R P P R C N P T T S S H C E 139

871 CAAGCAAGTGGAATACAACCTGACGGTCTATACCTTAGCCTTATACCTCACCGCTTAGGAACCGGAGCGTGAAGCGTAGTG  
Q A S G I Q L T V L Y L A L Y L T A L G T G G V K A S V 167

953 TCTCGGGTTTCGGGTTCGACCAATTTCGATGAGACCGAAACCAAGACGATGAAATGACATATTCTTCAACCGTTTCTTT  
S G F G S D Q F D E T E P K E R S K M T Y F F N R F F 194

1035 CTTTTGTATCAAGCTTGGCTCTCTTTTACTGTGACGGTCTTGTCTACGTACAAGACGATGTTGGACGCAATGGGGCTAT  
F C I N V G S L L A V T V L V Y V Q D D V G R K W G Y 221

1117 GGAATTTGCGCGTTTGCATCGTGCTTGCACTACGCGTTTCTTGGCCGGAACAAACCGCTACCGTTTCAAGAAGTTGATCG  
G I C A F A I V L A L S V F L G A G T N R Y R F K K I I G 249

1199 GTAGCCCGATGACGCAAGTTGTCGCGTTTATCGTGGCGCGTGGAGGAATAGGAAGCTCGAGCTGCCGGCAGATCCGTCCTA  
S P M T Q V A A V I V A A W R N R K L E L P A D P S Y 276

1281 TCTCTACGATGTGGATGATATTATTGCGCGGGAAGGTTCCGATGAAGGGTAAACAAAAGCTGCCACACACTGAACAATTTCCGg  
L Y D V D D I I A A E G S M K G Q K L P H T E Q F R 303

1363 tacgattcttttaattactcatcatttgccttcacgtacgtacgtccaaactcataatgatttttaagataaaaag  
1445 ttttaaaatttttcttttcttttccatttaagggttaattgaaataattgaggtactggttcccttaattaaatgtgatag  
1527 tatcagtagactttaaaaatgtgaaattaaaagtggtgtgtggtgtgaaataaactcgaaatgctgctgtcttttaatt  
1609 atagttgggacgtaaatattttctcattataatgctacttggcatttagaaatcattttttttagcagcattataaattat  
1691 taaaaggtgtgaaactatctttctttgtctttgtttacggggaaaataaatttttataaattatttactcgaccatacag  
1773 cgaaactggtccaggatacgaattatacagctaattacaacatttttggttttgggttcaaagtccttctttggcattt  
1855 tatattgtatatttttcccttaaaatcctatgtgtgtgcatgtgtatatcttgttagataaacggttaagttcggttca  
1937 tagaattaggtaggaataaaaagtagtctctcggaattagtcggaagataaagtaattcagataaaaagaattgaataagtt  
2019 atgcaagtgatgaactgaggtgaaatttccccatttcttcttggctataaaaaatttccacaccactcgtagaatatt  
2101 tggtttttacagaaagagtatcttcatcattgcggaaaattaaatacaccaatttgcatttttatctttatagattat  
2183 aattatgatttatgattctaatgccttataacttttaatacacatcacactgacacacatatatttatatttcccctga  
intron IV

2265 ctcgtaggttttccccccttaagtagcggatctttttatttttgctaacttaagtagatctttttatccatcaatccctat  
2347 gcatacctacatagaataaaaagaatccaaaggaatcaatttttctttataccagataattatgaaaatctatttagcat  
2429 cctcagatattggaatccctttctcggaataaaaactaaagaagaagagacgagaagtttagggtagggaggcagggtgt  
2511 ttctgttagtgaaaacagatacaaatctgtgtctgtagcactcactactttacaccttcacgggacacgggacaaagtttag  
2593 gcataaataagaacactagcgtcatgcagcacagcgtgtcttgctgtctcggtgcttcattaaatttcttcttctgtttggg

2675 tctctctaaaaaggtagttgaaaactaaagaaaaaacctttttacactttttccataaattcaaactcttaactttttttgcct  
 2757 acactatttttaaaagcaattacgtatttgtcatgcatgtgtgtgaaggatatatcatcttgaaaagattccacgtaactatc  
 2839 tccacaacatgttgattcaaatattcaatgcgtaagctatatccaaaaaagaaacaatgtattattgatatgaaatttca  
 2921 ataaatagggcacttcataattttctaagaacacacaaaaagatcttttggttgaaaaatcagatacttatctttaatcca  
 3003 atatatgtaaaatattttatctccattttacataatttactattatattcttaattattccttatatcatcttttgatgccaca  
 3085 gTTCATTAGATAAGGCAGCAATAAGGGATCAGGAAGCGGGAGTTACCTCGAATGTATTCAACAAGTGGACACTCTCAACACT  
           S L D K A A I R D Q E A G V T S N V F N K W T L S T L 330  
 3167 AACAGATGTTGAGGAAGTGAACAAATCGTGCGAATGTTACCAATTTGGGCAACATGCATCCTCTTCTGGACCGTCCACGCT  
           T D V E E V K Q I V R M L P I W A T C I L F W T V H A 357  
 3249 CAATTAACGACATTATCAGTCGCACAATCCGAGACATTGGACCGTTCATCGGGAGCTTCGAGATCCCTCCAGCATCGATGG  
           Q L T T L S V A Q S E T L D R S I G S F E I P P A S M A 385  
 3331 CAGTCTTCTACGTCGGTGGCTCTCTCTAACCACCGCGTCTATGACCGCGTCGCCATTTCGTCTATGCAAAAAGCTATTCAA  
           V F Y V G G L L L T T A V Y D R V A I R L C K K L F N 412  
 3495 CTACCCCATGGTCTAAGACCGCTTCAACGGATCGGTTTGGGGCTTTTCTTCGGATCAATGGCTATGGCTGTGGCTGCTTTG  
           Y P H G L R P L Q R I G L G L F F G S M A M A V A A L 439  
 3577 GTCGAGCTCAAACGCTCTTAGAACTGCACACGCTCATGGTCCAACAGTCAAACGCTTCCTCTAGGGTTTATCTACTCATCC  
           V E L K E R L R T A H A H G P T V K T L P L G F Y L L I P 467  
 3659 CACAATATCTTATTGTTCGGTATCGGCGAAGCGTTAATCTACACAGGACAGTTAGATTCTTCTTTCGAGAGAGTGCCCTAAAGG  
           Q Y L I V G I G E A L I Y T G Q L D F F L R E C P K G 494  
 3741 TATGAAAGGGATGAGCACGGGTCTATTGTTGAGCACATTGGCATTAGGCTTTTCTTCAGCTCGGTTCTCGTGACAATCGTC  
           M K G M S T G L L L S T L A L G F F F S V L V T I V 521  
 3823 GAGAAATTCACCGGAAAGCTCATCCATGGATTGCCGATGATCTCAACAAGGGCCGCTTTACAATTTCTACTGGCTTGTGG  
           E K F T G K A H P W I A D D L N K G R L Y N F Y W L V A 549  
 3905 CCGTACTTGTTCGCTTGAACCTTCTCATTTTCTCTAGTTTCTTCCAAGTGGTACGTTTACAAGGAAAAAGACTAGCTGAGGT  
           V L V A L N F L I F L V F S K W Y V Y K E K R L A E V 576  
 3987 GGGGATTGAGTTGGATGATGAGCCGAGTATTCCAATGGGTCAATTGATCATGAATTCATGATCATGATCATGATGATGAACAT  
           G I E L D D E P S I P M G H \* 590  
 4069 TATGCATGTCTATGTTGATATGTTTGAATTTGATTTTGAGTTTGTGTATATTTTATGATGCTTTTCTTAATTACCTCTT  
 4151 GTTTCGAGTCGACAAATAATGGACCGCGATAAGGGATAAAAGTTAAAGTTTCCCATCAGGTTCTGTATAAAAGCAGAA  
 4233 ATTGTAACATTTCTTAAGATTGACGCAATTAACGCTTTTTTGTCTTCAATTTCCATTTTTCATTTTTTCCAGTAAATAT  
 4315 CATACCATTCAACGTTTGTCTTCTCACTTGTCTTGGTATCGTTTGTCTTGAATATGTGAATAATTTGGACCAGAACTAGGC  
 4397 GTCTAGGCCACTAGCTAGTGCCTAGTGGACTATCAC

Figure 2-10. (continued).

The number on the left starts at the proposed start codon, and the numbers on the right refer to amino acid residues. Intron sequences are shown in lower cases. GATA boxes are underlined. TATA box is indicated with thick underline. Nitrate-dependent transcription motifs are double underlined.

also contains a putative TATA box (-138) and several GATA boxes. The open reading frame (ORF) of *AtNRT1.1* consists of 1770 bp, encoding 585 amino acids (64.9kD).

By comparison between the genomic DNA (AC010675) from the BAC clone (T17F3) and the cDNA (AF073361) sequences, the *AtNRT1.2* is interrupted by four introns (130, 135, 1365, and 75 bp from 5' to 3'), and the ORF which consists of five exons (3, 106, 219, 431, and 996 bp) encodes 585 amino acids with a predicted MW of 63.9 kD (Figure 2-11). The promoter region of *AtNRT1.2* also contains two nitrate-dependent transcription motifs, as well as putative TATA and GATA boxes.

In *AtNRT1.3* three introns (83, 78, 79 bp from 5' to 3') are located at the beginning of the gene, resulting in four exons of 3, 118, 218, and 1431 bp (Figure 2-12). The ORF encodes 590 amino acids (65.2 kD), which is same length as that of *AtNRT1.1*. Five potential nitrate-dependent transcriptional motifs were observed in the 5' upstream region between -893 and -635 bp.

The *AtNRT1.4* gene consists of 6 introns and 7 exons, spanning about 4.1 kb in the genome (Figure 2-13). The ORF of the gene is 22 bp longer than was originally reported (Hutzfeld and Saito, 1999). The full length of 1758 bp encodes a sequence of 586 amino acids with MW of 64.5 kD.

Among the four *AtNRT1* members, sequence identities (between 33 to 51 % at the protein level), were not particularly high (Table 2-1). *AtNRT1.1* and 1.2 are the most dissimilar, and *AtNRT1.3* and 1.4 are located between 1.1 and 1.2. The *AtNRT1* members share only 3-10 % identities with members of the *AtNRT2* family at the protein level.

Membrane spanning regions and topologies were also analyzed. Four programs (i.e., HMMTOP, TMAP, TMPred, and Toppred) predicted 12 TMS in all *NRT1* members. Two

[illegible]

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2842 AGGAAGCCTAAAGATACCTCCAGCTTCCTTACCGATCTTCCCGTCGTTTTTCATAATGATCCTCGCACCTATCTACGACCAT
   G S L K I P P A S L P I L P V V F I M I L A P I Y D H 406
2924 CTCATTATCCCATTCGCTAGAAAAGCTACCAAGACCGAAACAGGAGTCACTCATCTACAAAGAATCGGAGTAGGTTTAGTTC
   L I I P F A R K A T K T E T G V T H L Q R I G V G L V L 434
3006 TTTCGATATTAGCAATGGCGGTTGCAGCTCTAGTTGAGATTAAACGAAAGGGAGTGGCTAAAGACTCCGGCTTGCTTGACTC
   S I L A M A V A A L V E I K R K G V A K D S G L L D S 461
3088 GAAAGAAACCTTACCCGTGACTTTCCTATGGATCGCACTTCAGTATCTTTTCCTAGGGTCAGCCGATCTATTACACTAGCT
   K E T L P V T F L W I G L Q Y L F L G S A D L F T L A 488
3170 GGACTACTAGAGTATTTCTTCACGGAAGCACCTTCCTCAATGAGATCTCTCGCAACATCGCTCTCGTGGGCTTCTTTGGCTA
   G L L E Y F F T E A P S S M R S L A T S L S W A S L A M 516
3252 TGGGGTATTACCTAAGCTCAGTGATCGTGTCCATAGTAAACAGCATCACAGGAAGCTCAGGGAACACACCTTGGCTCAGAGG
   G Y Y L S S V I V S I V N S I T G S S G N T P W L R G 543
3334 AAAAAGCATAAACCGTTACAACTAGACTACTTCTATTGGCTAATGTGTGTTCTTAGTGCAGCTAACTTCTTGCACTACCTC
   K S I N R Y K L D Y F Y W L M C V L S A A N F L H Y L 570
3416 TTTTGGGCAATGCGTTACAAGTATAGATCAACTGGTTCAAGAAGCTAAAATTGGATGGGCAACTTTTGAGTTTTTTTTTTGT
   F W A M R Y K Y R S T G S R S * 585
3498 TTTTAATTAGAGCAGAAAATGTTTTTACTTGAGCGGCTGTTATATAAGAGATGATAAATGGGGGTAAGTAGTAATGTTGTGA
3580 TCAGGTATATATTGGGGTTTGAAGTTTTTATCGTTGGACAAGTAATGTACAGCGAATGATATTAACCTTGCTTTAAGTCTTTC
3662 TTTTGTT

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Figure 2-11. (continued).

The number on the left starts at the proposed start codon, and the numbers on the right refer to amino acid residues. Intron sequences are shown in lower cases. GATA boxes are underlined. TATA box is indicated with thick underline. Nitrate-dependent transcription motifs are double underlined.

-1344 TTTAATGTATTTGCTGCTTTCTTGACTTATATTGCTAACATCATTTCAAGTAGAGGGATCAATTCATTAACATGTACAGCTT  
 -1262 TTTAATCAGTGGATCTCTCTAAGTAAATATCTTCTCCATTTTCATGGATTTTTTTACATGTCATATTCACCTTGATTACAGT  
 -1180 CACTTTCGAATATTACTTATAACGGAATAATATGTTTTTTTTTGCTTTACACATCTCTGGTTGAATACGCAATTCGTAAT  
 -1098 TTCTTATGACACCTGAAATCTTGATCTTTCTTAAAGTATATAGACTAGCTTCTTGTGGATCTAACAAATCCTTTTAGCT  
 -1016 AAAAATAATCCATTAAACCTAGTTCCAAATGATTCAACATTTTGGTATACTAAATTAAGTGTATGTCTAGGGATGTTG  
 -934 TGAACAAAAACAGTCTGATTTAAACATACTCTTGGTGATATATATATATAGTCAATAATTTCTTAATCAAGTACAAATTGG  
 -852 TTCATGATTTTTTGATCCACCAAAACATTTCTAATGAAACGAAATACTCAATTAAGAACAACACTCACTCACTAATGATTGAAC  
 -770 ATTAATATGAACCATGTGATATTGAAGTGAAAGCACTAACTTTAATGCTTAACAAGTCAATCCCAAAATATTAGAGACCATGC  
 -688 ATATTGTACCAGTGAGAGTTTCAATCTTGAACCTTGTGTCTATACTCACTAAGAACTGTCCACGTGGTAGATCAAT  
 -606 AAACAACCACAATCTGGCACACATGATATAAGACAAAGATTTTGTATATGTCTCAATTAATGTAAATCTTAACAATAAA  
 -524 AAACAATTCTATAGTATGGATGGTAAGAGATTTGTGTAAGGAGAAGAGGACGTGGGAAAAATACAAGAAGCTAAAAA  
 -442 CTCATTGCAACCAACAAACAAATGGTATAAAGAGACAAAGAGAGTAAATGGACATGGGGAATTACAAGAAGCAGAAAACT  
 -360 TGCAACCACATGAAATAAACAATGTCTTTGTCTATAAGATAATGAAATCACACTGTTGTCTCAATGTTTGTATTAGATCTATC  
 -278 TGATCATATATCGTGAAACAACCAACACAAAGCAAGCCACATTTATATAAGACCCATCATATATCAATCAATCCCCA  
 -196 TCTCTCTTCACATGCCCATTTGAATCGTATCTCTCTCATTTCTCTTTTGTGTTGTTGGAGTTAGATAAAATCAAGGTCT  
 -114 AATAAAGTGCAAAACCTTATCGTATAACCTCACTACCATTTAACTCTCTCTCTTATAACTCAGCTTCTCTCTCTTTC  
 -32 TTCTCTCAGAGTCTAACTGCTCAAATAAGAAATGgtgagttaaaaggtttttctctctgtttttttttgtttctctgt  
  
 M intron I 1  
 51 ttcttatgttcttaattggttttgcctgtctctctaaagGTTTCATGTGTCATCATCTCATGGAGCCAAAGATGGCTCTGAAGAAG  
 V H V S S S H G A K D G S E E A 17  
 133 CCTATGATTACAGAGGAAACCCACCAGATAAGTCTAAAACCGGTGGATGGTTAGGCGCCGTTTAAATTTTAGgtaagtcctt  
 Y D Y R G N P P D K S K T G G W L G A G L I L G 41  
 215 aattcttaattgtgaaaaagagtgtactcctctgtttcttaacactcctctgtatcttcttaattgtgaaggGAGCGATCA  
 intron II S E L S 45  
 297 GAGAGAATATGCGTGATGGGCATATCAATGAATCTAGTGACGTACCTTGTGGAGATTTACACATCTCATCAGCTAAATCAG  
 E R I C V M G I S M N L V T Y L V G D L H I S S A K S A 73  
 379 CGACCATAGTCACCAACTTCATGGGAACCTTAACCTTCTAGGGCTTCTCGGTGGTTTTTGGGCTGACGCTAAATCGGTG  
 T I V T N F M G T L N L L G G F L A D P T S D 100  
 461 CTACAAGATGGTTGCAATCTCAGCTTCTGTACAGCTCTGgtaagctcttaaacacgtactttagctcgattggttgag  
 Y K M V A I S A S V T A L intron III 113  
 543 actttctagctcttaattccttgtttctgtgaaaacagGGAGTGTGCTTTTGACGGTGGCTACAATATCTCAAGCATGAGA  
 G V L L L T V A T T I S S M R 128  
 625 CCACCAATATGTGACGATTTTCAGGAGACTTCATCATCAGTGCATAGAAGCAACGGACACCGATTGGCTCTTCTCTATGTTG  
 P P I C D D F R R L H H Q C I E A N G H Q L A L L Y V A 156  
 707 CTCTCTATACCATAGCTCTAGGCGGAGGAGGAATCAAACCTCTCTGGTTTTGGGCTGACCACTGATAGTGA  
 L Y T I A L G G G I K S N V S G F G S D P T S D 183  
 789 TCCTAAAGAAGAGAAACAGATGATTTTCTTCTTCAACAGATTCTATTTCTCCATCAGCGTGGCTCTCTCTCGCCGTGATT  
 P K E E K Q M I F F F N R F Y F S I S V G S L F A V I 210  
 871 GCTCTGTTTACGTTTCAAGACAACGTCGGGAGAGGCTGGGGTTACGGGATCTCTGCCGCACTATGGTGGTTGCAGCCATTG  
 A L V Y V Q D N V G R G W G V I S A A T M V A I V 238  
 953 TTTTACTCTGCGGAACGAAACGGTACCGTTTCAAGAAACCTAAAGGAAGCCCTTTTACAACAATATGGAGGGTTGGTTTCTT  
 L L C G T K R Y R F K K P K G S P F T T I W R V G F L 265  
 1035 GGCTTGAAGAAGAAAGAGAGTTACCTGCGCATCCAAGTCTTTTGAACGGTTATGACAACACACCGTTCACACACA  
 A W K K R K E S Y P A H P S L L N G Y D N T T V T 292  
 1117 GAGATGCTTAAGTGTTTAGACAAAGCCGAATTTCCAAGAAGCAGAGCTCTCTAGCTCGAAGGACTTCGAAGAGAAGGATC  
 E M L K C L D K A A I S K N E S S P S S K D F E E K D P 320  
 1199 CGTGATCGTTTCGACTGTTACACAAGTCGAAGAAGTGAACCTCGTGATGAAATTGGTACCGATTGGGCAACGAACATTCT  
 W I V S T V T Q V E I V M K L V P I W I L 347  
 1281 TTTCTGGACGATTTACTCCAAATGACGACTTTACGGTGAACAGCGAGCTTTATGGACCGAAAACCTCGGATCTTTCACT  
 F W T I Y S Q M T T F T V E Q A T F M D R K L G S F T 374  
 1363 GTTCTGACAGCTCTTACTCTGCTTTTCTCTATCTCACAATTTCTCTCTTCACTTCCCTTAACGAGAGAGTCTTTGTTCTT  
 V P A G S Y S A F L I L T I L L F T S L N E R E V F P L 402  
 1445 TAACAAGAAGGCTCACAAAAAGCCTCAAGGAATCAACAGCTACAGAGAATCGGAGTAGGGCTAGTATTCTCAATGGCTGC  
 T R R L T K K P Q G I T S L Q R I G V G L V F S M A A 429  
 1527 AATGGCTGTAGCCGCGTTATAGAGAACGCTAGACGCGAGGACGCGTTAACAACGATAAGAAAAAAGCGGTTTGGTTG  
 M A V A A V I E N A R E A A V N N D K K I S A F V L 456  
 1609 GTTCCACAATATTTCTTAGTCGGTGCGGGTGAAGCCTTTGCTTACGTTGGACAGCTTGAGTTCTTTATAAGAGAAGCACCAG  
 V P Q Y F L V G A G E A F A Y V G Q L E F F I R E A P E 484  
 1691 AGAGGATGAAATCGATGAGCACCAGGATGTTTCTAAGCAGATATCGATGGGATCTTCTGAGTAGCTTGCTTGTTCGCT  
 R M K S M S T G L F L T I S M G F F V S S L V S L 511  
 1773 TGTGTAGAGGTTACAGACAAAAGCTGGCTTAGAAGTAACCTTAACAAGCGAGATTGAACTACTTCTACTGGTTACTTGT  
 V D R V T D K S W L R S N L N K A R L N Y F Y W L L V 538  
 1855 GTCTTGGGAGCATTGAACCTTCTGATTTTTATTGTGTTGGCATGAAACATCAGTATAAAGCTGATGTGATTCTGTTGTTG  
 V L G A L N F L I F I F A M K H Q V K A D V I T V V 566  
 1937 TGACATGATGATTGAGTGGAGAAGGAGTGACGAGAAAGAGAGCTCTGAATTTGAGCTTAAGGACATTCCTTGAACCA  
 T D D D S V E K E V T K K E S S E F E L K D I P \* 590  
 2019 GATTTAGAAGAGAAAAAACCTCTGGTTTTGTTATTTGCTTTGAAGATTTCATTTTGTCTTTGTATTTTAGTATATTGT  
 2101 GTGATGAAATAGAAAGATTATATGTTTGGATTGTAATTTTTTTTGAAGATTTTAAAAATTTAGTTACTATAGTTT

Figure 2-12. DNA and deduced amino acid sequence of the *AtNRT1.3* gene.

The number on the left starts at the proposed start codon, and the numbers on the right refer to amino acid residues. Intron sequences are shown in lower cases. GATA boxes are underlined. TATA box is indicated with thick underline. Nitrate-dependent transcription motifs are double underlined.



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2839 AAGATCTTGGGCTTATGGAATCTGCACTGTCTCTATGGCTATAGCTATTGTAATATTCCTGTGTGGGACTAAGAGATACCGT
   R S W A Y G I C T V S M A I A I V I F L C G T K R Y R 247
2921 TATAAAGAGAGCCAAGGAAGTCCCGTTGTGCAATATTTTCAGGTCATAGCAGCTGCGTTCCGAAAGAGGAAAATGGAAC
   Y K K S Q G S P V V Q I F Q V I A A A F R K R K M E L P 275
3003 CTCAAAGCATTGTTTATCTTTATGAAGATAACCCCTGAAGGCATTAGAATTGAACATACTGATCAGTTTCagtgagtttctca
   Q S I V Y L Y E D N P E G I R I E H T D Q F H 298
3085 gttcaatctttacaaagaggaagtaaattaaagtaaattttgtatctataaaaaatcttggtgtgtagCTTGTGGACAAGGCG
   intron V L L D K A 303
3167 GCCATAGTTGCAGAAGGAGATTTTGAACAAACCCTTGATGGAGTTGCAATCCCAACCCTTGAAGCTAAGCTCAGTGACCA
   A I V A E G D F E Q T L D G V A I P N P W K L S S V T K 331
3249 AAGTTGAGGAAGTAAAAATGATGGTTAGGCTTTTGCCTATTTGGGCAACAACATAATTTTGGACAACATATGCCCAAAT
   V E E V K M M V R L L P I W A T T I I F W T T Y A Q M 358
3331 GATTACATTCTCTGTTGAGCAAGCTTCAACTATGAGACGTAACATTGGAAGCTTTAAGATCCCAGCTGGTTCCCTCACCCTG
   I T F S V E Q A S T M R R N I G S F K I P A G S L T V 385
3413 TTTTCGTTGCGGCTATTCTCATAACTCTAGCTGTCTACGACCGTGCCATAATGCCTTTTGAAGAAATGGAAGGAAAAC
   F F V A A I L I T L A V Y D R A I M P F W K K W K G K P 413
3495 CAGgtgacataagattcatagattcatatattctttttcatatatgtctctcagctatatattcacatgcattaaca
   G intron VI 414
3577 tttcagGTTTCTCTAGCCTACAAAGAATAGCTATTGGATTGGTCTTATCAACCGCTGGAATGGCAGCTGCAGCTCTAGTAGA
   F S S L Q R I A I G L V L S T A G M A A A A L V E 439
3659 GCAAAAGCGTTTATCCGTTGCGAAATCTAGTTTCACAAAAACATTGCCTATAAGTGTGTTTTACTTGTTCACAATTCTTC
   Q K R L S V A K S S S Q K T L P I S V F L L V P Q F F 466
3741 TTAGTAGGAGCTGGGGAAGCCTTTATCTACACTGGCCAACCTTGATTCTCTCATAACACAATCGCCTAAGGAATGAAACTA
   L V G A G E A F I Y T G Q L D F F I T Q S P K G M K T M 494
3823 TGAGCACTGGACTCTTCTTGACCACTTTATCACTAGGTTTCTTTGTGAGCAGTTTCTTGGTCTCAATCGTCAAGAGGGTCAC
   S T G L F L T T L S L G F F V S S F L V S I V K R V T 521
3905 TTCAACTTCTACTGATGTAGGATGGCTGGCTGATAACATTAACCGCCGACTCGATTACTTTTATTGGCTTTTACTGATT
   S T S T D V G W L A D N I N H G R L D Y F Y W L L V I 548
3987 CTCAGTGGAATTAACCTTCGTTGTCTATATCATATGTCCTTTGTGGTTTAAGCCAACGAAGGGTAAAGACTCAGTAGAGAAG
   L S G I N F V V Y I I C A L W F K P T K G K D S V E K E 576
4069 AAAATGGCAAGGGATTTCAGTTGAAGACTGCTGAATTTTGTGTTTGAATAATTGTCTTGTGTACAATATTATCTTGCAACA
   N G K G F S V E D C * 586
4151 ATGTCGCTTTTCCAATTATGTTTCTTGTATATTAAGCAATTGGCTTGTGTTTATGATTTCCTCCCGGTCTCAAAATATTGTGCTT
4233 TACCGTTTATGTTATTCACCTCTTTTGTGATCGTGAATAAATTAATTAACGAAGTATACTTTAATTTTCTGTTTAC
4315 GTCCTGTCGCGATCCGATTAATCTCTTTTTCGATTAACTTTTAGGTTTATGCTTTAGTAGGTCGAGAAGATGAGA
4397 TCCACATTTAGGCCTTTAGCTATTTCATATCTTTTACCTTTGTGATCTAGTGTCCGAACATTGTAGAACTGTGGAGAACA
4479 TCTTGCAAAATTAAAGATGGTCTTA

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Figure 2-13. (continued).

The number on the left starts at the proposed start codon, and the numbers on the right refer to amino acid residues. Intron sequences are shown in lower cases. GATA boxes are underlined. TATA box is indicated with thick underline. Nitrate-dependent transcription motifs are double underlined.



PTR signature sequences: PTR1: [GA]-[GAS]-[LIVMFYWA]-[LIVM]-[GAS]-D-x-[LIVMFYWT]-[LIVMFYW]-G-x<sub>3</sub>-[TAV]-[IV]-x<sub>3</sub>-[GSTAV]-x-[LIVMF]-x<sub>3</sub>-[GA] between TMS2 and TMS3, and PTR2: [FYT]-x<sub>2</sub>-[LMFY]-[FYV]-[LIVMFYWA]-x-[IVG]-N-[LIVMAG]-G-[GSA]-[LIMF] [FYT]-x<sub>2</sub>-[LMFY]-[FYV]-[LIVMFYWA]-x-[IVG]-N-[LIVMAG]-G-[GSA]-[LIMF] within TMS5, are well conserved among the four AtNRT1 members (Figure 2-14). The PTR family was not included within the MFS because of insufficient homology between the two families (Pao et al., 1998). However, PTR members possess some features in common with those of MFS: The four AtNRT1 members possess sequences of 592 amino acids on average, which are 70 residues longer than those of AtNRT2 proteins, and they are also predicted to have 12 TMS. The AtNRT1 members commonly have cytosolic N- and C-termini and a relatively long cytosolic loop, approximately 100 amino acids, between TMS6 and TMS7. In the loops at least one phosphorylation site was found in each protein. A conserved protein kinase C (PKC) phosphorylation site was also found at the end of TMS6. Putative N-glycosylation sites were found between TMS1 and TMS2 of AtNRT1.1 and 1.2. Because predicted TMS1 of AtNRT1.2, M-34 to A-53, is shifted upstream compared to AtNRT1.1, the N-54 becomes a possible glycosylation site (Figure 2-14).

TMHMM and MEMSTAT were evaluated as the finest performance prediction programs for known and un-known proteins (Moller et al., 2001). However, both programs predicted that only one third of NRT members should possess 12 TMS. Rather, Toppred, considered to be one of the less powerful performance programs, predicted that all of 11 members should have 12 TMS and cytoplasmic N-terminus (Table 2-2). These

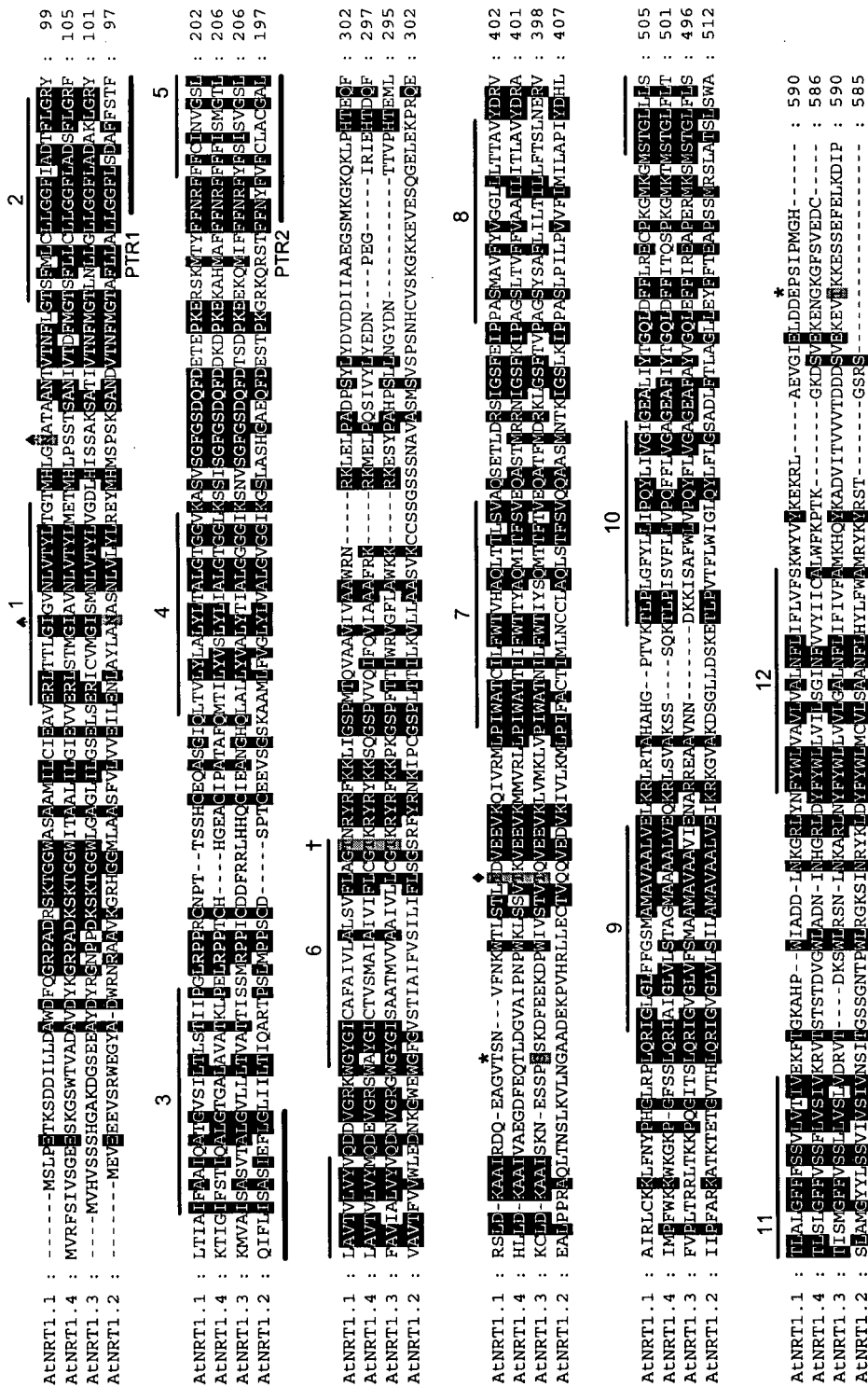


Figure 2-14. Amino acid sequence alignments of AtNRT1 family.

Amino acid sequence alignments of AtNRT1 family. Sequences were initially aligned with CLUSTAL W and finely adjusted and output with GeneDoc (Nicholas and Nicholas, 1997). Amino acid residues in black background indicate identities more than 50 % (i.e., 3 out of 4 sequences). 12 predicted TMS regions of AtNRT1.1, determined by HMMTOP program (Tusnady and Simon, 1998), were indicated by numbered lines above the sequences. Other members' TMS locations were close to those of AtNRT1.1 except TMS1 of AtNRT1.2 (see RESULT5). Potential protein kinase C and casein kinase 2 phosphorylation, and N-glycosylation sites, indicated with (+), (♦), and (▲), respectively, were searched with the ProSite database (<http://www.expasy.ch/prosite/>). The threonine/serine residues, indicated with asterisk, are recognition sites for both PKC and CK2. Conserved sequence regions, PTR1 and 2 in (b), are indicated in thick lines under the sequences.

discrepancies might be due to insufficient information of the MFS proteins. Clearly larger data sets and experimental evidence are essential for more accurate predictions.

Introns found in the *AtNRT* gene families carry conserved sequences at 5' and 3' ends, G-T-[AT]-[ATC] and [CT]-A-G, respectively. These sequences match with the consensus sequences for splicing in eukaryotes (Keller and Noon, 1984; Padgett et al., 1986). The position and length of introns become a key factor for designing primers of RT-PCR. This detail will be discussed in the next chapter.

# 3

## Expression patterns of Nitrate Transporter *AtNRT* Genes

### 3.1 Introduction

It has been demonstrated that genes belonging to the *NRT2* family are induced in response to nitrate provided externally. Among lower eukaryotes, this family includes representation in *Aspergillus nidulans* *NrtA* and *NrtB* (Unkles et al., 2001), *Chlamydomonas reinhardtii* *CrNRT2.1* and *2.2* (Quesada et al., 1998), and the diatom *Cylindrotheca fusiformis* *NAT1* and *NAT2* (Hildebrand and Dahlin, 2000). In higher plants, the barley *HvNRT2.1* (*BCH1*) was one of the earliest identified *NRT2* members that showed rapid induction in response to nitrate provision. In this species the transcript was detectable within 30 min of  $\text{NO}_3^-$  provision (Trueman et al., 1996). Subsequently, cloned *NRT2* genes from other species also showed nitrate inducibility with 1 to 4 h for the peak of induction. These include *Arabidopsis thaliana* *AtNRT2.1* and *AtNRT2.2* (Filleur and Daniel-Vedele, 1999; Lejey et al., 1999; Zhuo et al., 1999), *Nicotiana plumbaginifolia* *NpNRT2.1* (Quesada et al., 1997;

Krapp et al., 1998), *Glycine max* *GmNRT2* (Amarasinghe et al., 1998), and *Lycopersicon esculentum* *LeNRT2* (Ono et al., 2000). However, additional members of the *NRT2* family from *A. thaliana*, identified from searches of the *Arabidopsis* genome data bank, *AtNRT2.3-2.7*, have not been characterized yet.

*NRT1* family genes, on the other hand, respond to nitrate provision in two ways, either they are nitrate-inducible, or nitrate-constitutive. *AtNRT1.1* (*CHL1*), the first nitrate transporter gene isolated from higher plants, was induced by nitrate and acidic pH (Tsay et al., 1993). Nitrate-inducible *NRT1* genes have also been identified from *Brassica napus* *BnNRT1.2* (Zhou et al., 1998), *G. max* *GmNRT1.2* (Yokoyama et al., 2001), and *L. esculentum* *LeNRT1.2* (Lauter et al., 1996; Ono et al., 2000). By contrast, nitrate-constitutive genes, such as *AtNRT1.2*, are expressed independently of external nitrate. These genes were also observed in *G. max* *GmNRT1.3* (Yokoyama et al., 2001), *L. esculentum* *LeNRT1.1* (Lauter et al., 1996; Ono et al., 2000), and *Oryza sativa* *OsNRT1* (Lin et al., 2000). The expression patterns of *AtNRT1.3* and *1.4* are unknown.

Significant portions of incoming  $\text{NO}_3^-$  proceed from roots to shoots to be reduced and metabolized and/or stored (Marschner, 1995). Indeed, in some species virtually all nitrate reduction occurs in leaf tissue, and hence  $\text{NO}_3^-$  absorption by leaf cells is of crucial importance. Yet, there is little information concerning  $\text{NO}_3^-$  transport systems in shoots. Although loading of  $\text{NO}_3^-$  into xylem vessels is considered to be passive, the absorption of  $\text{NO}_3^-$  from the leaf apoplast by leaf mesophyll cells is necessarily against the electrochemical potential gradient (Glass and Siddiqi, 1995). This  $\text{NO}_3^-$  flux might be anticipated to involve LATS based upon typical values of xylem  $\text{NO}_3^-$  (Glass and Siddiqi, 1995). Notwithstanding the importance of absorbing  $\text{NO}_3^-$  into leaf cells, expression levels

of *AtNRT1.1*, *1.2*, and *2.1* in shoots were shown to be significantly lower than those in roots (Tsay et al., 1993; Huang et al., 1999; Zhuo et al., 1999). However, Guo et al. (Guo et al., 2001) showed strong expression of *AtNRT1.1* in shoots using GFP/GUS fusion lines. The foregoing emphasizes the importance of  $\text{NO}_3^-$  transport systems in shoots.

Therefore, I have characterized the expression patterns of 11 *AtNRT* family members in both roots and shoots, in response to the provision of  $\text{NO}_3^-$  following a 7-day period of  $\text{NO}_3^-$  deprivation. The transcript abundances were analyzed quantitatively by RT-PCR.

## 3.2 Materials and Method

### Plant growth condition

*Arabidopsis* plants (ecotype Columbia) were grown hydroponically under non-sterile conditions as described in earlier papers (Gibeaut et al., 1997; Lejay et al., 1999). Briefly, ¼ inch thick Styrofoam was fitted to an 8L container as a floating platform to support plant growth. Each platform contained 30 holes (diameter 1.5 cm) covered with nylon mesh at the bottom surface and these holes were filled with clean sand. Seeds were imbibed in a cold room at 4 °C for 3-5 days, and sown directly on the moistened sand in the platform. The nutrient solution which was used to support plant growth contained: 1mM  $\text{KH}_2\text{PO}_4$ , 0.5mM  $\text{MgSO}_4$ , 0.25mM  $\text{CaSO}_4$ , 20µM Fe-EDTA, 25µM  $\text{H}_3\text{BO}_3$ , 2µM  $\text{ZnSO}_4$ , 2µM  $\text{MnSO}_4$ , 0.5µM  $\text{CuSO}_4$ , 0.5µM  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ , and 0.5mM  $\text{NH}_4\text{NO}_3$ . For the induction study, 5-week-old plants were transferred to -N solution for one week (other nutrients remained as before), then re-induced with 0.5mM  $\text{Ca}(\text{NO}_3)_2$  for from 0 to 72 hours. pH of

the nutrient solution was maintained with  $\text{CaCO}_3$  around 6.2. The walk in environment chamber was maintained under the following conditions: light/dark=8/16 hrs, 25/20 °C; RH=70 %. Light was provided from fluorescent tubes (VITA-LITE,  $150 \mu\text{E m}^{-2}\text{sec}^{-1}$ ). All flux determinations and plant harvesting for RNA extraction were undertaken at 4 hours after the light period began, and all pretreatments were appropriately staggered to meet this requirement. All experiments were repeated at least twice. The means of those experiments were used as physiological data, and some data such as gel pictures were representatives of the experiments.

#### **RNA isolation and relative quantitative RT-PCR**

RNA was isolated using TRIzol Reagent (Life Technology, Grand Island, NY) according to the manufacturer's method followed by additional chloroform isolation and sodium acetate precipitation steps. Total RNA concentrations were determined by UV spectrophotometry. RT-PCR was carried out using OneStep RT-PCR Kit (Qiagen Inc., Mississauga, ON) with QuantumRNA 18S Internal Standards (Ambion, Inc., Austin, TX) under the following conditions: 50 °C for 30 min; 95 °C for 15 min; 19-37 cycles (Table 3-1) of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min; 72 °C for 10 min. Total RNA, 25 to 250 ng (Table 3-1), was added to the reaction mixture containing 400  $\mu\text{M}$  dNTP, 0.6  $\mu\text{M}$  of gene specific primers (Table 3-1), 0.4  $\mu\text{M}$  of the QuantumRNA 18S primers, 0.2  $\mu\text{g}$  of hexamers, as well as buffer and enzymes according to the manufacturer's protocol, and RNase-free water was added to make a final volume of 12.5  $\mu\text{l}$ . The RT-PCR products were electrophoresed on 1.3 % agarose gels and stained with SYBRGold (Molecular Probes, Inc., Eugene, OR) for

Table 3-1. Gene specific primers and conditions<sup>a</sup> of RT-PCR

Gene	Primer	Product size (bp)	Shoot		Root	
			RNA <sup>b</sup> (ng)	Cycle number	RNA <sup>b</sup> (ng)	Cycle number
AtNRT2.1	Forward	770	250	28	25	21
	Reverse					
AtNRT2.2	Forward	401	250	33	25	21
	Reverse					
AtNRT2.3	Forward	366	250	37	250	27
	Reverse					
AtNRT2.4	Forward	520	250	33	250	27
	Reverse					
AtNRT2.5	Forward	503	250	26	250	30
	Reverse					
AtNRT2.6	Forward	505	250	32	250	30
	Reverse					
AtNRT2.7	Forward	691	250	28	125	19
	Reverse					
AtNRT1.1	Forward	809	250	21	250	26
	Reverse					
AtNRT1.2	Forward	463	250	23	25	26
	Reverse					
AtNRT1.3	Forward	609	250	21	25	26
	Reverse					
AtNRT1.4	Forward	621	250	21	250	26
	Reverse					

<sup>a</sup> Detail conditions are described in Materials and Methods.<sup>b</sup> Amount of total RNA used in single RT-PCR.



30 minutes. The signals of the targeted gene products on the stained gels were captured and densitometry analyzed with an AlphaImager™ 1200 (Alpha Innotech Corp., San Leandro, CA). For each gene, the optimization of RT-PCR was strictly carried out as follows: 1. Preliminary RT-PCR trials to determine the specificities of the gene specific primers and the presence of the gene expression (e.g. product size and its sequence, and approximate expression levels among the samples); 2. Determination of the PCR cycle number which gave linear range of gene amplification, choosing one sample in which the target gene was expected to be the most abundant. After the RT step, aliquots were taken every two cycles in the PCR step. Signal intensities were then plotted versus cycle numbers on a log scale. A cycle number in the linear range was chosen for the subsequent experiments (Table 3-1). 3. The amounts of the 18S rRNA internal standard primers were determined according to the manufacture's protocol in order to amplify both the target gene and the 18S at a similar level.

### **3.3 Results**

#### **Expression of Two *NRT* families by Relative Quantitative RT-PCR**

A unique feature of the transport of nitrate, compared to other inorganic nutrients is that nitrate ( $\text{NO}_3^-$ ) is both a substrate for transport and also for the induction of nitrate transport systems at the gene and at the physiological levels (Glass and Siddiqi, 1995; Forde, 2000). Therefore, as the first step in characterizing members of the two  $\text{NO}_3^-$  transporter families, their responses to induction by  $\text{NO}_3^-$  were examined by means of time-course experiments.

Five-week-old *Arabidopsis* plants were nitrogen starved for 7 days, in order to de-induce  $\text{NO}_3^-$  transport (Siddiqi et al., 1989). Preliminary experiments showed that  $\text{NO}_3^-$  was undetectable in roots by 4 days after removing exogenous  $\text{NO}_3^-$ , while shoot  $\text{NO}_3^-$  was reduced by >70 % by 7 days. The starved plants were then transferred to nutrient solution supplemented with 1mM  $\text{NO}_3^-$  for up to 3 days. Gene expression in response to this renewed  $\text{NO}_3^-$  provision was analyzed using relative quantitative RT-PCR method.

Designing the gene specific primer is the first and perhaps the most important step of the entire RT-PCR method. Some online programs are helpful to seek ideal primer sets by using information such as GC contents,  $T_m$  value, primer length, size of the product, and location on the gene. The final decision, however, has to be based on the need to select unique sequences. Table 3-1 shows the gene specific primer sets used in the study. The primers were designed either to flank half of a primer lay in one exon and the second half in the next exon to eliminate genomic DNA amplification, or to flank a region that contains intron(s) to indicate genomic DNA contamination (i.e., genomic DNA would be amplified in larger product size than that of cDNA due to intron(s)). The product sizes of the gene specific primers were aimed to be more than 300 bp since the internal standard primer sets produced about 300 bp fragment (Table 3-1). The specificities were confirmed by the sizes and sequences of the products. PCR conditions including amount of RNA template and PCR cycle numbers were optimized after several trials.

Figure 3-1 shows a relative expression level among the *AtNRT* family genes. The values were calculated by the amount of RNA used for RT-PCR, and the PCR cycle numbers (Table 3-1). Although quantitative relative RT-PCR does not count absolute copy number of an RNA transcript, it still provides a simple estimation of the relative expression

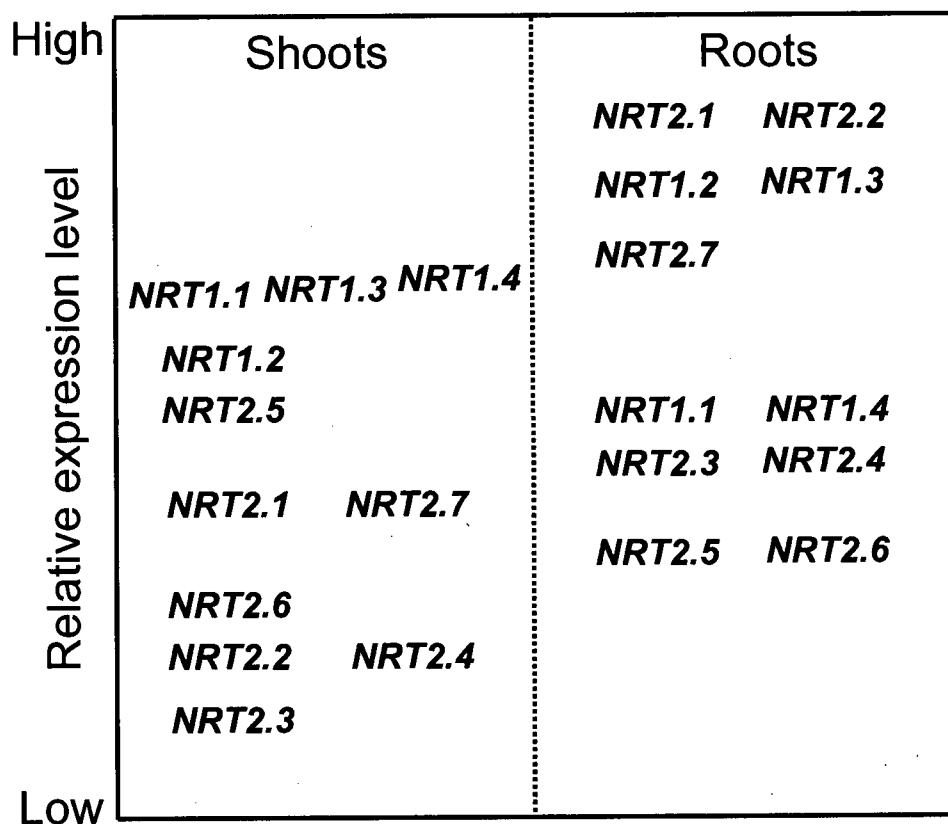


Figure 3-1. Estimated relative expression levels of *AtNRT* genes.

Relative value =  $1 / (a \times 2^n)$ , where *a* is amount of RNA used in the RT-PCR; *n* is PCR cycle number. Y-axis is expressed in logarithmic scale.

level among the genes. Typically, expression levels of all genes, with the exceptions of *AtNRT1.1*, *AtNRT1.4* and *AtNRT2.5*, were higher in roots than shoots. Within root tissues, the following four genes (listed in order of decreasing transcript abundances) were more highly expressed than in shoots, and more highly expressed than all other genes whether in roots or shoots:  $AtNRT2.1 \geq 2.2 > 1.2 \cong 1.3$ . On a relative scale, based upon the number of PCR cycles required and quantities of template RNA used to obtain a similar signal, and assigning an arbitrary value of 100% for root expression levels of *AtNRT2.1*, *AtNRT 2.2*, *AtNRT1.2* and *AtNRT1.3*, their expression levels in shoots and expression levels of all other genes in roots or shoots were ~5 to 10%. By contrast, within shoot tissues, all members of the *AtNRT1* family were expressed in greater abundance than *AtNRT2* genes (Figure 3-1).

Despite diverse levels of transcript abundances it was possible to detect expression patterns of all eleven  $\text{NO}_3^-$  transporter genes (Figures 3-2 to 3-6). From the responses to  $\text{NO}_3^-$  exposure, genes were categorized into three groups, namely, nitrate-inducible, nitrate-repressible, and nitrate-constitutive. In all of the following, plants that had been grown for 5 weeks with ammonium nitrate were N-deprived for 1 week and then re-exposed to 0.5 mM  $\text{Ca}(\text{NO}_3)_2$  for up to 72h.

### **Nitrate-Inducible Genes**

Three genes in this category, *AtNRT2.1*, *AtNRT2.2* and *AtNRT1.1* showed very strong induction (>3 to 5-fold increases) in root tissues following exposure to 1 mM  $\text{NO}_3^-$ , while other genes showed only modest increases in roots or shoots under the same conditions. In

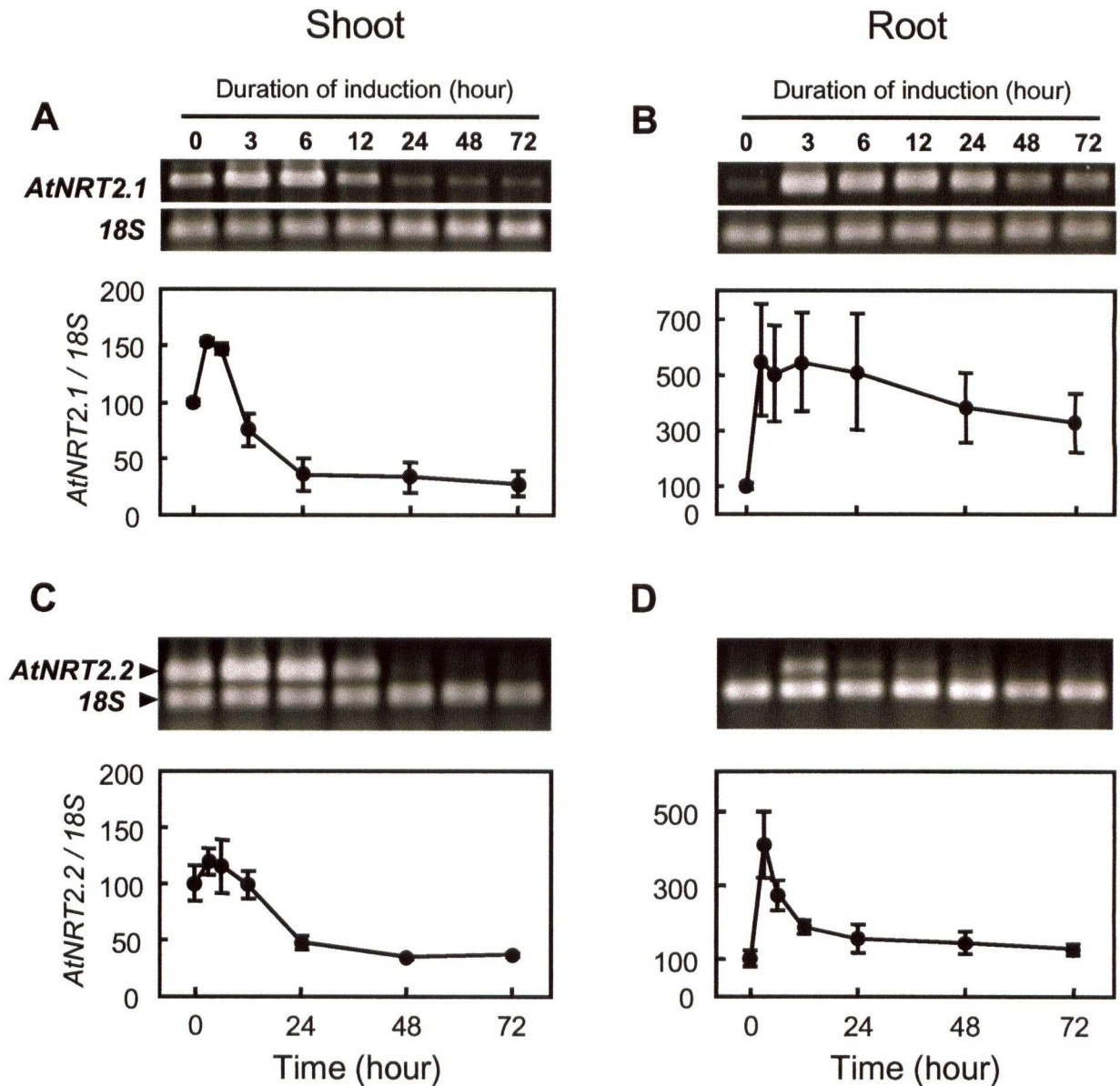


Figure 3-2. Expression patterns of nitrate-inducible *AtNRT2* genes.

RT-PCR products were obtained from 6-week-old *Arabidopsis* plants, which were grown hydroponically for 5 weeks and supplied with 0.5 mM  $\text{NH}_4\text{NO}_3$ . Plants were N deprived for 1 week (0h), and then re-supplied with 0.5 mM  $\text{Ca}(\text{NO}_3)_2$  for 3-72h. Relative values were obtained by the ratio of the gene specific amplicon over the 18S amplicon. The values shown are means of three RT-PCR replicates. Bars indicate SE. *AtNRT2.1* in shoot (A), root (B), *AtNRT2.2* in shoot (C), root (D).

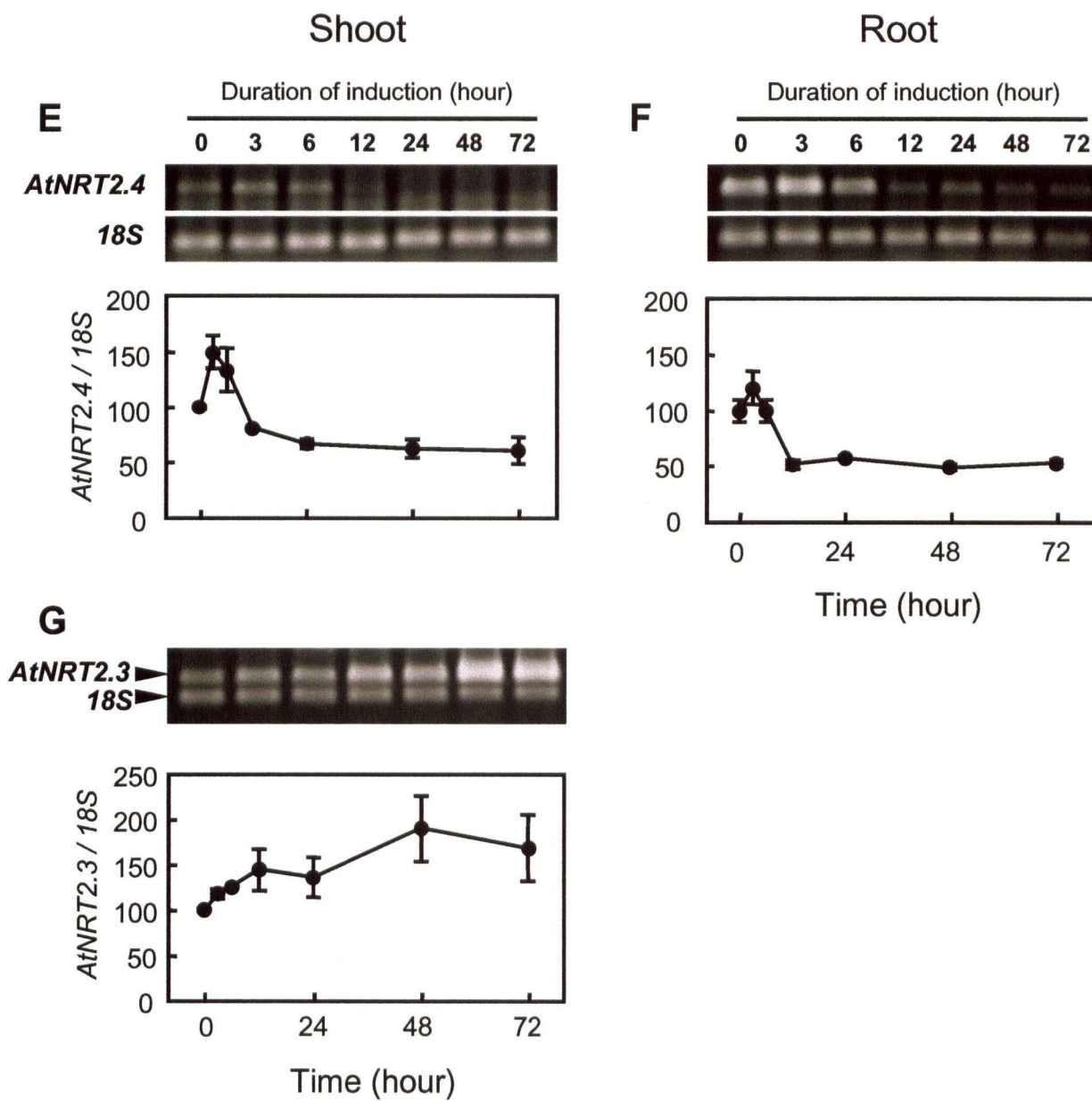


Figure 3-2 (continued).

*AtNRT2.4* in shoot (E), root (F); *AtNRT2.3* in shoot (G).

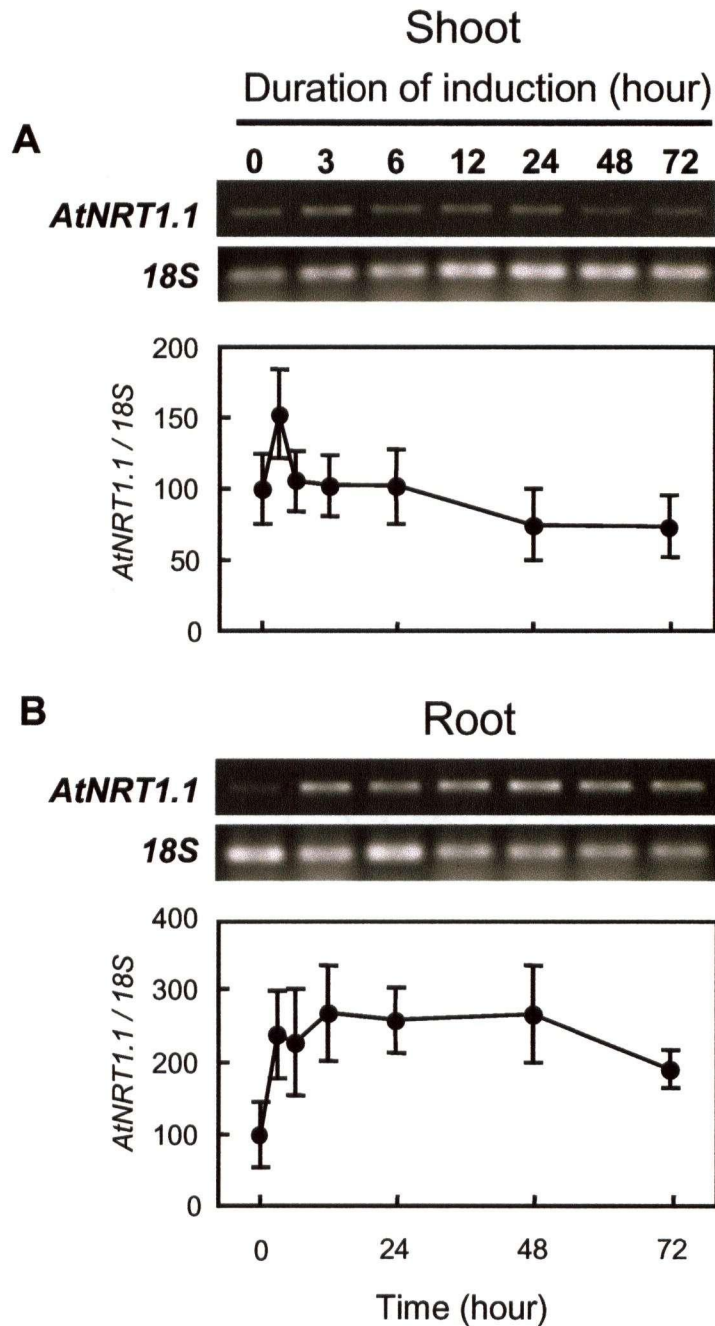


Figure 3-3. Expression patterns of nitrate-inducible *AtNRT1* genes.

RT-PCR products were obtained from 6-week-old *Arabidopsis* plants, which were grown hydroponically for 5 weeks and supplied with 0.5 mM  $\text{NH}_4\text{NO}_3$ . Plants were N deprived for 1 week (0h), and then re-supplied with 0.5 mM  $\text{Ca}(\text{NO}_3)_2$  for 3-72h. Relative values were obtained by the ratio of the gene specific amplicon over the 18S amplicon. The values shown are means of three RT-PCR replicates. Bars indicate SE. *AtNRT1.1* in shoot (A), root (B).



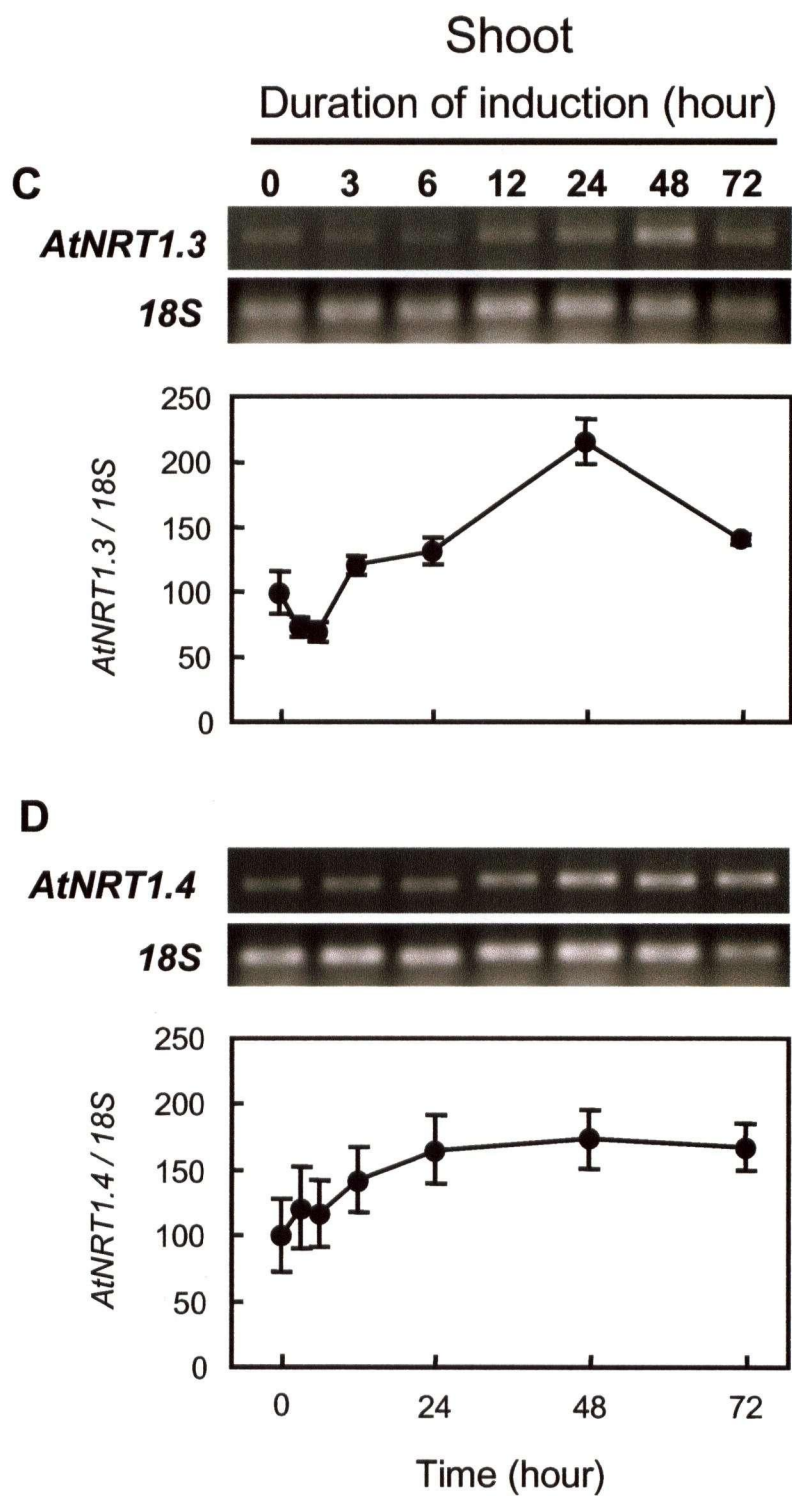


Figure 3-3 (continued).

*AtNRT1.3* in shoot (C); *AtNRT1.4* in shoot (D).



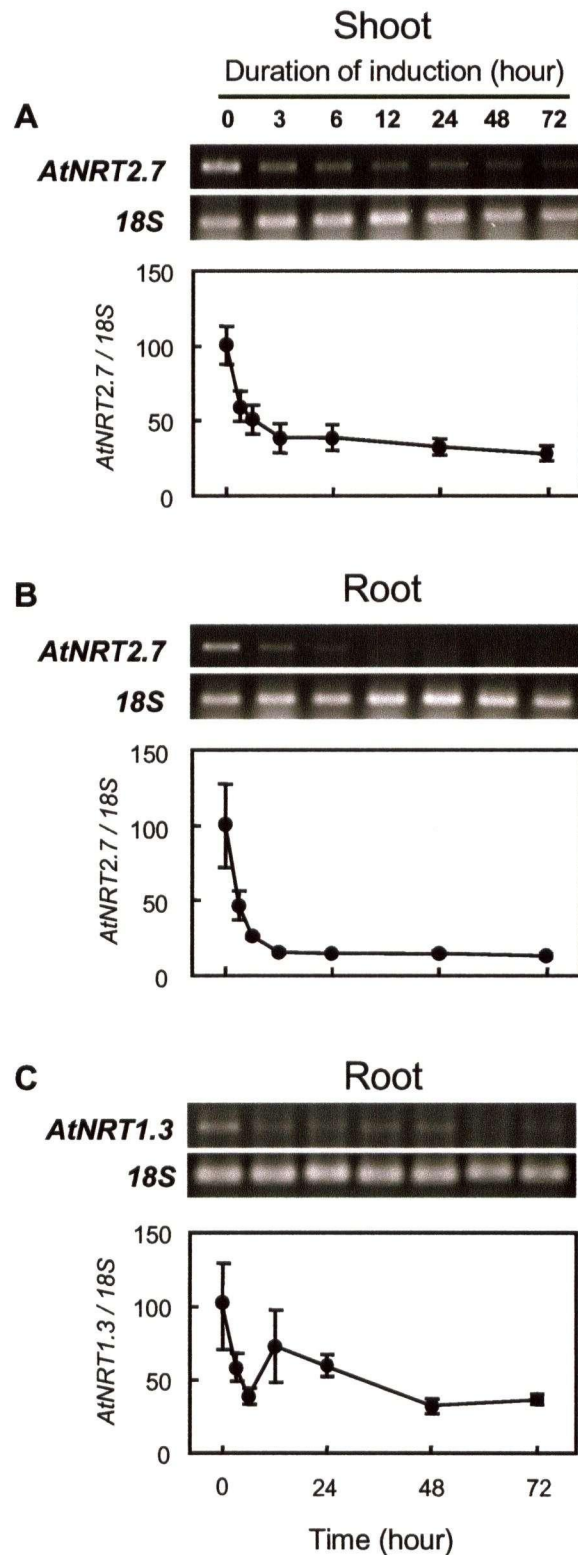


Figure 3-4. Expression patterns of nitrate-repressible *AtNRT* genes.

RT-PCR products were obtained from 6-week-old *Arabidopsis* plants, which were grown hydroponically for 5 weeks and supplied with 0.5 mM  $\text{NH}_4\text{NO}_3$ . Plants were N deprived for 1 week (0h), and then re-supplied with 0.5 mM  $\text{Ca}(\text{NO}_3)_2$  for 3-72h. Relative values were obtained by the ratio of the gene specific amplicon over the 18S amplicon. The values shown are means of three RT-PCR replicates. Bars indicate SE. *AtNRT2.7* in shoot (A), root (B); *AtNRT1.3* in root (C).

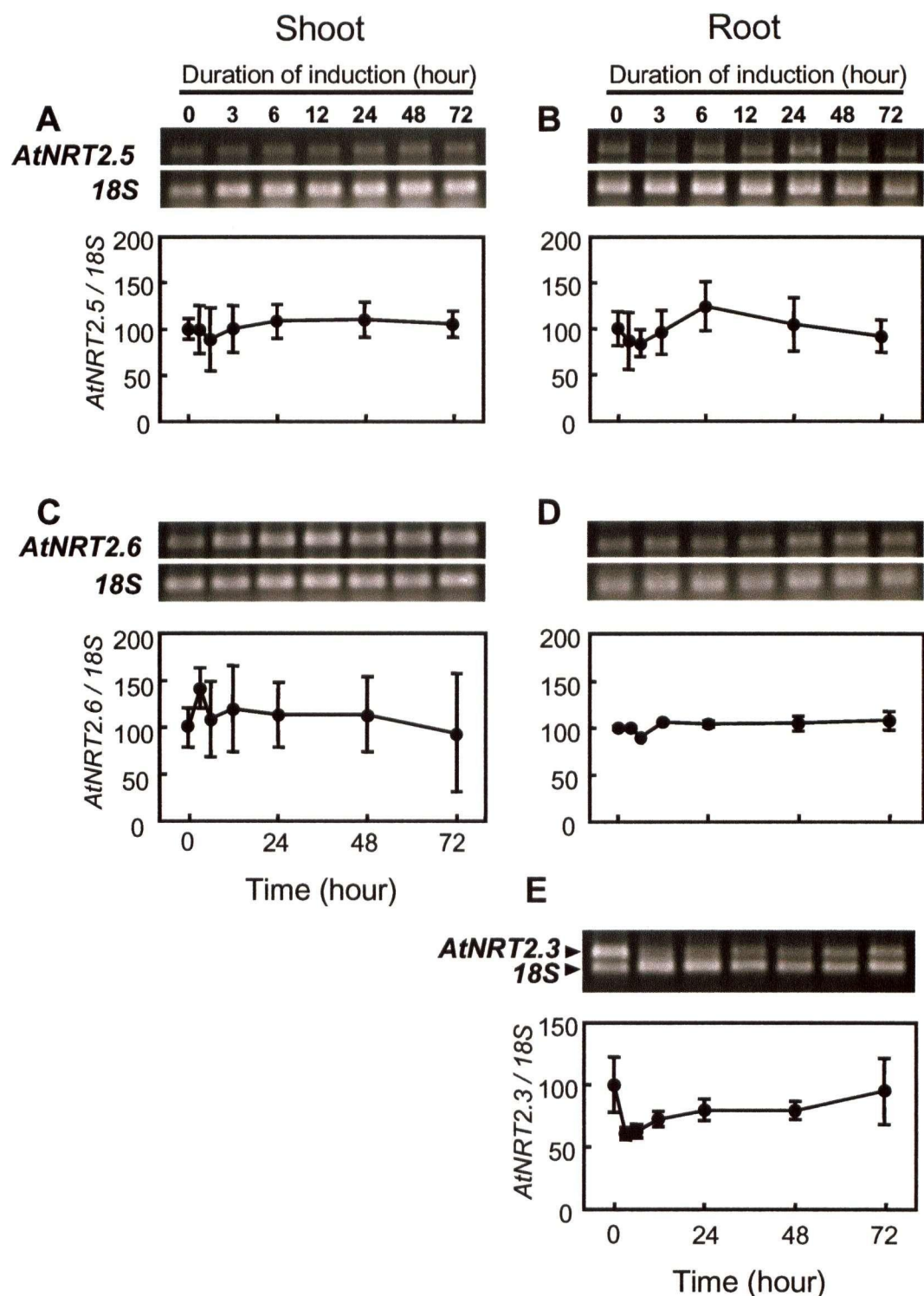


Figure 3-5. Expression patterns of nitrate-constitutive *AtNRT2* genes.

RT-PCR products were obtained from 6-week-old Arabidopsis plants, which were grown hydroponically for 5 weeks and supplied with 0.5 mM  $\text{NH}_4\text{NO}_3$ . Plants were N deprived for 1 week (0h), and then re-supplied with 0.5 mM  $\text{Ca}(\text{NO}_3)_2$  for 3-72h. Relative values were obtained by the ratio of the gene specific amplicon over the 18S amplicon. The values shown are means of three RT-PCR replicates. Bars indicate SE.

*AtNRT2.5* in shoot (A), root (B); *AtNRT2.6* in shoot (C), root (D); *AtNRT2.3* in root (E).

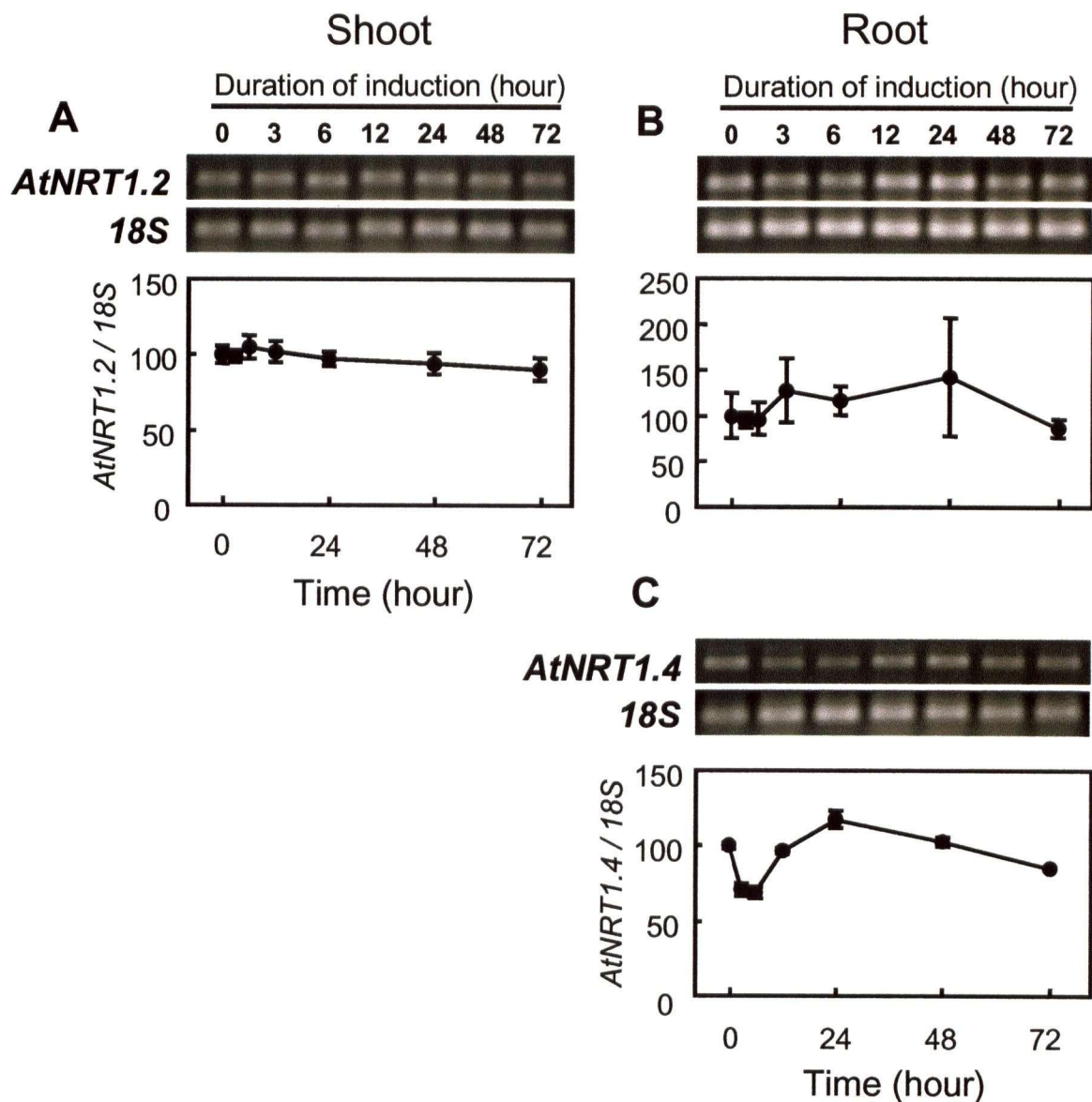


Figure 3-6. Expression patterns of nitrate-constitutive *AtNRT1* genes.

RT-PCR products were obtained from 6-week-old *Arabidopsis* plants, which were grown hydroponically for 5 weeks and supplied with 0.5 mM  $\text{NH}_4\text{NO}_3$ . Plants were N deprived for 1 week (0h), and then re-supplied with 0.5 mM  $\text{Ca}(\text{NO}_3)_2$  for 3-72h. Relative values were obtained by the ratio of the gene specific amplicon over the 18S amplicon. The values shown are means of three RT-PCR replicates. Bars indicate SE.

*AtNRT1.2* in shoot (A), root (B); *AtNRT1.4* in root (C).

the *AtNRT2* family, *AtNRT2.1* showed the strongest induction by  $\text{NO}_3^-$  in roots (Figure 3-2B). By three hours of  $\text{NO}_3^-$  provision, the expression level had reached > 5 fold that of 0h-plants. This peak was sustained for up to 24h, followed by a gradual reduction to about half of the peak level by 72h. In shoots, on the other hand, there was only a small enhancement in the expression of *AtNRT2.1* after 3h of exposure to  $\text{NO}_3^-$ , followed by rapid down-regulation after 6h, until by 24 h the level was lower than it had been at 0h. Transcript level in shoots was less than 1% of that observed in roots based on the fact that shoot PCR required 7 times the number of PCR cycles and 10 times more RNA template than root PCR (Figure 3-2A, Table 3-1). *AtNRT2.2* was also induced by  $\text{NO}_3^-$  both in roots and shoots. As was the case for *AtNRT2.1*, this was more pronounced in roots where 4 fold increases in transcript abundance were evident by 3h. However, after this peak, expression levels were quickly down-regulated and returned to a value that was close to the original level by 24h (Figures 3-2C and 3-2D). This rapid down-regulation was different from that observed for *AtNRT2.1*, although the expression level of *AtNRT2.2* was initially similar to *2.1* as we described earlier. *AtNRT2.2* in shoots as well as *AtNRT2.4* in both in shoots and roots showed similar expression patterns which were characterized by modest increases culminating in peaks at 3h followed by declines that stabilized by 24h (Figures 3-2C, 3-2E, and 3-2F).

*AtNRT1.1* also showed a strong induction by  $\text{NO}_3^-$  in roots. The induction peaked rapidly, as early as the third hour of  $\text{NO}_3^-$  provision, and reached a maximum level that was 2.5 times that of the 0h. This high level of transcript was sustained from 12h to 48h (Figure 3-3B). The expression of *AtNRT1.1* in shoots showed a similar pattern to that of *AtNRT2.4*, namely a small spike at 3 hours, followed by a return to a level that was as low as the

original level (Figure 3-3A). Interestingly, unlike *AtNRT2.1*, the expression level of *AtNRT1.1* was equal to, or even higher in shoots than roots considering both the PCR cycle numbers and RNA template provided (Table 3-1, Figures 3-3A, and 3-3B).

There were three genes, *AtNRT1.3*  $\cong 1.4 > 2.3$ , which were induced by nitrate only in shoots (Figure 3-2G, 3-3C, and 3-3D). The expression patterns of those genes in the roots will be described later. *AtNRT2.3* was induced slowly, reaching peak expression that was 90% higher than at time 0 h, only after 48 h of  $\text{NO}_3^-$  provision (Figure 3-2G). *AtNRT2.3* expression level in the shoots was much lower than that in the roots (based on a requirement for 10 times as many PCR cycle numbers as in the roots) (Table 3-1). In fact, within shoot transcript abundance of this gene was among the lowest of all of the *NRT* family of genes. *AtNRT1.3* expression level was also gradually induced, increasing to more than 2 fold at 48h, although there was a slight decline during the first 6 hours. However, there was subsequently a significant down-regulation, which began at 48h, reducing transcript abundance to a level that was similar to that at 24 h (Figure 3-3C). Another *NRT1* family member, *AtNRT1.4* also had a slow induction pattern, reaching the plateau after 48h, a level that was maintained for the next 48 hours (Figure 3-3D).

### Nitrate-Repressible Genes

In contrast to  $\text{NO}_3^-$ -inducible genes, there were some genes whose transcript abundances were actually reduced by nitrate provision. *AtNRT2.7* conformed to this pattern, in both roots and shoot, while this pattern was only observed in roots for *AtNRT1.3*. Highest levels

of *AtNRT2.7* were evident prior to the provision of nitrate (0h). By 3-6 h after exposure to 1mM  $\text{NO}_3^-$ , transcript abundances had declined to 50% (shoots) and 25% (roots) of the 0h values (Figures 3-4A and 3-4B). Although both shoots and roots shared a similar response pattern, roots responded more rapidly than shoots. It is also notable that the transcript abundance in roots was more than 100 times higher than that of the shoots at 0h (Table 3-1, Figures 3-1). *AtNRT1.3* also revealed a repressible pattern in the roots, although gene expression was induced by  $\text{NO}_3^-$  in the shoots (Figures 3-3C and 3-4C). The initial response to nitrate provision was as rapid as that of *AtNRT2.7* in the roots. However, after 6h, expression level decreased, reaching a value that was about 30 % of the initial (0h) value by 48h (Figure 3-4C).

### **Nitrate-Constitutive Genes**

The third group of nitrate transporter genes is described as constitutively expressed. A characteristic pattern of this group is that substantial transcript abundance was already present even under  $\text{NO}_3^-$ -starved conditions (0h), and the expression levels did not change substantially during 72 h of exposure to  $\text{NO}_3^-$ . *AtNRT2.5* and 2.6 in the NRT2 family, and *AtNRT1.2* in the NRT1 family showed such a constitutive expression pattern both in shoots and roots, wherein the fluctuations of expression levels were less than  $\pm 50\%$  (Figures 3-5 and 3-6). Interestingly, the expression level of *AtNRT2.5* in the shoot was higher than that in the roots (Figures 3-1, 3-5A, and 3-5B). This is unique in the *AtNRT2* family because all other members had greater transcript abundance in roots (Figure 3-1). Although *AtNRT1.2*

expression level in shoots was ~10% of that in roots, this gene was among the most highly expressed of shoot-expressed genes. Given that the *AtNRT1.2* is constitutively expressed, overall, this is one of the most highly expressed nitrate transporter genes throughout the plant during all stages of our investigation.

As mentioned earlier, *AtNRT2.3* showed a nitrate-inducible pattern in the shoots, but its expression in roots was essentially constitutive, although a slight down-regulation was observed during the first 6 hours (Figure 3-5E). Besides differences in root/shoot expression patterns, expression levels were also significantly different (i.e., root levels were typically ~10 times higher than those of the shoots) (Figure 3-1). The expression of *AtNRT1.4* in the roots also showed a constitutive pattern (Figure 3-6C). This insensitivity to  $\text{NO}_3^-$  did not match its shoot expression where the gene was induced by  $\text{NO}_3^-$  provision (Figure 3-3D). Similar to *AtNRT2.5* and *AtNRT1.1*, *AtNRT1.4* was the third gene whose expression levels were higher in shoots than in roots (Figure 3-1).

### 3.4 Discussion

The present study using RT-PCR clearly showed that all 11  $\text{NO}_3^-$  transporter genes were expressed in roots and shoots, whereas previous reports have claimed that expression levels of nitrate transporters in the shoots were either extremely low or undetectable. This applies specifically to *AtNRT2.1* (Filleur and Daniel-Vedele, 1999; Zhuo et al., 1999), *AtNRT2.2* (Zhuo et al., 1999), *AtNRT1.1* (Tsay et al., 1993), and *AtNRT1.2* (Huang et al., 1999). Our successful demonstration of shoot expression of these genes, though in low abundance, is



probably due to the greater sensitivity of the RT-PCR method but might also result from differences in plant growth conditions among different laboratories. In previous reports plants were grown in sterilized systems where sucrose was added in the media (Tsay et al., 1993; Huang et al., 1999; Zhuo et al., 1999). Sucrose additions to growth media have been documented to produce significant effects at both the physiological and molecular levels. Hanisch and Breteler (1981) showed that exogenous sucrose application restored the decreased nitrate uptake rate and nitrate reductase activity to 83 % and 108 % of the level of the intact plants, respectively, in decapitated dwarf bean roots. This indicated that carbon metabolites normally provided through photosynthesis could be compensated for by sugar supplement via root tissues. In *Arabidopsis* 1 % sucrose in the media stimulated transcripts of *AtNRT1.1* and *AtNRT2.1*, and nitrate influx during dark periods (Lejay et al., 1999). On the other hand, photosynthetic genes were down-regulated by sugars in isolated maize mesophyll protoplasts (Sheen, 1990). Furthermore, Krapp and Stitt (1995) demonstrated that a cold-girdle treatment on spinach leaves led to an increase of sugar contents in the leaves, and inhibition of the rate of photosynthesis and expression of photosynthetic genes. In addition, when sucrose is provided via roots, any carbon limitation upon  $\text{NO}_3^-$  reduction and assimilation is removed and in contrast to plants growing without sucrose, it is reasonable to expect that there might be greater root than shoot assimilation of nitrate. Therefore, it is obvious that the presence of sucrose in the growth media has effects on N- and C-metabolism, the localization (root versus shoot) of these activities, and its expression levels of genes involved in these processes at the gene and physiological levels, although the mechanisms of the interactions between sucrose and nitrate transporters are not clear (Coruzzi and Bush, 2001).



Although the patterns of, and extents of, expression levels of the genes were variable, we grouped all members into three categories in order to organize the information generated. Most of those genes investigated had similar expression patterns in roots and shoots. However, in specific cases, such as *AtNRT2.3*, and *1.3*, expression patterns were quite different between roots and shoots (Figures 3-2 to 3-5). Another example of different expression patterns within the same plants was observed through a split-root experiment. The expression level of *AtNRT2.1* in one portion of the roots under steady state condition was up-regulated by N-depriving the other portion of the roots where *NRT2.1* expression was slowly down-regulated (Gansel et al., 2001). The result indicated that *NRT2.1* was controlled not only by local N-supply but also shoot-root signals of N demand (Gansel et al., 2001). Interestingly, the authors observed a different response with respect to *AtAMT1.1* expression which seemed to be controlled primarily by local conditions.

In the present study, the N-pool size within the plant tissues changed during the period following re-provision of nitrate. These changes of nitrate concentration as well as amino acid pools are probably important in the regulation of some *NRT* genes, resulting in different expression patterns within the plant. To date, since little is known about the nitrate transporters and transport systems in shoots, we are not able to define the roles of specific *NRT* genes in shoots, other than the stated importance of absorbing  $\text{NO}_3^-$  into leaf cells from the leaf apoplasm. Yet, further proposed functions of the *NRT* nitrate transporters will be discussed in chapter 5.

On the basis of correlations between physiological patterns of  $\text{NO}_3^-$  influx and corresponding patterns of gene expression, it has been suggested that the *NRT2* proteins probably play a major role in inducible HATS in *Arabidopsis* (Zhuo et al., 1999; Lejay et

al., 1999), *N. plumbaginifolia* (Krapp et al., 1998), soybean (Amarasinghe et al., 1998), and barley (Glass et al., 2001). In this study *AtNRT2.1*, *2.2* and *2.4* showed nitrate-inducible patterns, suggesting that these genes might be involved in iHATS activity (Table 3-2). However, it is evident from the foregoing results, that not all members of the *NRT2* family of genes can be characterized as nitrate-inducible. The present findings demonstrate that “nitrate-inducible” is not a universal characteristic of the NRT genes. Rather it appears that two other characteristics, nitrate-constitutive, and nitrate-repressible must be included in the characteristics of these gene families. It is also apparent that *Arabidopsis* has substantial  $\text{NO}_3^-$  uptake capacity even prior to exposure to  $\text{NO}_3^-$  (Zhuo et al., 1999). Therefore, genes, which show nitrate-constitutive properties such as *AtNRT2.3*, *2.5*, and *2.6*, might have roles in cHATS activity (Table 3-2). *AtNRT2.4* showed significant transcript abundance even in un-induced plants, although this gene was considered to be nitrate-inducible because of its expression pattern (Figure 3-2F). According to physiological studies by Aslam et al. (1992) and Kronzucker et al. (1995), the constitutive flux of  $\text{NO}_3^-$  into roots of barley and white spruce was increased by exposure to  $\text{NO}_3^-$ . Therefore a feature of the gene(s) encoding this  $\text{NO}_3^-$  flux would be (a) constitutive expression that is (b) increased by exposure to  $\text{NO}_3^-$ . Thus, *AtNRT2.4* might also be involved in the cHATS. Wang et al. (Wang and Crawford, 1996) isolated a mutant, which was impaired only in the cHATS transport in roots of *Arabidopsis*. It will be interesting to see if this mutation is located in one of the above-proposed candidates.

In the same way, low-affinity nitrate transporter (*AtNRT1*) family members are predicted to have a role in iLATS and cLATS, according to their nitrate-inducible, and nitrate-constitutive patterns, respectively (Table 3-2). A *chl1* mutant study showed that

Table 3-2. *AtNRT* Genes and hypothesized nitrate transport systems

Gene	Response to Nitrate (root)	Transport System
<i>AtNRT2.1</i>	Inducible	iHATS
<i>AtNRT2.2</i>	Inducible	iHATS
<i>AtNRT2.3</i>	Constitutive	cHATS
<i>AtNRT2.4</i>	Inducible	iHATS, cHATS
<i>AtNRT2.5</i>	Constitutive	cHATS
<i>AtNRT2.6</i>	Constitutive	cHATS
<i>AtNRT2.7</i>	Repressible	?
<i>AtNRT1.1</i>	Inducible	iLATS , HATS
<i>AtNRT1.2</i>	Constitutive	cLATS
<i>AtNRT1.3</i>	Inducible	iLATS
<i>AtNRT1.4</i>	Constitutive	cLATS

$\text{NH}_4\text{NO}_3$  grown mutant plants had about 75 % less nitrate influx, compared to WT in the LATS range (Touraine and Glass, 1997), and this defect was found even at cHATS and iHATS activities (Wang et al., 1998b; Liu et al., 1999). As a consequence it has been suggested that *AtNRT1.1* may encode both high- and low-affinity transporters. In the present study *AtNRT1.1(CHL1)* and *1.3* in roots showed nitrate-inducible patterns. Therefore, these genes are candidates for iLATS activity (Table 3-2). *AtNRT1.2* showed a nitrate-constitutive expression pattern and this is consistent with an earlier report, which demonstrated that transgenic plants expressing antisense *AtNRT1.2* showed reduced levels of low-affinity nitrate transport (Huang et al., 1999). Given that *AtNRT1.4* also displayed a similar expression pattern, these two genes are predicted to be involved in cLATS activity (Table 3-2).

The third category of nitrate-repressible genes represents the most puzzling group. This response is largely unknown at the physiological level, except that it might be speculated that when  $\text{NO}_3^-$  is removed from the external medium there begins a net transfer of  $\text{NO}_3^-$  from vacuole to cytoplasm (Zhen et al., 1991; Glass and Siddiqi, 1995). Normally this transfer exhausts the vacuolar reserve within 3-4 d (van der Leij et al., 1998 and references therein; Okamoto, unpublished data). When  $\text{NO}_3^-$  is re-supplied, the  $[\text{NO}_3^-]$  of the vacuole restored. It is possible that genes encoding transporters involved in the transfer of  $\text{NO}_3^-$  from vacuole to cytoplasm are down-regulated when  $\text{NO}_3^-$  is re-supplied in order to restore vacuolar  $[\text{NO}_3^-]$  to its normal level. However, it is unclear from the molecular perspective whether members of the NRT2 family might function as tonoplast transporters since there is no indication of the appropriate signal peptide to direct expression of the transporter to the tonoplast (Chapter 2).

# 4

## Tissue-Specific Expression Patterns of *AtNRT* Genes

### 4.1 Introduction

It is reasonable to presume that nitrate absorption occurs mainly from the rhizosphere where most nutrients are available. In the present study, I have documented that six genes out of the seven *NRT2* family members are expressed more dominantly in roots than shoot (Chapter 3). The expression patterns of, and levels of expression genes of the *NRT* families were diverse. However, whole root and whole shoot analysis leave many questions unanswered. For example, how is the expression of *NRT* genes localized within different tissues? Is there any correlation between the expression patterns from total RNA analysis of roots or shoots and tissue-specific expression patterns?

*AtNRT1.1(CHL1)* was the first gene among the *NRT* families of genes to be investigated with respect to its tissue-specific expression by using *in situ* hybridization (Huang et al., 1996). *AtNRT1.1* mRNA accumulation was observed primarily in the epidermal tissues in young roots, while mainly in the cortex or endodermal cells in mature roots. Similar results were also obtained by *GUS/GFP* fusion lines, where the 5' flanking

and partial coding regions of the *AtNRT1.1* were fused in frame with either *green fluorescent protein* (*GFP*) or  $\beta$ -*glucuronidase* (*GUS*) reporter DNAs (Guo et al., 2001). The authors, furthermore, found that *AtNRT1.1* was expressed in nascent organs in both roots and shoots, including flowers. The findings were also verified with immunolocalization using polyclonal antibodies raised against the *AtNRT1.1* protein (Guo et al., 2001). In contrast to *AtNRT1.1*, *AtNRT1.2* was highly expressed in the epidermis and root hairs regardless of the stage of root development (Huang et al., 1999). These locations are consistent with the belief that *NRT1* genes are involved in  $\text{NO}_3^-$  uptake. As well, it is possible that *AtNRT1.1(CHL1)* has a role in early organ development (Guo et al., 2001).

Among cloned *NRT2* genes, *NpNRT2.1* from *Nicotiana plumbaginifolia* was the only gene that has been analyzed for its tissue-specific expression pattern. The accumulated mRNA was localized in the epidermal and endodermal cells of the root tip, while lateral root primordia were the main targets in mature roots (Krapp et al., 1998). To date, no publication is available detailing the tissue localization of the *NRT2* gene expression in *Arabidopsis*. Therefore, this work was particularly focused on the *NRT2* family genes.

To investigate *NRT2* gene expression at the cellular level, transgenic plants were produced, which carry the promoter regions of *AtNRT2* genes fused to *GUS* reporter genes. Four gene constructs (i.e., *AtNRT2.1-GUS*, *2.2-GUS*, *2.4-GUS*, and *2.6-GUS*) showed *GUS* signals. Predominant expression was observed in roots as expected, but each gene had an unique expression pattern. *AtNRT2.1* and *AtNRT2.6* were also expressed in shoots. The significance of each expression pattern will be discussed.

## 4.2 Materials and Method

### Construction of *NRT2-GUS* Fusion Genes

The *NRT2.1* promoter region was obtained from a plasmid pCRN2.1pro which contained 1.2 kb upstream flanking sequences and the start codon of *AtNRT2.1*. To fuse the promoter region to the GUS reporter gene, a HindIII-NcoI fragment from pCRN2.1pro was cloned into the corresponding sites of pBI320X. A HindIII-SacI fragment from the pBI320X, consisting of the *NRT2.1* promoter region, GUS, and NOS3', was then ligated into the corresponding sites of a binary vector pMOG402.

The promoter regions from the rest of *NRT2* gene family members were cloned by PCR. Primers used to amplify each 5' flanking region were: N2.2P5 (5'-AAGCTTCAACAGAGGGGAACACCGGCCACG), and N2.2P3 (5'-GGGCTCATCAGTAGAACCCATGGATTTTAAAGC) for *NRT2.2*, N2.3P5 (5'-), and N2.3P3 (5'-ACAAACATTAAAATCCAGTGGCAGCCATGGTAA) for *NRT2.3*, N2.4P5A (5'-TCTAAGCTTCCAGTTTAAATTTCTATATAATTGAG), and N2.4P3 (5'-CATCGGGCCATGGTGTGAATATTT) for *NRT2.4*, 2.5P5-1 (5'-AAGCTTTCCATCCTCACCTGCAGAAGCAC), and 2.5P3-1 (5'-GCAAAACAAGACATGGTTAGTTTGTATCCAAAA) for *NRT2.5*, N2.6P5 (5'-AAGCTTTCTAAAATATGAGTTTACGTTCCAA), and N2.6P3 (5'-GTGAGCCATGGATCTTTAGTTCAAAGA) for *NRT2.6*, and N2.7P5 (5'-AAGCTTCTGATCCCCGATCTCAAGTATTGACT), and N2.7P3 (5'-CCATGGTTGTGATCTTTGTGAAGGTTCAAGAGAATTTTG) for *NRT2.7*. These primer

sets amplified the promoter regions of 1.2-2.0 kb. PCR was carried out using Expand High Fidelity PCR System (Roche, Laval, QC) under the following conditions: 94 °C for 2 min; - 10 cycles of 94 °C for 15 s, 65 °C for 30 s, 72 °C for 2 min; 20 cycles of 94 °C for 15 s, 65 °C for 30 s, 72 °C for 2 min + cycle elongation of 5 s for each cycle; 72 °C for 7 min. The fragments were cloned into pCR2.1. A NcoI-XhoI fragment in the pCR2.1 was introduced to pBI320.X, and resulting fusion gene, *NRT2.x-GUS-3'NOS*, was cloned into pBIN+ (*NRT2.1*, 2.2, and 2.4) or pMOG402 (*NRT2.3*, 2.5, 2.6, and 2.7).

### **Plant Transformation**

Binary vectors, which contained the fusion genes, were transformed into *Agrobacterium tumefaciens* strain GV3102. Transformation into *Arabidopsis* plants was carried out according to Clough and Bent (Clough and Bent, 1998). Overnight cultures of *Agrobacterium* (1L LB) grown at room temperature ( $OD_{600} = 0.8-1.0$ ) were harvested and re-suspended in 500mL of inoculation media, containing 5% sucrose and 0.005% Silwet L-77 (Lehle Seeds, Round Rock, TX). *Arabidopsis* plants (ecotype Columbia) were grown in soil (Redi-Earth Potting Soil, Grace-Sierra Horticultural Co., Lansing, MI) until flowering stage in a controlled-environment chamber with 24/18°C at 16/8h of light/dark cycle. Whole shoots of 4-5 week-old plants (flowering stage) were dipped in the inoculation media containing appropriate *Agrobacterium* strain for 60 sec twice. The whole plants were then covered with a plastic bag to maintain moisture for the next two days. Seeds (T1 generation) were harvested when siliques turned yellow to brown. T1 seeds were selected on respective selection substrates (i.e., 25mg L<sup>-1</sup> kanamycin for *NRT2.1*, 2.2, 2.4 on petri



plates; 25mg L<sup>-1</sup> Basta with 0.02% Silwet L-77 for *NRT2.3*, 2.5, 2.6, 2.7 applied by spray on soil grown plants). Basta selection was also performed on petri plates with 10mg L<sup>-1</sup> Basta. The selection media for plates contained ½ MS medium (Murashige and Skoog, 1962), 1% sucrose, 0.8% agar, 0.5g MES in 1L, pH 5.7. Subsequent GUS analysis was based on the T2 generation.

### **GUS Staining**

T2 seeds were germinated on selection plates after surface sterilization, and resistant plants were transferred onto growth plates (i.e., same as selection plates without kanamycin or Basta) for up to 2 weeks for vegetative stage or 3 weeks for flowering stage. The plants were then grown on a N-free plate (nutrient composition was the same as for the hydroponic solution in Chapter 3, omitting sucrose) for 1 week before plants were induced with 1mM NO<sub>3</sub><sup>-</sup>.

Harvested plant materials were washed with water and placed in a 24-well plate or in small petri dishes. X-Gluc solution (0.5 mM 5-bromo-4-chloro-3-indolyl-β-D-glucoronide cyclohexylamine salt (Rose Scientific Ltd., Edmonton, AB), 100mM sodium phosphate buffer pH 7, 0.1% Triton X-100, 2mM ferricyanide (K<sub>3</sub>Fe(CN)<sub>6</sub>), 2mM ferrocyanide (K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O)) was added until whole plants were submerged. After vacuum infiltration, the solutions with plants were incubated at 37°C for 4-5 hours or overnight. The plants were “bleached” with a series of EtOH solutions (20% to 95%).

### **Cross-Sectioning and Histochemical Analysis**

GUS stained roots were investigated more thoroughly after sectioning in transverse and/or longitudinal planes. The root tissues fixed in 80% EtOH were dehydrated in a scintillation vial as follows: 30 min in 90% EtOH, 30 min in 95% EtOH, 3 times 30 min in 100% EtOH (molecular sieve-treated to get rid of traces of H<sub>2</sub>O). Subsequently, the tissues were embedded in Spurr's resin (J.B. EM Service Inc. Dorval, QE) prepared by following the manufacture's manual. The specimens were given an additional 100% EtOH change (1ml) before the embedding process. The concentration of resin was raised gradually as follows: one drop of resin was added into the vial and gently mixed for 5 min; two drops of resin were added and mixed for 5 min; the last step was repeated; two drops of resin were added and mixed for 45 min. One half of total volume of the resin/EtOH solution ( $\approx 0.55$  ml) was removed and replaced with the same volume of resin (mixing for 90 min). This was repeated once more and left on a rotary shaker overnight. On the subsequent day, the solution was replaced with 100% resin and mixed for 5h. Then the resin was refreshed once, and the solution was mixed overnight without cap, but with aluminum foil as a cap to let EtOH evaporate from the solution. After the incubation period, the solution was replaced with new resin. Finally, the tissues in the solution were transferred to an aluminum-weighing dish with fresh resin, and arranged in the appropriate orientation under a dissecting microscope. The polymerization of resin was achieved in an oven at 60 °C overnight. A trimmed polymerized block was sectioned in 2-4  $\mu\text{m}$  thickness with a microtome (Reichert, Model OM-U3, Austria). Images of the samples were captured in digital formats with a SPOT system (Diagnostic Instruments Inc. Sterling Heights, MI).

## 4.3 Results

### Tissue-Specific Expression Patterns of NRT Genes

To visualize the specific expression patterns of *AtNRT2* genes within the tissue, the promoter regions of each member of the family were fused to *GUS* reporter gene. *AtNRT2.3*, *2.5* and *2.7* were unable to be used as subjects for further analysis either because no GUS activity was shown or an insufficient number of positive transformants were observed. Therefore only *AtNRT2.1*, *2.2*, *2.4*, and *2.6* were investigated in detail. A transgenic line which carried *AtNRT1.1(CHL1)-GUS* (Guo et al., 2001), donated by the authors was also analyzed for comparison. Since *AtNRT2.1*, *2.2*, *2.4*, and *1.1* are nitrate-inducible, the plants were first N-deprived for 1 week, and then, re-induced by 0.5 mM  $\text{Ca}(\text{NO}_3)_2$  for 6 h (see detail in Materials and Method). More than eight lines of transformants from each construct were examined, and the studies that showed the reproducible results, were repeated at least three times. The pictures in the figures are representative of those experiments.

#### *AtNRT2.1*

In 3-week-old plants, blue GUS staining was found in entire roots, with stronger staining in the mature region of the root and in the root tip. By contrast, staining was minute or absent from the root cap and distal region close to the hypocotyls zone (Figures 4-1). The gradient of GUS activity is evident along the younger part of the root (Figure 4-1A). From the meristematic to elongation zones GUS staining is lighter than in the mature region. In the

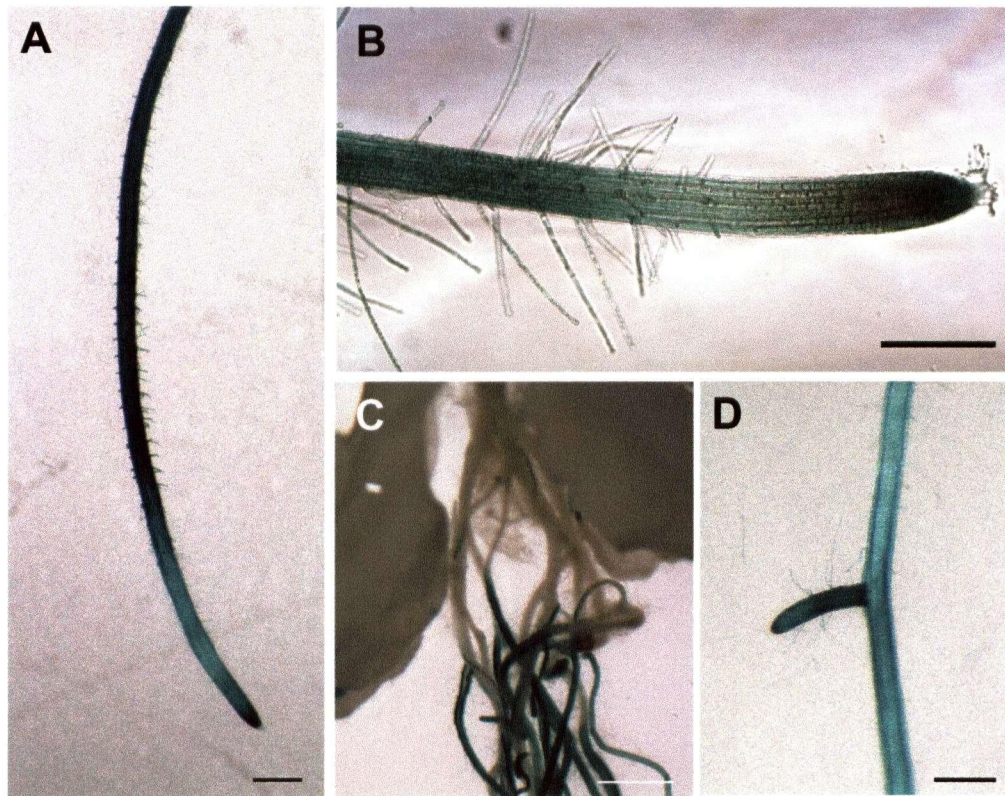


Figure 4-1. Analysis of GUS activity in *AtNRT2.1* promoter-*GUS* Arabidopsis plants.

GUS histochemical staining in a root of an *AtNRT2.1* promoter-*GUS* plant.

Bar = 0.5 mm.

Close up of root tip. Bar = 200  $\mu$ m.

Basal region of a plant. Bar = 1 mm

Close up of a lateral root near the basal region. Bar=0.5 mm.

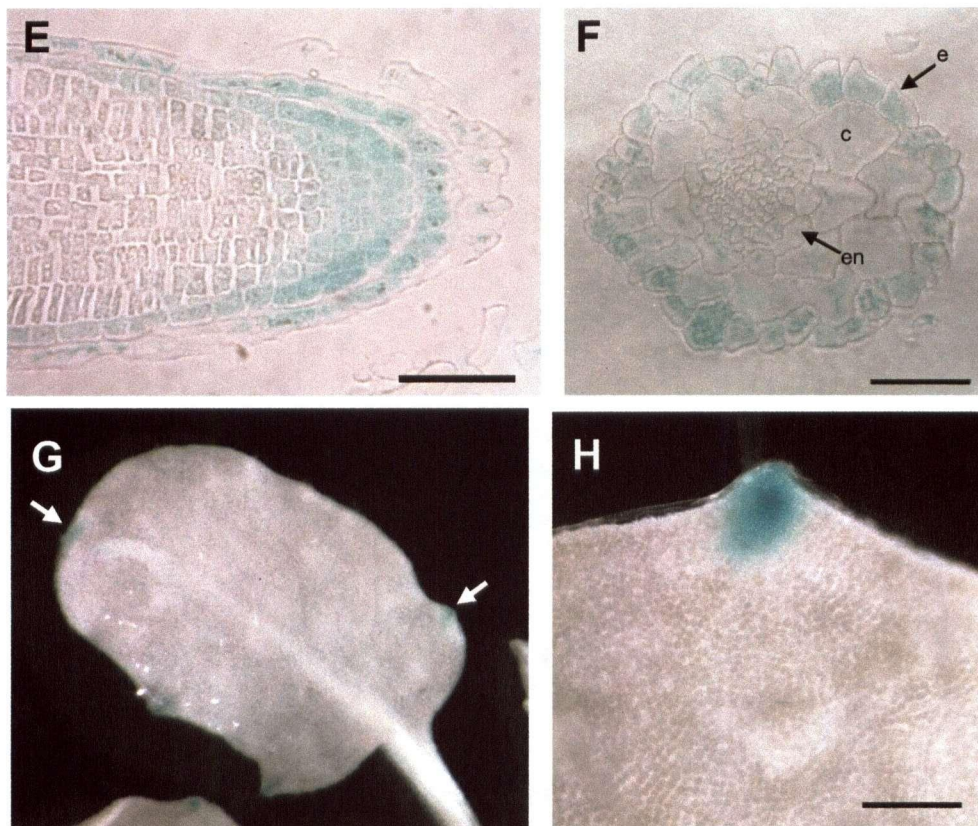


Figure 4-1. (continued).

(E) Longitudinal section close to a root tip. Low level of GUS expression in the outer most layer of the root cap. Bar = 50  $\mu$ m.

(F) Cross-section of a root (5 mm from a root tip). c, cortical cells; e, epidermal cells; en, endodermal cells. Bar = 50  $\mu$ m.

(G) Fully expanded leaf. GUS staining is seen in hydathodes indicated with arrows.

(H) High magnification near a leaf hydathode. Bar = 200  $\mu$ m.



more mature roots, developing lateral roots showed stronger GUS activity than the parental root (Figure 4-1D).

To look at more detail of the localization, sectioning of the fixed samples was carried out (Figures 4-1 D and E). GUS activity was seen in epidermal cells, cortex, and endodermal cells, although the strongest signals were observed in the epidermal cells. In shoots GUS staining was especially evident in leaf hydathodes (Figures 4-1G and F).

#### ***AtNRT2.2***

GUS expression in an *AtNRT2.2* promoter-*GUS* plant is shown in Figure 4-2. GUS staining was found in recently developed roots, but was absent in the tissue close to the root tip (Figure 4-2A). When lateral roots appeared, GUS activity was found in the newly developing root, whereas the parental root showed only faint GUS staining (Figure 4-2B). Once lateral roots developed to a certain stage where root hairs start appearing, the blue GUS staining in the parental root completely disappeared (Figure 4-2C). However, the intensity of GUS activity was not uniform. Figure 4-2D shows this irregular pattern along a root, and the GUS staining was primarily in epidermal cells and root hairs. There was no GUS activity in shoots including leaves, stems and flowers (data not shown).

#### ***AtNRT2.4***

GUS activity was primarily seen in developing or recently developed roots (Figure 4-3). Similar to that of *AtNRT2.2*, GUS staining in *AtNRT2.4-GUS* plants was absent in the region of the primary root where lateral roots grew (Figure 4-3A). In a developing lateral root GUS staining was strong, although in the regions of the apical meristem and the root

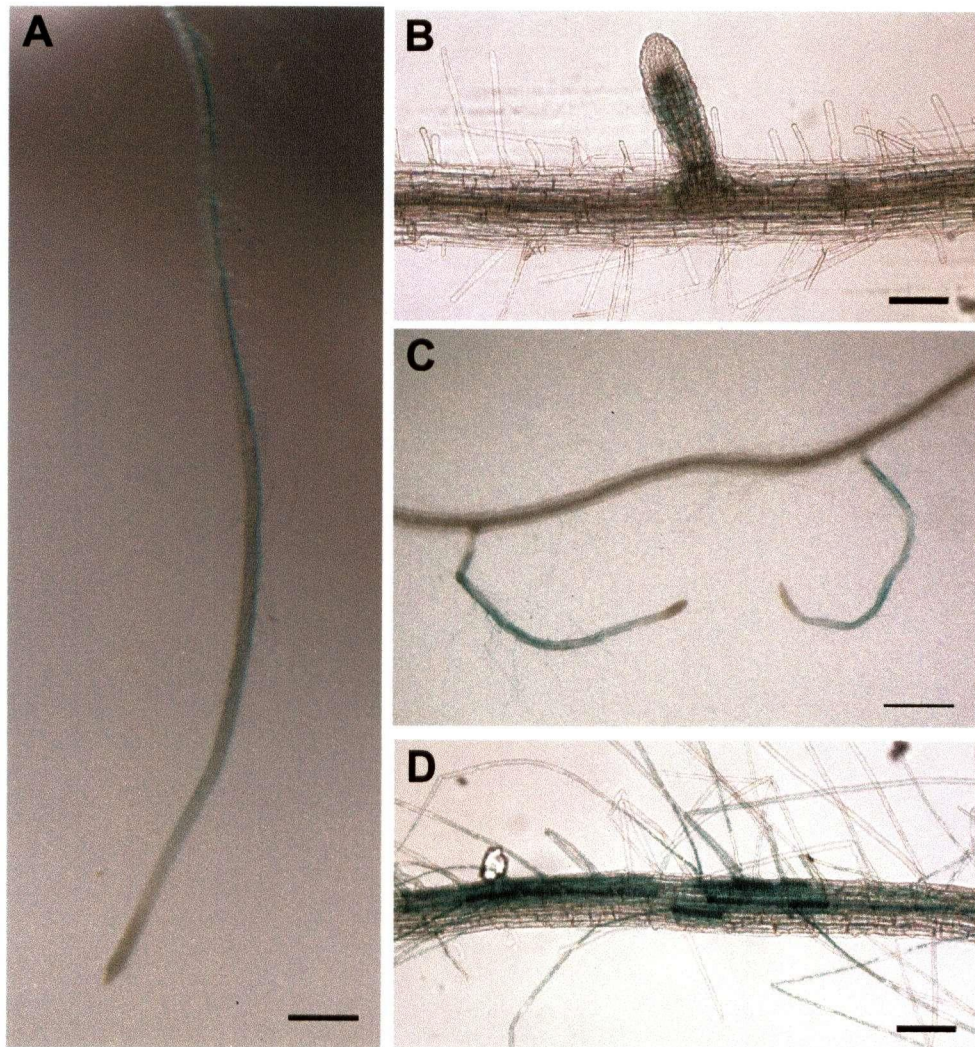


Figure 4-2. Analysis of GUS activity in *AtNRT2.2* promoter-*GUS* Arabidopsis plants.

(A) GUS histochemical staining in a root of an *AtNRT2.2* promoter-*GUS* plant. Bar = 0.5 mm.

(B) GUS staining in a lateral root. Bar = 100  $\mu$ m.

(C) GUS staining is localized in secondary roots. Bar = 1 mm.

(D) High magnification of GUS staining in the root hair zone. Bar = 100  $\mu$ m.



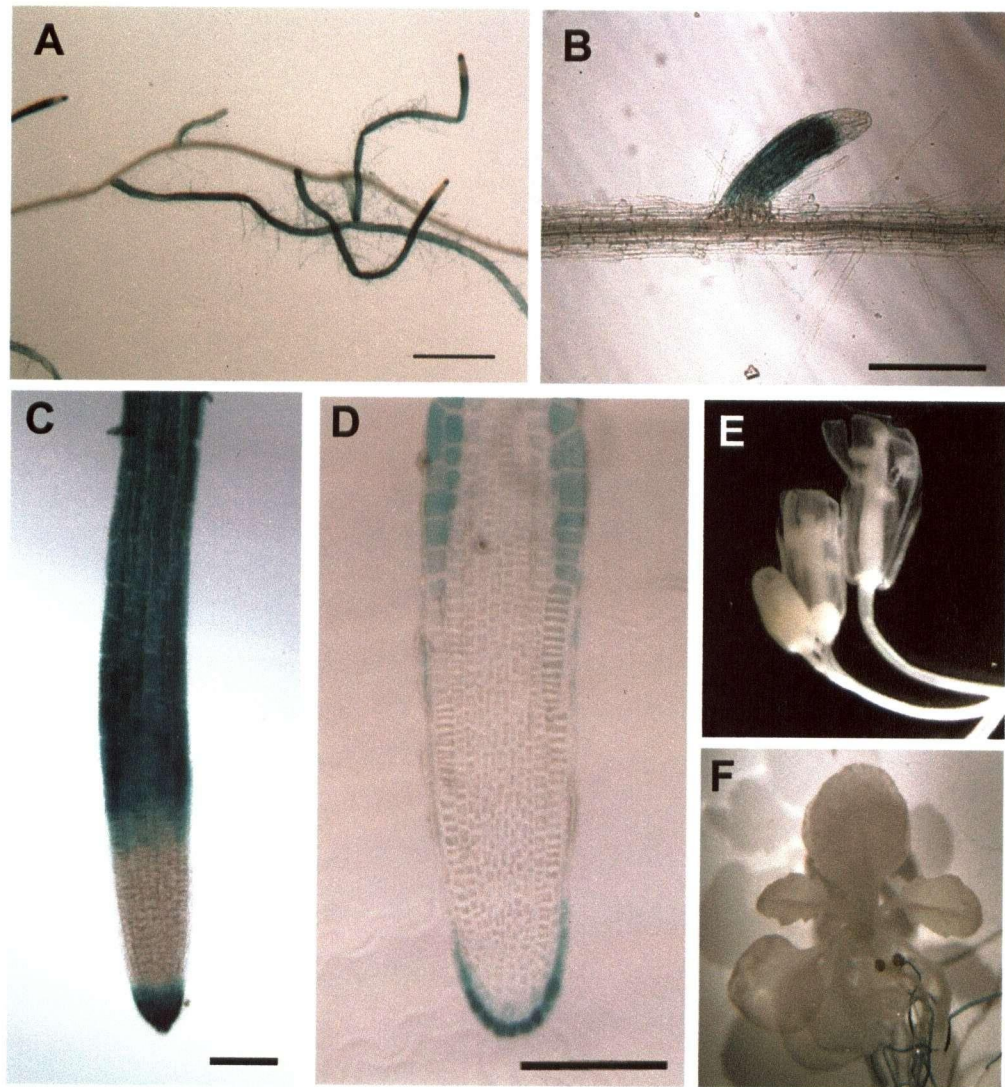


Figure 4-3. Analysis of GUS activity in *AtNRT2.4* promoter-*GUS* Arabidopsis plants.

(A) GUS histochemical staining in roots of an *AtNRT2.4* promoter-*GUS* plant. Bar = 2 mm.

(B) Lateral root. Bar = 200  $\mu$ m.

(C) GUS staining in root close to a root tip. Bar = 100  $\mu$ m.

(D) Longitudinal section of root close to a root tip. Bar = 100  $\mu$ m.

(E) and (F) No GUS activity in flowers (E), or leaves (F).



tip were not the targets for the expression (Figure 4-3B). Developed roots showed GUS activity in the root tip region, where the staining was placed in one to two layers of cells from the surface i.e., the root cap (Figures 4-3C and D). Shoot tissues showed no GUS activity (Figures 4-3E and F).

#### ***AtNRT2.6***

GUS activity in *AtNRT2.6-GUS* plants was seen both in roots and shoots (Figure 4-4). In roots, the zone of maturation (including root hairs) showed the strongest GUS staining, although the expression was absent from the basal region, and from the root tip and zone of elongation (Figures 4-4A and B). Cross-sections of the region close to the root tip showed that GUS activity was primarily seen in the epidermis, endodermis, and pericycle (Figure 4-4C).

In shoots, GUS staining was specifically seen in the anthers of flower buds (Figures 4-4D). Under N-starvation the anthers of opened flowers also showed GUS activities, while N-fed plants seemed to have less or no GUS activity in the anthers (Figures 4-4E and F). Higher magnification and dissection analysis revealed that GUS staining was localized in pollen grains (Figures 4-4F and G).

#### ***AtNRT1.1(CHL1)***

*AtNRT1.1* promoter-*GUS* plants obtained from Guo et al. (2001) were also induced by 1 mM  $\text{NO}_3^-$  for 6 h, and subjected to GUS staining. Strong GUS activities were noticeable in root tips and leaves (Figure 4-5). GUS expression in a newly developed root was primarily seen in outer layers (Figures 4-5A and B). The layers were verified as epidermal cells and

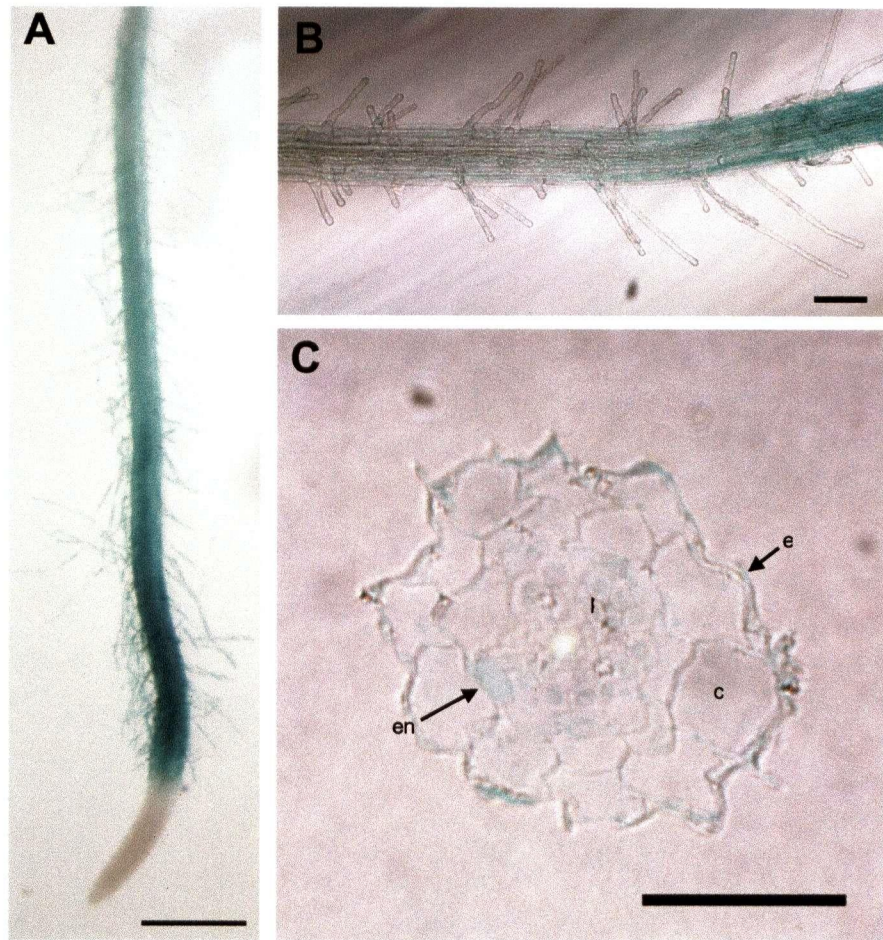


Figure 4-4. Analysis of GUS activity in *AtNRT2.6* promoter-*GUS* Arabidopsis plants.

(A) GUS histochemical staining in a root of an *AtNRT2.6* promoter-*GUS* plant. Bar = 0.5 mm.

(B) Middle section of a root. GUS staining is fading out toward the basal (to the left in the picture) section. Bar = 100  $\mu$ m.

(C) Cross section of a root 1 cm from the tip. c, cortical cells; e, epidermal cells; en, endodermal cells; p, pericycle. Bar = 50  $\mu$ m.



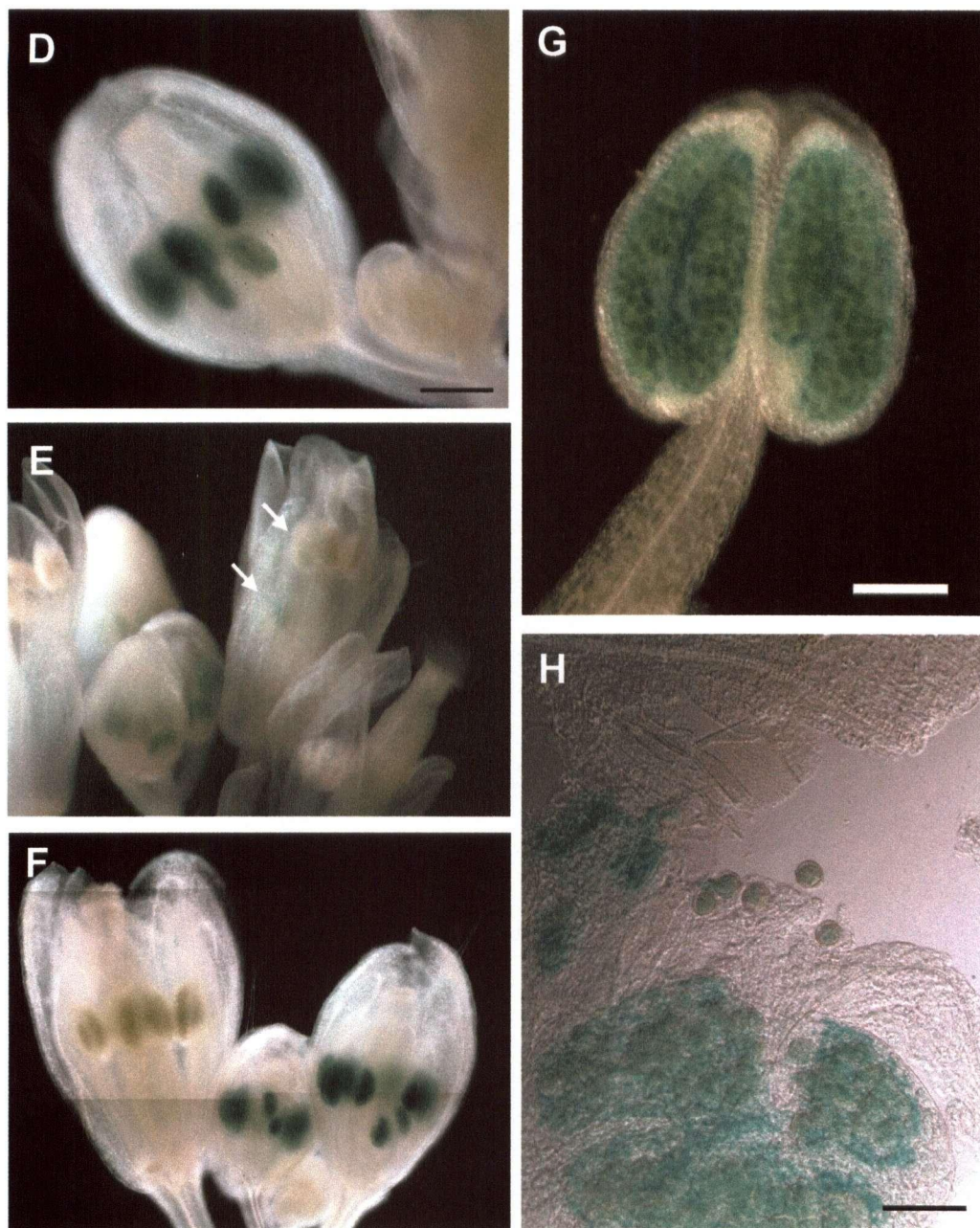


Figure 4-4. (continued).

(D) GUS staining in a flower bud of an *AtNRT2.6* promoter-*GUS* plant. GUS activity is seen in anthers. Bar = 200  $\mu$ m.

(E) GUS staining in uninduced plants. Flower buds as well as opened flowers (indicated with arrows) show some GUS activities.

(F) GUS expression in nitrate-induced plants. GUS staining is concentrated in developing flower buds, and diminished in opened flowers.

(G) Higher magnification of an anther from a flower bud. GUS staining is seen in pollen grains. Bar = 100  $\mu$ m.

(H) Higher magnification of pollen grains from a dissected anther. Bar = 50  $\mu$ m.



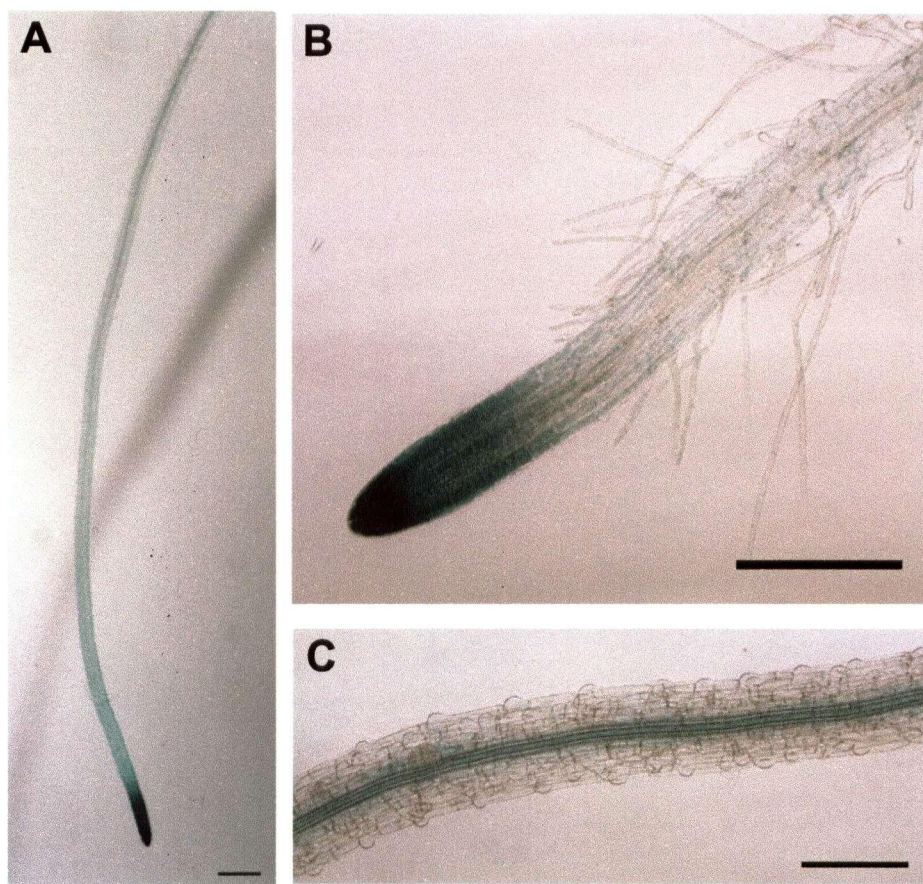


Figure 4-5. Analysis of GUS activity in *AtNRT1.1* promoter-*GUS* Arabidopsis plants.

(A) GUS histochemical staining in a root of an *AtNRT1.1* promoter-*GUS* plant. Bar = 0.5 mm.

(B) Strong GUS staining around a root tip. Bar = 200 μm.

(C) High magnification of mature section of a root. GUS staining is seen in the epidermal cells. Bar = 200 μm.

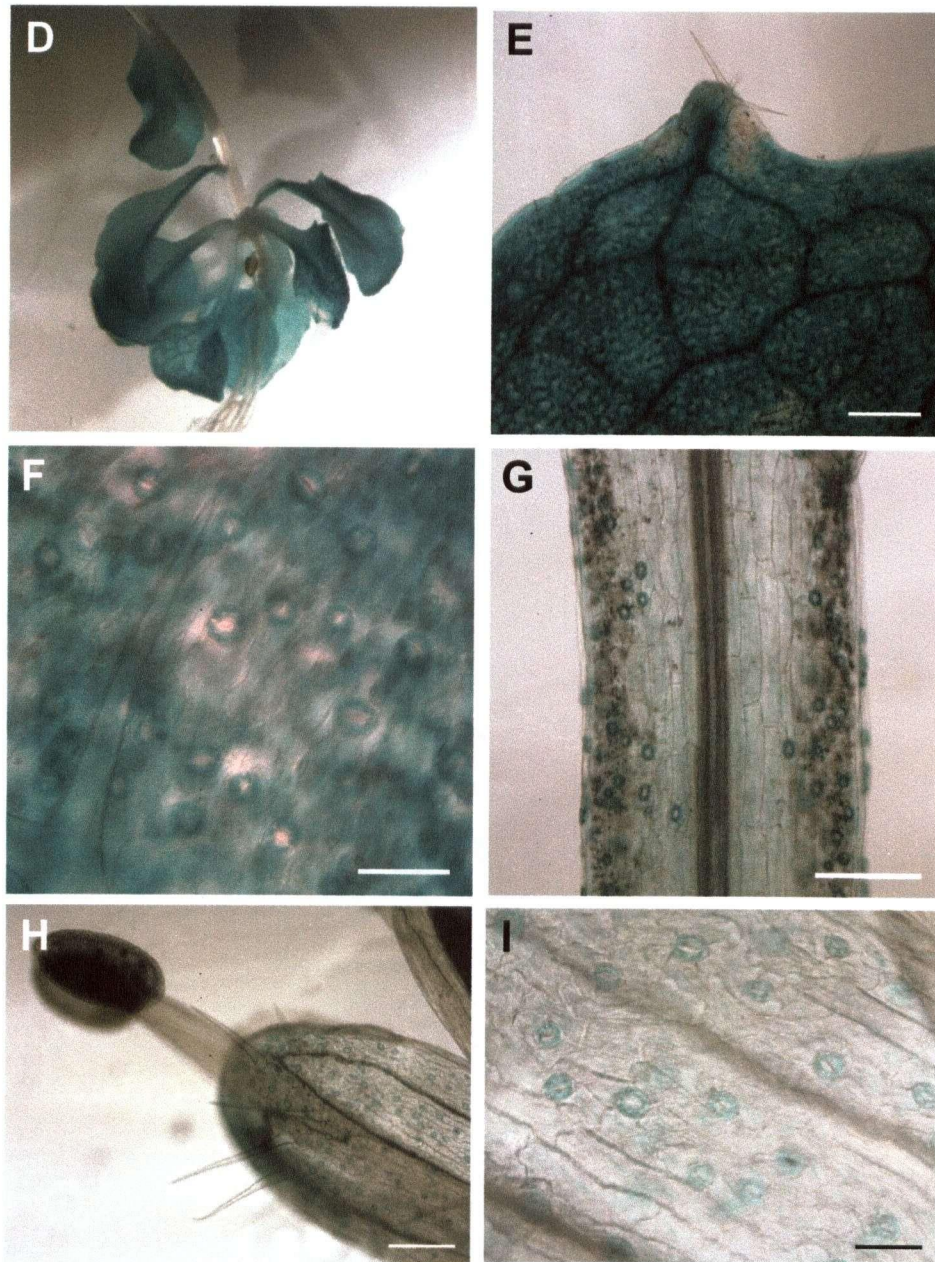


Figure 4-5. (continued).

- (D) GUS staining in leaves of an *AtNRT1.1* promoter-*GUS* plant.
  - (E) Close up of a leaf. GUS staining is stronger along the vascular system.
  - (F) Higher magnification of leaf surface.
  - (G) GUS staining in a petiol.
  - (H) GUS staining in a sepal.
  - (I) Higher magnification of a sepal.
- Bars in (E), (G), and (H) = 200  $\mu$ m, (F), and (I) = 50  $\mu$ m.



cortex in previous studies (Huang et al., 1996; Guo et al., 2001). The GUS activities in these regions gradually disappeared toward the basal of the roots. Instead, the center region of the root took over the localization of GUS expression (Figures 4-5A and C). The region was claimed to correspond to endodermal cells by *in situ* hybridization (Huang et al., 1996).

In shoots, GUS activity was seen in whole leaves, and stronger (dark staining) in the vascular system (Figures 4-5D and E). On the surface of a leaf, GUS activity was also observed in guard cells (Figure 4-5F). This characteristic was clearly visible in petioles and sepals (Figures 4-5G to I).

#### 4.4 Discussion

Analysis of GUS activity controlled by *AtNRT* promoter genes revealed that each gene member of *AtNRT* families has a unique tissue-specific expression pattern. Every *AtNRT* gene, examined in the present GUS study showed GUS expression in roots. This evidence is consistent with the belief that *NRT* genes are involved in  $\text{NO}_3^-$  uptake. *AtNRT2.1*, *2.2*, and *2.4* showed high sequence similarity, and similar expression patterns as nitrate-inducible genes as revealed by RT-PCR with total tissue RNA (Chapters 2 and 3). This highly homologous trio also displayed some similarities in the GUS expression patterns. For example, strong GUS activities were observed in epidermal cells and root hairs for all three genes. *AtNRT2.1* and *AtNRT2.2* had less or no GUS activity in meristematic and elongation zones (Figures 4-1B and 4-3C), while *AtNRT2.2* and *AtNRT2.4* showed no GUS expression in the older regions of roots where lateral roots emerged (Figures 4-2C and 4-

3A), and *AtNRT2.1*-GUS expression was localized in both young and mature root regions (Figure 4-1). This expression pattern perhaps explains why *AtNRT2.1* showed the highest expression level among the *NRT2* gene family by RT-PCR in whole root studies (Chapter 3). No GUS staining was found in leaves of *AtNRT2.2*, *2.4*, and *2.6*, although RT-PCR could show the expression patterns of the genes in the shoots (Chapter 3). These discrepancies could be due to their lower gene expression levels (Table 3-1, Figure 3-1), and GUS reporter system might not be sensitive enough to visualize the expression patterns. In *Nicotiana plumbaginifolia*, using *in situ* hybridization, high-affinity nitrate transporter *NpNRT2* was expressed in root tip, and epidermal and endodermal cells in developing roots, while in older roots the transcript was primarily localized in the lateral root primordial, and there was little expression in the parental root (Krapp et al., 1998). This tissue-specific expression pattern is similar to that of *AtNRT2.4*, although Northern blot analysis showed that *NpNRT2* expression pattern was more similar to that of *AtNRT2.1*. These results therefore indicate that expression patterns revealed from total RNA and from tissue-specific methods may differ among species. Characterization of additional *NRT2* homologues in *N. plumbaginifolia* or other species will solve this issue.

Plant roots experience biochemical gradients both in the longitudinal and the radial axes. Zhen et al. (1991) observed a longitudinal gradient of nitrate concentration in barley roots. The five-day-old roots were grown in 10 mM  $\text{NO}_3^-$  and divided into three regions i.e., from the tip, 0-2.5 cm, between 2.5 and 5.0 cm, and >5.0 cm. The middle section (2.5-5.0 cm) had 113.9 mM  $[\text{NO}_3^-]$ , whereas the root tip and >5.0 cm regions possessed 90.9 and 93.3 mM, respectively. Siebrecht et al. (1995) also measured  $[\text{NO}_3^-]$ , as well as,  $\text{NO}_3^-$  uptake and nitrate reductase (NR) activity along the barley root axis. Net  $\text{NO}_3^-$  uptake rates

by root tips of 7-day-old plants were half of those of the middle and basal zones. However, nitrate absorbed by root tips was translocated to older root zones, and induced  $\text{NO}_3^-$  uptake there. Furthermore, within 20 mm from the tip maximum NR activity (on a weight basis) was seen at 1mm behind the apex (Siebrecht et al., 1995). These results indicate that nitrate absorption and metabolism in the root tip region may impact upon the rest of the root, although  $\text{NO}_3^-$  uptake *per se* was not high.

Among low-affinity nitrate transporters *AtNRT1.1* was expressed in both mature and lateral root tips (Guo et al., 2001; this study), and *AtNRT1.2* was expressed in epidermal cells in the root tips (Huang et al., 1999). As well, among high-affinity transporters, *AtNRT2.1* and *2.4* were expressed in the root tips (the present work). These multiple genes expressed in the root tips might indicate the significance of this region for  $\text{NO}_3^-$  uptake or  $\text{NO}_3^-$  sensing. In the soil environment nutrient availability varies depending on mass flow, nutrient diffusion, and interception by root growth (Marschner, 1995), resulting in great soil heterogeneity in nutrient availability (Jackson and Caldwell, 1993; Wolt, 1994). The root tip region, as a “frontier”, may have more chance to deal with higher nitrate concentrations in the media than mature regions of the roots, which create a gradient of the nutrient concentration surrounding the roots as a result of withdrawing  $\text{NO}_3^-$ . In this case, the region close to the root tips might be well served by low-affinity nitrate transporter(s), while older regions of the roots are the zones where high-affinity transporter(s) becomes more important. High-affinity transporters *AtNRT2.2* and *2.6* might be involved only in the latter case, since both genes were specifically expressed in older regions of the roots (Figures 4-2A and 4-4A).



There are two pathways for nutrients to traverse the root in the radial direction. One is called the symplasmic pathway where nutrients enter root tissues through epidermal cells and move through plasmodesmata within the cytoplasm toward the stele. The other pathway is apoplastic. The solutes enter the cell wall space and can travel through cell walls and intercellular spaces until reaching endodermal cells. Because a suberized Casparian strip is located in endodermal cell walls, solutes are blocked from entering the stele via the apoplast. Therefore the nutrients need to be absorbed at endodermal cells. All of *AtNRT* genes in the present study were expressed in the epidermal cells, and *AtNRT2.1*, *2.6* and *1.1* (Huang et al., 1996) were also expressed in endodermal cells, indicating that all these genes are involved in symplasmic transport, and the latter group of genes may contribute in apoplastic transport.

Interesting findings involved the expression patterns of *NRT* genes in shoots. Quantitative relative RT-PCR using total tissue RNA extractions revealed that the *AtNRT1* family of genes and *AtNRT2.5* had substantial levels of expressions in shoots (Chapter 3). This was consistent with the results from transgenic lines which carry *AtNRT1.1* promoter-*GFP/GUS* genes. *AtNRT1.1(CHL1)* mutant analysis indicated that *AtNRT1.1* is not only involved in  $\text{NO}_3^-$  uptake, but also in the growth of nascent organs both in roots and shoots (Guo et al., 2001). However, the role of gene expression in the guard cells as revealed by GUS expression remains unsolved. It is well known that potassium ion fluxes in and out of the guard cells to open/close the stomata by osmotic effects. Nitrate, an anion, may act as a counter ion when  $\text{K}^+$  moves into the guard cells in order to maintain electrical neutrality across the plasma membrane.

GUS staining in pollen grains of *AtNRT2.6-GUS* plants was a novel finding (Figures 4-4D to H). Pollen is rich in nitrogen (N), ranging from 2.5 to 61 % of protein contents (Roulston et al., 2000), and/or micro-molar concentrations of amino acids can be stored (Schwacke et al., 1999). In fact, flowers are an important diet for some animals because of the high protein content of pollen grains (van Tets and Hulbert, 1999). N-sources in pollen are most likely utilized for pollen development and for producing enzymes which are essential for pollen germination and pollen tube formation. In tomato pollen, proline is a dominant free amino acid (> 70 %), and a proline transporter *LeProT1* was observed to be expressed in mature and germinating pollens (Schwacke et al., 1999). On the other hand, GUS activity in an *AtNRT2.6-GUS* plant was found primarily in pollen grains in flower buds, suggesting that nitrate might be important for pollen development. One might argue that plants can grow and reproduce without nitrate as a N-source. However, nitrate can be stored at concentrations exceeding 100 mM in tissues (Glass and Siddiqi, 1995), whereas ammonium, another major N-source, may be toxic when accumulated to this level (Brito et al., 2001). Therefore, given that pollen requires large amounts of N during its entry developmental stage, it might be advantageous for plants to have nitrate transporter in pollen in order to gain high concentration of N when it is available. Further investigation needs to be done to address this hypothesis.

In conclusion, the *NRT* genes investigated in this study and reported data of *AtNRT1.2* showed different tissue-specific expression patterns. In other words, the *NRT* gene family members are not genetically redundant. Rather, each nitrate transporter seems to be involved in a specific localized function. Of course some regions of roots are targets for more than a one gene (e.g., *AtNRT1.1*, *2.1*, and *2.4* in the root tips; *AtNRT2.1*, *2.4*, and

2.6 in epidermis and cortex cells in mature regions of roots). Each encoded transporter might have different  $K_m$  and  $V_{max}$  values (as distinct from the differences between high- and low-affinity transporters). *Aspergillus nidulans* contains two high-affinity nitrate transporters, *NrtA* and *NrtB*, which show 61 % identity (Unkles et al., 1991, 2001). Mutant analysis revealed that  $K_m$  values for the NrtA and NrtB transporters were 108  $\mu\text{M}$  and 11  $\mu\text{M}$ , respectively (Unkles et al., 2001). *Chlamydomonas reinhardtii* also has two high-affinity nitrate transporters, *CrNRT2.1* and *CrNRT2.2*, with  $K_m$  values of 1.6 and 11.0  $\mu\text{M}$ , respectively (Galvan et al., 1996). The role of such closely related proteins (apparently) differing only in their kinetic properties is still unclear.

# 5

## Functional Aspects of Nitrate Transporters

### 5.1 Introduction

Nitrate transport systems fall into three groups, constitutive high-affinity transporter system (cHATS), inducible high-affinity transporter system (iHATS), and low-affinity transporter system (LATS). The cHATS and iHATS typically operate in the range of 10 to 250  $\mu\text{M}$   $\text{NO}_3^-$ , while the LATS only becomes evident above these concentrations. At such concentrations total uptake rates for  $\text{NO}_3^-$  are the sum of these three transporter activities (Siddiqi et al., 1990; Glass et al., 1992). The induction pattern of HATS would vary depending on external  $\text{NO}_3^-$  concentration, growth condition, and species or even varieties (Siddiqi et al., 1989). A typical  $\text{NO}_3^-$  induction period to maximize  $\text{NO}_3^-$  influx is 6-12 hours in barley (Siddiqi et al., 1989; Vidmar et al., 2000). In spruce it took three days to induce maximum  $\text{NO}_3^-$  influx (Kronzucker et al., 1995). 3-week-old *Arabidopsis* plants (ecotype Columbia), grown on ammonium citrate, showed a peak of  $\text{NO}_3^-$  influx from 100  $\mu\text{M}$   $\text{NO}_3^-$  after 3 h induction by 1mM  $\text{NO}_3^-$  (Zhuo et al., 1999), whereas 6-week-old

*Arabidopsis* (ecotype Wasselewskija) displayed a peak  $\text{NO}_3^-$  HATS influx after 12 h of induction with 4mM  $\text{NO}_3^-$  (Cerezo et al., 2001).

The transcript abundances of *AtNRT2.1* and the patterns of high-affinity nitrate influx showed high correlations, suggesting that *AtNRT2.1* is primarily responsible for iHATS activity (Zhuo et al., 1999; Lejay et al., 1999). This conclusion was substantiated by the recent finding that a T-DNA mutant, lacking *AtNRT2.1* and a part of 2.2, lost about 70% of high-affinity  $\text{NO}_3^-$  uptake capacity compared to the WT plants, while LATS transport was unaffected (Filleur et al., 2001). The green alga *Chlamydomonas reinhardtii* has at least three genes that are involved in high-affinity nitrate transport, *CrNRT2.1*, *CrNRT2.2*, and *NAR2*, located within the same gene cluster containing other nitrate-regulated genes (Quesada et al., 1994; Galvan et al., 1996). *CrNRT2.1* and *CrNRT2.2* show high homology with *NrtA*, a high-affinity nitrate transporter in *Aspergillus nidulans*, while *NAR2* is a much smaller gene sharing no sequence homology with the *NRT2* or *NRT1* families. A *Chlamydomonas* mutant lacking *CrNRT2.1*, *CrNRT2.2*, and *NAR2* was incapable of high-affinity  $\text{NO}_3^-$  transport. When this null mutant was transformed with various combinations of these three genes, it was reported that high-affinity  $\text{NO}_3^-$  transport was restored by combination of *NAR2* together with either *CrNRT2.1* or 2.2. Thus *NAR2* appears to be obligatory for high-affinity transport by *NRT2.1* and *NRT2.2*, although the manner of the interaction between these two gene families is unknown.

$\text{NO}_3^-$  influx in the LATS range increases linearly along with external  $[\text{NO}_3^-]$ . As well, LATS was considered to be repressible but not inducible (Glass and Siddiqi, 1995). In *Arabidopsis*, however, LATS appeared to have two components, namely a constitutive LATS (cLATS) and an inducible LATS (iLATS) (Huang et al., 1999). Recent observations

with *chl1* mutants exhibiting defective LATS, suggest that  $\text{NO}_3^-$  uptake at concentrations typical of the HATS range ( $<250 \mu\text{M}$ ) is also reduced, leading the authors to propose that the so-called LATS transporters, formerly considered to be low-affinity, may function both as LATS and HATS (Huang et al., 1996; Wang et al., 1998).

To characterize the functions of particular gene products, one common approach is the use of heterologous expression systems. *NrtA* from *A. nidulans* was expressed in *Xenopus* oocytes, and caused cell membrane depolarization when oocytes were subjected to  $\text{NO}_3^-$  treatment, suggesting that  $\text{NO}_3^-$  was transported across the cell membrane (Zhou et al., 2000). However, plant *NRT2* genes failed to cause membrane depolarization or nitrate uptake when expressed in oocytes (Forde, 2000). As well, expressing *AtNRT2.1* in *A. nidulans* failed to complement a nitrate transport defective mutant (*nrtA nrtB*) (Okamoto et al., unpublished). These results suggest that the plant *NRT2* nitrate transporters may not function by themselves. Similar experiments were undertaken to express *CrNRT2.1*, a high-affinity  $\text{NO}_3^-$  transporter from *C. reinhardtii* in *Xenopus* oocytes (Zhou et al., 2000). When *CrNRT2.1* mRNA was injected into oocytes,  $\text{NO}_3^-$  transport activity was not detected. However, when *CrNRT2.1* was co-expressed with *NAR2*, the oocytes showed  $\text{NO}_3^-$  uptake. Interestingly, expressing *NAR2* alone in oocytes resulted in increased mortality of oocytes (Zhou et al., 2000b). These circumstantial lines of evidences suggest that some *NRT2.1* transporter(s), but apparently not the *A. nidulans* transporter, require co-expression of *NAR2*-like proteins in order to be functional. Interestingly, *AtNRT1.1*- or *AtNRT1.2*-injected *Xenopus* oocytes were able to show membrane depolarization and (presumably)

nitrate uptake activities by themselves (Tsay et al., 1993; Huang et al., 1999). This further emphasizes differences between these two families of nitrate transporters.

The aim of this Chapter is to present physiological responses of *Arabidopsis* plants to nitrate induction treatments, and to confirm the presence of each physiologically defined nitrate transporter systems (i.e., iHATS, cHATS, iLATS, and cLATS) by  $^{13}\text{NO}_3^-$  tracer experiments. Also, the occurrence and expression patterns of *NAR2* homologues in *A. thaliana* were investigated. Finally an outlining hypothesis of a mechanism for  $\text{NO}_3^-$  transport depending upon co-regulation of NRT2 and NAR2 proteins will be discussed.

## **5.2 Materials and Method**

### **Plant growth condition**

Same as for Chapter 3.

### **$^{13}\text{NO}_3^-$ influx experiments**

Nitrate influx using  $^{13}\text{NO}_3^-$  was measured as described before (Zhuo et al., 1999). The basic components of the solution for pretreatment, influx, and washing were the same as those of the growth media. The plants were pretreated with solution containing either 100  $\mu\text{M}$  or 5 mM  $^{14}\text{NO}_3^-$  for 5 min, then transferred for 10 min to the influx solution which had each  $\text{NO}_3^-$  concentration labeled with  $^{13}\text{NO}_3^-$ . After the influx period, plant roots were washed with “cold” solution (same as pretreatment) for 3 min to remove  $^{13}\text{NO}_3^-$  from the cell wall,

followed by counting  $^{13}\text{N}$  contents with a  $\gamma$ -counter (MINAXI  $\gamma$  Auto-Gamma 5000 series, Packard Instruments, Meriden, CT).

### **RNA isolation and RT-PCR**

RT-PCR was performed according to the method described in Chapter 3. Primers sets, *NAR2.1*Forward: 5' AGGACCAGGTGTTGTTTGGATGCC, *NAR2.1*Reverse: 5' ACTGAAACAGATGGAGGCAATATCTAGGGA, amplifying a product size of 421bp. The amount of total RNA used in the PCR was 250  $\mu\text{g}$  in the total volume of 12.5  $\mu\text{l}$  of the reaction mixture. PCR cycle numbers for *AtNAR2.1* were 21 and 30 for roots and shoots, respectively.

### **Bioinformatics**

Phylogenetic analysis:

CLUSTAL W or X was used for an initial amino acid sequence alignment analysis (Thompson et al., 1994, 1997). The alignments were then finely adjusted and gaps were removed with Bioedit (Hall, 1999). Phylogenetic analysis was carried out with PAUP 4.0b5 (<http://www.lms.si.edu/PAUP>) with following condition: bootstrap method with heuristic search; otimality criterion = distance; starting tree(s) obtained via neighbor-joining; branch-swapping algorithm = tree-bisection-reconnection (TBR). TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>) was employed to output the phylogenetic trees.

Other methods:



Same as for Chapter 2.

## 5.3 Results and Discussion

### $\text{NO}_3^-$ Influx by HATS and LATS

Nitrate influx by *Arabidopsis* roots was measured at low (100  $\mu\text{M}$ ) and high (5 mM) external  $\text{NO}_3^-$  concentrations, representative of the high- and low-affinity  $\text{NO}_3^-$  transporter systems (HATS, and LATS, respectively), using  $^{13}\text{NO}_3^-$ , at intervals of time after the initial exposure to 1mM  $\text{NO}_3^-$ . Prior to exposure to 1 mM  $\text{NO}_3^-$ , there was already a substantial HATS influx of 2.5  $\mu\text{mol g}^{-1}\text{FW h}^{-1}$  at 100  $\mu\text{M}$   $\text{NO}_3^-$ . This is considered to be due to the constitutive HATS (cHATS) (Figure 5-1A). After provision of 1 mM  $\text{NO}_3^-$  in the external media, influx via the HATS increased continuously for 6h, peaking at a value that was 2.5 times the constitutive value. After 12 hours, the flux steadily decreased down to 2  $\mu\text{mol g}^{-1}\text{FW h}^{-1}$  by 72h, a value that was similar to the original (0h) value (Figure 5-1). Figure 5-2A shows that nitrate influx from media containing 5 mM external  $\text{NO}_3^-$  also increased initially and subsequently declined. Both HATS and LATS are considered to contribute to  $\text{NO}_3^-$  influx at 5 mM (Glass et al., 1992; Lejay et al., 1999). To estimate LATS-mediated influx as distinct from the HATS influx, flux values due to the HATS activity were subtracted from those measured at 5 mM, corresponding to both HATS and LATS influx, presuming that the root influx from 100  $\mu\text{M}$  was close to the  $V_{\text{max}}$  for HATS influx, and that the HATS contribution remained unchanged at the two external concentrations (Figure 5-2B). These

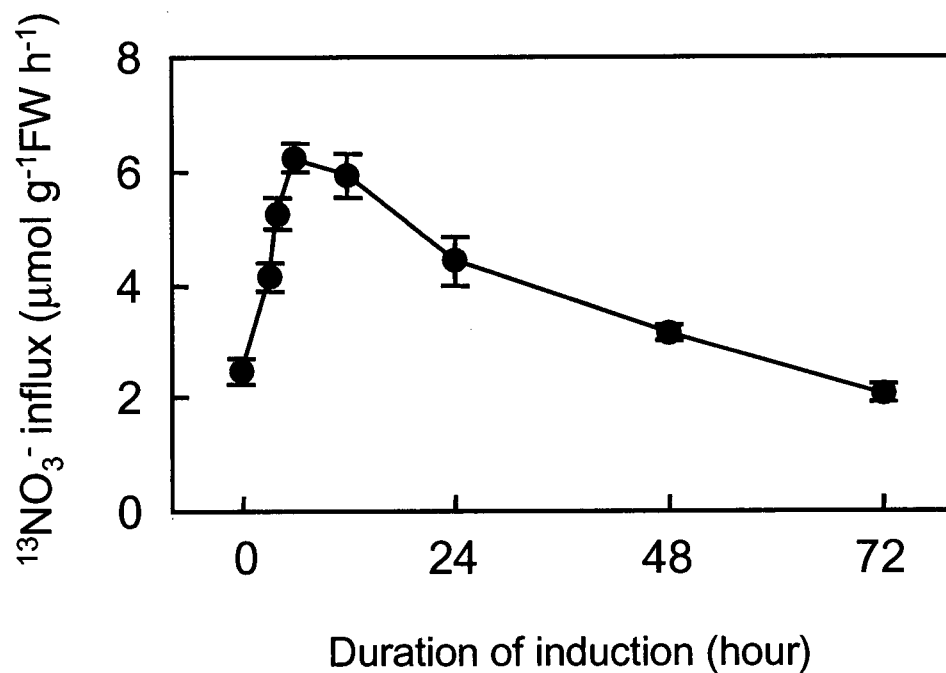


Figure 5-1. Time-course of  $^{13}\text{NO}_3^-$  influx into *Arabidopsis* roots at high-affinity range.

High-affinity nitrate influx measured with  $100 \mu\text{M NO}_3^-$ . 6-week-old plants were N deprived for 7 days before being transferred to  $1\text{mM NO}_3^-$  solution for 0 to 72 hours. The values are the means of 8 replicates, and vertical bars indicate SE.

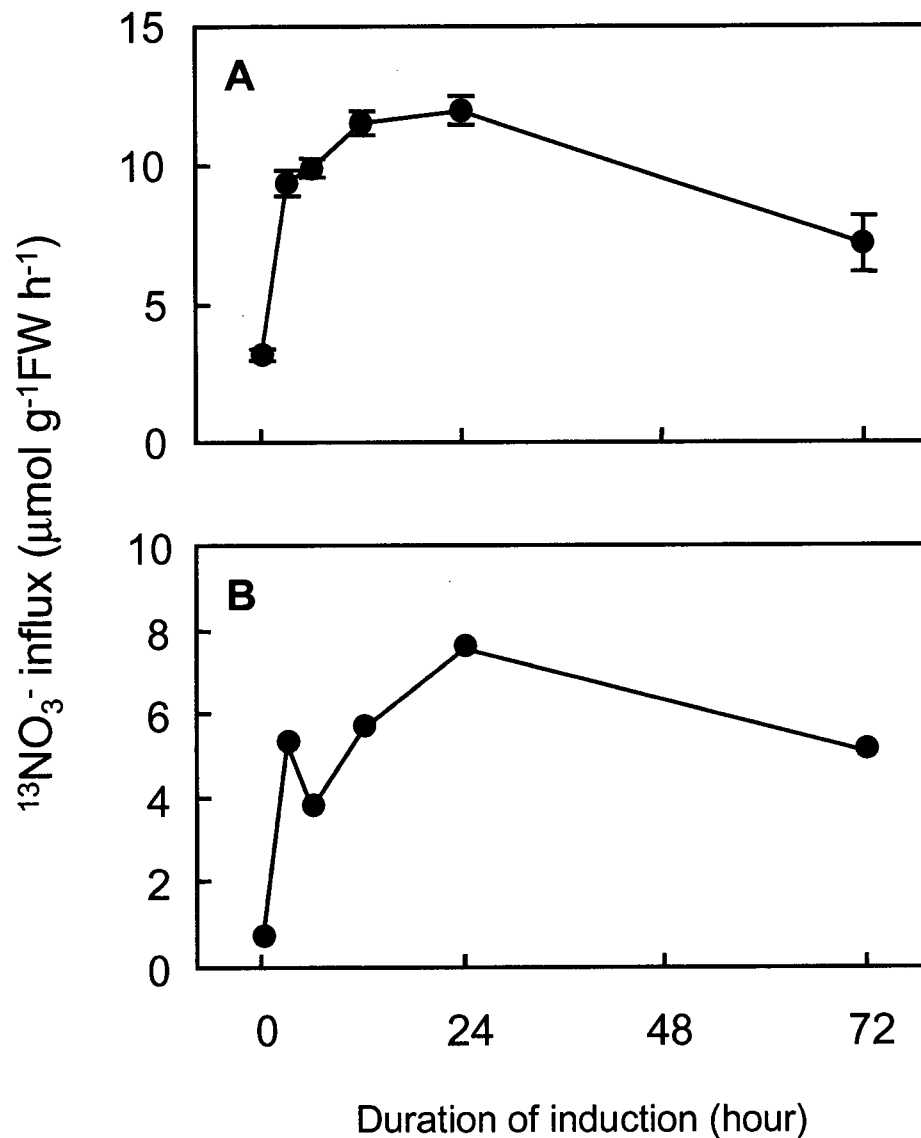


Figure 5-2. Time-course of  $^{13}\text{NO}_3^-$  influx into *Arabidopsis* roots at low-affinity range.

(A) Low-affinity nitrate influx measured with 5 mM  $\text{NO}_3^-$ . 6-week-old plants were N deprived for 7 days before being transferred to 1mM  $\text{NO}_3^-$  solution for 0 to 72 hours. (B) LAT5 activity at 5 mM  $\text{NO}_3^-$ . To estimate LAT5 mediated nitrate influx, mean values at 100  $\mu\text{M}$  (data from Figure 5-1) subtracted from those at 5mM. The values are the means of 8 replicates, and vertical bars indicate SE.

“corrected” values for LATS influx were initially  $< 1 \mu\text{mol g}^{-1}\text{FW h}^{-1}$  (at 0 h) but showed a rapid induction after  $\text{NO}_3^-$  provision, reaching a peak of  $8 \mu\text{mol g}^{-1}\text{FW h}^{-1}$ , at 24 h. This was followed by a slow down-regulation (Figures 5-2A and 5-2B). In summary, the cHATS influx (0h-plants) was higher than the constitutive LATS (cLATS). Induction of both HATS and LATS by  $\text{NO}_3^-$  was rapid, but times of peak activity and flux values were different. Furthermore, down-regulation in HATS appeared earlier and faster than that in LATS.

## **AtNRT Families and High- and Low-affinity Nitrate Transport System**

### **iHATS Transport**

It is evident from the foregoing results, that not all members of the *NRT2* family of genes can be characterized as nitrate-inducible. On the basis of correlations between physiological patterns of  $\text{NO}_3^-$  influx and corresponding patterns of gene expression, it has been suggested that the *NRT2.1* protein probably plays a major role in inducible HATS in *Arabidopsis* (Zhuo et al., 1999; Lejay et al., 1999), *N. plumbaginifolia* (Krapp et al., 1998), soybean (Amarasinghe et al., 1998), and barley (Glass et al., 2001). Our present data also showed that *AtNRT2.1* transcript abundance in roots corresponded closely with the temporal patterns of  $^{13}\text{NO}_3^-$  influx in the HATS range following provision of  $\text{NO}_3^-$  to  $\text{NO}_3^-$ -deprived plants (Figures 3-2B and 5-1). In fact, *AtNRT2.1* was the only one which showed statistically significant positive correlation among *NRT* genes (Table 5-1).

Table 5-1. Coefficients of determination ( $r^2$ ) for the relationships between *AtNRT* gene expression levels and two nitrate transport systems.

Gene	$r^2$ *	
	HATS	LATS
<i>AtNRT2.1</i>	0.55**	0.62
<i>AtNRT2.2</i>	0.22	<u>0.04</u>
<i>AtNRT2.3</i>	<u>0.62</u> **	<u>0.17</u>
<i>AtNRT2.4</i>	0.01	0.26
<i>AtNRT2.5</i>	<u>0.03</u>	0.18
<i>AtNRT2.6</i>	<u>0.22</u>	0.19
<i>AtNRT2.7</i>	<u>0.11</u>	<u>0.72</u> **
<i>AtNRT1.1</i>	0.32	0.77**
<i>AtNRT1.2</i>	0.04	0.16
<i>AtNRT1.3</i>	<u>0.00</u>	<u>0.27</u>
<i>AtNRT1.4</i>	<u>0.06</u>	0.06

\* Regressions were based on measurements of seven intervals (HATS; data from Figure 5-1), or six intervals (LATS; data from Figure 5-2) during nitrate induction period of 72 h. Negative correlations were underlined.

\*\* $P < 0.05$

Recently, *atnrt2*, a T-DNA knockout mutant in which *AtNRT2.1* and the 3' end of *AtNRT2.2* were deleted, has been characterized (Filleur et al., 2001; Cerezo et al., 2001).  $^{15}\text{NO}_3^-$  influx associated with iHATS activity of this *Arabidopsis* mutant was reduced to ~30 % of that of WT plants, while the LATS activity was relatively intact (Filleur et al., 2001). Although the data presented did not allow the authors to categorically distinguish which of the two genes, *AtNRT2.1* or *AtNRT2.2*, was the predominant contributor for iHATS at this stage, they were, nevertheless, able to conclude that these genes represent the major players (Cerezo et al., 2001). Our findings support this conclusion and further, as shown in Figure 3-2B, suggest that the pattern of *AtNRT2.1* expression most closely corresponds with the pattern of iHATS influx. *AtNRT2.2* expression pattern did not match the iHATS profile except during the first 3 hours. After 3 hours of  $\text{NO}_3^-$  provision the expression of *AtNRT2.2* was rapidly reduced compared to *AtNRT2.1* (Figures 3-2D and 5-1). Thus, *AtNRT2.1* appears to be a more likely candidate for iHATS influx.

Despite the identification of two distinct families of genes, *NRT1* and *NRT2*, encoding low- and high-affinity  $\text{NO}_3^-$  transporters, respectively, it has been suggested that *AtNRT1.1* (CHL1) should receive the status of a dual-affinity nitrate transporter (Crawford and Glass, 1998; Liu et al., 1999; Wang et al., 1998). This proposal is based on two observations: (1) Defective  $\text{NO}_3^-$  transport by both the HATS and LATS in *AtNRT1.1* mutants grown on  $\text{NH}_4\text{NO}_3$  (but not on  $\text{KNO}_3$ ), and (2) Heterologous expression of *AtNRT1.1* in *Xenopus* oocytes resulted in both HATS and LATS activities (Touraine and Glass, 1997; Wang et al., 1998; Liu et al., 1999). Since our observations were also based on  $\text{NH}_4\text{NO}_3$ -grown plants, *AtNRT1.1* protein might also be involved in the measured iHATS activity (Figures 3-3B and 5-1).

### cHATS Transport

Figure 5-1 shows that *Arabidopsis* plants have substantial cHATS activity in un-induced plants (i.e.,  $2.5 \mu\text{mol g}^{-1}\text{FW h}^{-1}$ ), as was the case in soybean plants (Amarasinghe et al., 1998) and in Steptoe barley (King et al., 1993). On the other hand, other barley varieties and spruce tend to have relatively small cHATS capacities (Siddiqi et al., 1990; Glass and Siddiqi, 1995; Kronzucker et al., 1995). Despite the disruption of *AtNRT2.1* and *AtNRT2.2* in the *atnrt2* mutant (Filleur et al., 2001), cHATS activity was almost the same as that of the WT in uninduced plants, suggesting that *AtNRT2.1* and *AtNRT2.2* make no contribution to cHATS activity. In  $\text{NO}_3^-$ -treated plants, this cHATS was even slightly increased by  $\text{NO}_3^-$  treatment (Filleur et al., 2001; Cerezo et al., 2001), consistent with earlier reports in barley and white spruce (Aslam et al., 1992; Kronzucker et al., 1995). Surprisingly, when grown on 1 mM  $\text{NH}_4\text{NO}_3$  for 5 weeks, and then on 1 mM  $\text{NO}_3^-$  for 1 week,  $^{15}\text{NO}_3^-$  influx in the mutant was maximally reduced at  $\sim 25 \mu\text{M}$ , while at lower (10  $\mu\text{M}$ ) and higher concentrations (100  $\mu\text{M}$ )  $^{15}\text{NO}_3^-$  influx was not as strongly reduced. The authors interpreted these observations to suggest that transporters other than *AtNRT2.1* and *AtNRT2.2* contribute to influx at very low  $\text{NO}_3^-$  concentrations (Cerezo et al., 2001; Cerezo et al., 2001).

CHATS candidates might be expected to satisfy two characteristics: (1) relatively high transcript abundance prior to exposure to  $\text{NO}_3^-$ , and (2) modest up-regulation of this transcript following induction by  $\text{NO}_3^-$ , the latter based on the documented increase of cHATS influx following this treatment (Aslam et al., 1992; Kronzucker et al., 1995).

Although several members of the NRT2 and NRT1 family, e.g. *AtNRT2.3*, *2.4*, *2.5*, *2.6* and *2.7* satisfy the first criterion, only *AtNRT2.4* also meets the second criterion, making it a viable candidate for the cHATS activity. Wang et al. isolated a mutant, which was impaired only in the cHATS transport in roots of *Arabidopsis* (1998). It will be interesting to see if this mutation is located in one of the above-proposed candidates.

### LATS Transport

Low-affinity transporter systems were originally thought to be either constitutive or repressible on the basis of flux analysis in barley (Glass and Siddiqi, 1995). However, the *AtNRT1.1* (*CHL1*) gene, considered to encode a low-affinity  $\text{NO}_3^-$  transporter in *Arabidopsis*, was induced by nitrate (Tsay et al., 1993). These contradictory findings may have been resolved by the demonstration that *Arabidopsis* possessed both an inducible- and a constitutively-expressed member of the *NRT1* family (Huang et al., 1996; Lejay et al., 1999; Liu et al., 1999). Our present findings confirm this, and also suggest that one member of the *NRT1* family is nitrate-repressible. However, the function of a nitrate-repressible *NRT1* gene is unclear at present given that our physiological evidence demonstrates only cLATS and iLATS (Figures 5-2A and 5-2B). As expected, *AtNRT1.1* showed a  $\text{NO}_3^-$ -inducible expression pattern in roots, and the pattern corresponded well with the observed LATS activity (Figures 3-3B and 5-2B). *AtNRT1.1* also showed the highest correlation ( $r^2$  values 0.77) against the LATS activity (Table 5-1). Other members of the *NRT1* family were either constitutively (*AtNRT1.2* and *1.4*), or repressively expressed (*AtNRT1.3*), indicating that these three transporters are unlikely to be major contributors to the iLATS. Rather *AtNRT1.2* and/or *AtNRT 1.4* may encode the cLATS.



According to Liu and Tsay (personal communications) the AtNRT1.1 (CHL1), a putative dual affinity transporter, switches between high and low-affinity through protein phosphorylation. This surprising finding adds even greater complexity to the existing situation vis á vis the 7 NRT2 nitrate transporters and the 4 NRT1 transporters. In the present study, although at the transcript level *AtNRT2.1* and *AtNRT1.1* behaved similarly, there were distinct differences between patterns of HATS and LATS influx: (1) the iHATS peaked at 18 hours earlier than the LATS; (2) the iHATS influx was reduced to the original influx value after 72 h of  $\text{NO}_3^-$  exposure, whereas LATS remained considerably higher ( $5.1 \mu\text{mol g}^{-1}\text{FW h}^{-1}$ ) than its original value ( $<1 \mu\text{mol g}^{-1}\text{FW h}^{-1}$ ) even after 72 h (Figures 5-1 and 5-2B). Thus at the physiological and molecular levels, the two nitrate transport systems (Wang and Crawford, 1996) not only co-exist but they appear to be differently regulated. Nevertheless, both HATS and LATS have constitutive and inducible systems.

### **Shoot and Root Differences and Similarities**

Roots are considered to be the main organ for nitrogen uptake by terrestrial plants, and have consequently been the focus of attention in studies of  $\text{NO}_3^-$  transport. By contrast, only a few studies have reported on ion transport by shoot tissue. Nitrate translocated to shoots is released from vascular tissue to the leaf apoplasm before being re-absorbed by leaf cells. In consideration of the thermodynamics of  $\text{NO}_3^-$  transport even when apoplastic  $\text{NO}_3^-$  is in the high mM range ( $>20 \text{ mM}$ ) the  $\text{NO}_3^-$  re-absorption step in leaves would be active, requiring a source of free energy (presumably the proton motive force) and appropriate transporters (Glass and Siddiqi, 1995). Based upon typical analyses of apoplastic  $[\text{NO}_3^-]$ ,

leaf re-absorption would most likely be mediated by low-affinity nitrate transporters. In fact, all members of *AtNRT1* family showed relatively high expression level in the shoots in our present study. Furthermore, a recent study of *AtNRT1.1* showed high levels of gene expression to be present in the developing organs of entire plants including leaves and flowers (Guo et al., 2001). These observations provide support for the rationale that nitrate uptake by shoot tissues is vitally important for the assimilation of  $\text{NO}_3^-$  by leaf tissues. Nevertheless, if this is mediated by low-affinity transporters, the question remains: what is the relevance of the observed expression patterns of NRT2 transporters in shoots? In *Limium* and *Bromus*, the concentrations of leaf apoplastic nitrate varied from 0.11 mM to 2.38 mM in N-deficient and N-replete plants, respectively (J. Schjoerring, personal communication). This suggests the possibility that as apoplastic  $\text{NO}_3^-$  declines from mM to  $\mu\text{M}$  concentrations, as for example under field conditions as external supplies of  $\text{NO}_3^-$  are depleted, high-affinity transport systems may be necessary to scavenge apoplastic  $\text{NO}_3^-$  from the latter range of concentration. This argument suggests that both the NRT2 and NRT1 transporters may participate in the re-absorption of  $\text{NO}_3^-$  by leaf cells.

#### **Gene Structures and Functions- Can we predict functions from sequence homology?**

Using the differential patterns of gene expression and documented patterns of  $^{13}\text{NO}_3^-$  influx, we have attempted to attribute known transport functions (cHATS, iHATS, cLATS, and iLATS) to particular nitrate transporter genes identified from *A. thaliana*. Although we are as yet unable to ascribe functional roles to all members of the *NRT1* and *NRT2* families of genes, in a limited number of cases e.g. *NRT1.1* and *NRT2.1* and *NRT2.2* (Cerezo et al.,

2001) there is sufficient evidence to allow reasonable predictions concerning functions to be made. Genes with high sequence homology might be anticipated to perform similar functions. Figure 5-3 shows amino acid sequence comparisons among known *NRT2* family members from various organisms. In *Arabidopsis* nitrate-inducible genes *AtNRT2.1*, 2.2, and 2.4 have high sequence similarity, and it is reasonable to assume that their functions might also share some similarities. Another homologous duo *AtNRT2.3* and 2.6 also showed some similarities in the expression patterns, although the functions are unknown (Figures 3-5D and 3-5E). Most of the cloned *NRT2* family members from higher plants (e.g. from *N. plumbaginifolia*, soybean, *Arabidopsis*, and tomato (Amarasinghe et al., 1998) were nitrate inducible (Figure 5-3), and exhibited high sequence homology with *AtNRT2.1* and 2.2 proteins. Thus these transporters may function as iHATS (Quesada et al., 1997; Trueman et al., 1996; Zhuo et al., 1999).

Similarly, nitrate-inducible *AtNRT1.1* has high sequence similarity with *BnNRT1.2* that is also induced by nitrate (Muldin and Ingemarsson, 1995; Zhou et al., 1998). Likewise, *AtNRT1.2* from *A.thaliana* (Huang et al., 1999; this work), *OsNRT1.1* from *Oryza sativa* (Lin et al., 2000) and *GmNRT1.3* from *Glycine max* (Yokoyama et al., 2001) are constitutive genes and these three genes encode proteins that have closely related amino acid sequences (Figure 5-4). Therefore, these transporters may serve as cLATS. Clearly greater details of function will be necessary in order to develop useful predictions.

### **Gene Structures and Predicted Protein Sequences of *AtNAR2* Genes**

*NAR2* genes may be involved in nitrate transport system in higher plants as has been demonstrated for the alga *C. reinhardtii*. A homology search was performed using the

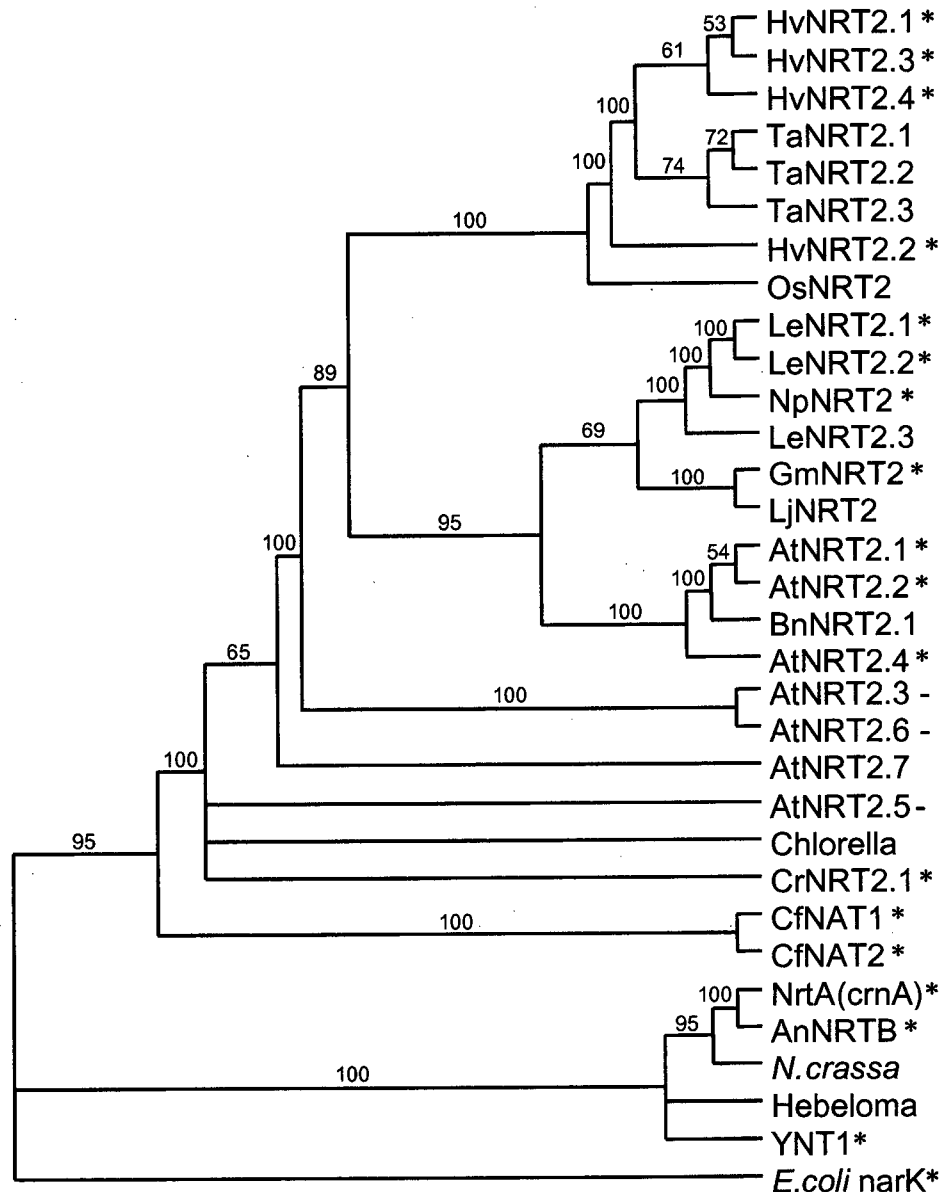


Figure 5-3. Sequence relationships of NRT2 family.

Amino acid sequences were aligned with CLUSTAL X and BIOEDIT, the neighbor-joining trees were generated with PAUP 4.0b8. Bootstrap values are shown in branching positions. Nitrate-inducible and nitrate-constitutive genes are indicated with asterisks and (-), respectively.

Sequence sources: *Arabidopsis thaliana* (accession numbers: see Table 1); *Aspergillus nidulans*, NrtA(crnA)(M61125), NrtB(AF453778); *Brassica napus*, BnNRT2(CAC05338); *Chlamydomonas reinhardtii*, CrNRT2.1(Z25438); *Chlorella sorokiniana* (AY026523); *Cylindrotheca fusiformis*, CfNAT1(AF135038), CfNAT2(AF135039); *Escherichia coli*, nark(X15996); *Glycine max*, GmNRT2(AF047718); *Hebeloma cylindrosporum* (AJ238664); *Hordeum vulgare*, HvNRT2.1(U34198), HvNRT2.2(U34290), HvNRT2.3(AF091115), HvNRT2.4(AF091116); *Lotus japonicus*, LjNRT2(AJ292342); *Lycopersicon esculentum*, LeNRT2.1(AF092655), LeNRT2.2(AF092654), LeNRT2.3(AY038800); *Nicotiana plumbaginifolia*, NpNRT2(Y08210); *Neurospora crassa* (b8g12\_170 in the MIPS *N. crassa* database; <http://mips.gsf.de/>); *Oriza sativa*, OsNRT2(AB008519); *Pichia angusta*, YNT1(T43154); *Triticum aestivum*, TaNRT2.1(AF288688), TaNRT2.2(AF332214), TaNRT2.3(AY053452).

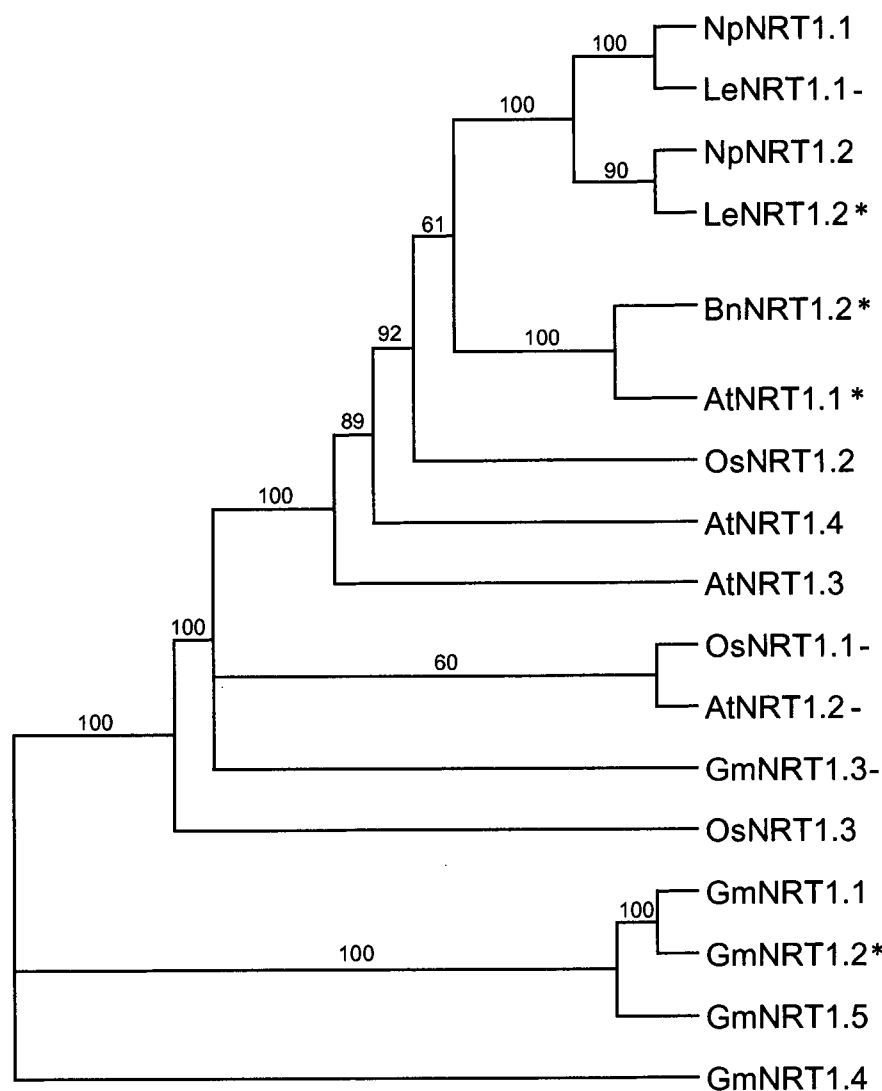


Figure 5-4. Sequence relationships of NRT1 family.

Amino acid sequences were aligned with CLUSTAL X and BIOEDIT, the neighbor-joining trees were generated with PAUP 4.0b8. Bootstrap values are shown in branching positions. Nitrate-inducible and nitrate-constitutive genes are indicated with asterisks and (-), respectively.

Sequence sources: *Arabidopsis thaliana* (accession numbers: see Table 1); *Brassica napus*, BnNRT1.2(U17987); *Glycine max*, GmNRT1.1(BAB19756), GmNRT1.2(BAB19757), GmNRT1.3(AB052786), GmNRT1.4(BAB19759), GmNRT1.5(BAB19760); *Lycopersicon esculentum*, LeNRT1.1(X92853), LeNRT1.2(X92852); *Nicotiana plumbaginifolia*, NpNRT1.1(CAC00544), NpNRT1.2(AJ277085); *Oriza sativa*, OsNRT1.1(AF140606), OsNRT1.2(AC037426), OsNRT1.3(AP003263).

sequence of the CrNAR2 protein against the Arabidopsis gene database, and two homologues were found, namely *AtNAR2.1* and *AtNAR2.2* (Figures 5-5 and 5-6). *AtNAR2.1* is located on chromosome 5. It is interrupted by one intron (291 bp), and encodes 210 amino acids with MW of 23.4 kD. In the promoter regions there are two nitrate-dependent transcriptional motifs at -78 and -325, a putative TATA box, and several GATS boxes. *AtNAR2.2* also consists of one intron (298 bp) and two exons (142, 485 bp), coding polypeptides of 209 amino acids (23.3 kD) on chromosome 4. The nitrate-dependent transcriptional motif was only observed far upstream (-1322) of the *NAR2.2* gene. This may not be functioning because of the location (i.e., typically -140 to -250 bp upstream in Arabidopsis; Hwang et al., 1997). SignalP (Nielsen et al., 1999) predicted that both NAR2 proteins possess signal peptides, cleaving between amino acid 22 and 23. These predictions were also supported by PSORT program (Nakai and Kanehisa, 1992). One transmembrane region was predicted at the C-termini, leaving a long hydrophilic N-terminal end that is exoplasmic (Figure 5-7). NAR2.1 and 2.2 proteins share 61 % of identity and 76 % of similarity. In N-terminus TonB-dependent receptor protein signatures "TonB-Box", [DENF]-[ST]-[LIVMF]-[LIVSTEQ]-V-x-[AGP]-STANEQPK, were observed in NAR2.2 in high degree, and in NAR2.1 with less conservation (Figure 5-7). The function of TonB, a periplasmic protein in Gram-negative bacteria, has been suggested in following way. TonB, dimerizing at C-termini interacts with outer membrane receptor proteins providing energy, and the receptor proteins execute high-affinity binding and transport of substrates through the plasma membranes into the periplasmic space (Chang et al., 2001). The substrates include iron, colicin, and vitamin B12 (Bassford et al., 1976;

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-1500 CAGAGAAGAGGTACGAGTAAAAGAAACAAAGTGACTTAGGAAACGCCATTGTTGGAGACTGCTCACTGGAAGATAGAGAGT
-1418 CGTGAGAGACAGTGATAAAGCGTATCAAGTCATATAGAGGGTCTTCTTATCTTTTCTTTAAACATGTGAGGGTTGAGTTAAT
-1336 TATGCGGGCTGATTATAGAGTTTTTAAATTGAATTTACGATTGTTTTTTCTTACATATAAATGCAATCTATATTGTGTTT
-1254 GGAATAACCCATCTAATATTACTCCATGTATTAACATAAAATATTTGTCATGTTTGGTAGATCAACTTTTTGAATGATCAC
-1172 AGACCTACAAACATCAACCCCTCTAATTATCCAATTTTACCATAATCCACGAGCTCTTAAATTCATTTTTAATATATATAAT
-1090 TAAATAGTTCAAACAGTTTAAACTTTGTGACCAAGTTAAATATTTAAATAGTTTGACTTTGTGATCAACATATTTAAAT
-1008 ATAATCAATATTTTCAATTTTAAAGCGGAAAAATCAGTCTTACAAATATATTTCTGATAGACACACCTATAATTCCAAATTT
-926 TTGACTTTTTAAAAACAAACAAAAAAATCTCATTAAATACCACTACATACGTTTTTACAAAAATACCATTAAAGATATTTTTT
-844 TCAAATACAAAAAACTAAACTTAACTTTAAACCTATAAAACACTAAATCCCTATAAAGTTTATATTCAGTATAAACCT
-762 TAAAGTTCAACCCCTAAATCCTGAAAGCAAGCCCTAAACCCAACTCAAACCTTTTAAATATAAATCCTTAAACAAAATCA
-680 TTTTGTAGTCTTATGGTATTTTAGAGTTAACAGTTTGTAGTTGATTTTTTGAAAAAAAACACTAGTGATAGGTTTTTAGA
-598 ATAAAACTTAATTTAGTGGTATTGAGGATATTTCTCTAATAAAAAATTATTCACAAAAAATATGTATTAAAGCAAAGTGCTA
-516 TGCTTATTCATGCAATCTTTTTTGAAAAAAAATATTTCTAACCGATTAGTATATCTCTAGAGGATTCATAGAAAAAG
-434 AGGATACAATTACAATATGTAGAGTATCTTATAGGTGACGTAACCATGAAATATAGAATTCTTTGGAATCTGAACTGAATT
-352 ATTCAGTTGATAAATGATAAAACAATACTCATATCTCATCTTTGGCATGTTTAGGAGCCATCTCTCAGCTGGAGGAGAC
-270 AACACAGACACTTGTGCCAGAGAGGGAACACAATCTTCCCAAGTCTTCGAAAGGGTAATCTCAGACCAAAACCGTTGACTTTG
-188 TTCTCATTAATTGTTTATGCTAAACCACAAGATATTTGGATCAAAAGTATTACCATAAATCACTATATAAGACATAAATGT
-106 GCACCCCACTTTTCTTATCAATCATAAAGTCAGCAAAACACAAGGCATATTCCTCTTCTCTCCTCAGCCTTATTTTCTG
-24 ATATTCAGTTTCAAGGATATATCCATGGCGATCCAGAAGATCCTCTTTGCTTCACTTCTCATATGCTCATGATCCCAATCCA
      M A I Q K I L F A S L L I C S L I Q S I 20
59 TCCACGGGGCGGAAAAAGTAAGACTCTTCAAAGAGCTGGCAAAAGGTGCACCTTGATGTCACCACATAAACCCAGCCGAGAAGG
      H G A E K V R L F K E L D K G A L D V T T K P S R E G 47
141 ACCAGGTGTTGtatgtataattctcttcaaacatctatcatttgctacttcattagccatgccttataaatgtgcgattt
      P G V V 51
223 tgctctaataccatgtagatttttaagctttatgaatgttaagacaaattttatgagtgttgaaacattagttctcttt
      intron I
305 agtcgtttattacaacatcattagatcttatcctattaattagaattacttagaattaagtgatatgaatgatagtagatgc

387 taatatcaattttacaagaataagttggaattaatattttgaatgtttggtcttttagTTTTGGATGCGGCAAGGATACGTTG
      L D A G K D T L 59
469 AACATTACATGGACGCTAAGCTCGATTGGGTCTAAAGAGAGGCTGAATTTAAGATCATCAAAGTTAAGCTATGCTACGCTC
      N I T W T L S S I G S K R E A E F K I I K V K L C Y A P 87
551 CACCTAGCCAAGTTGACCGACCATGGCGCAAAACCCATGACGAGTCTTCAAAGACAAGACCTGCCACACAAGATCATAGC
      P S Q V D R P W R K T H D E L F K D K T C P H K I I A 114
633 CAAGCCTTATGACAAAACACTTCAATCACTACTTGGACTCTTGAGCGTGACATCCCCACCGGAACCTACTTCGTTTCGTGCC
      K P Y D K T L Q S T T W T L E R D I P T G T Y F V R A 141
715 TACGCGGTTGATGCCATTGGCCATGAAGTTGCCTATGGACAGAGACCGACGATGCCAAGAAAACCAATCTTTCAGCGTTT
      Y A V D A I G H E V A Y G Q S T D D A K K T N L F S V Q 169
797 AGGCTATCAGTGGCGCCACGCGTCCCTAGATATTCCTCCATCTGTTTTCAGTGTCTTCTCCGTCGTGCTTGTGCTCTT
      A I S G R H A S L D I A S I C F S V F S V V A L V V F 196
879 CTTTGTCAATGAGAAGAGGAAGGCCAAGATAGAGCAAAGCAAATGAGTCGTTTACTTTGCGTATTGTGACGTTGAACCCAA
      F V N E K R K A K I E Q S K * 210
961 AAAAAGTTGACTTTGAACTTCTTGTTTACCAATTCCTTTTGTCTTGTGACACTTCTTCTTTCTTATATGCTTTTATTTA
1043 TGTGTTTGTACAATTAAGCCATTGATATGGAACAAAATACTACCGGTGGTGTGCGTTTGTGTGCTTTATATATTTTTT
1125 ATACACCTTTTGATATATTTCTTACACACTTTTTTCACAAAGATTGATATTTGCATAATTTGTGGTAATCATCTCAAATAC
1207 GGTATATATACTTACCTCTTACTTTCAAAGAGAAATCATAAGTAACTAAGCAATAATCTATATATAGGGAATTATGAT
1289 ATAAATTTATCCAAATTTTCAAAATATTAACAAA

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Figure 5-5. DNA and deduced amino acid sequence of the *AtNAR2.1* gene.

The number on the left starts at the proposed start codon, and the numbers on the right refer to amino acid residues. Intron sequences are shown in lower cases. GATA boxes are underlined. TATA box is indicated with thick underline. Nitrate-dependent transcription motifs are double underlined.





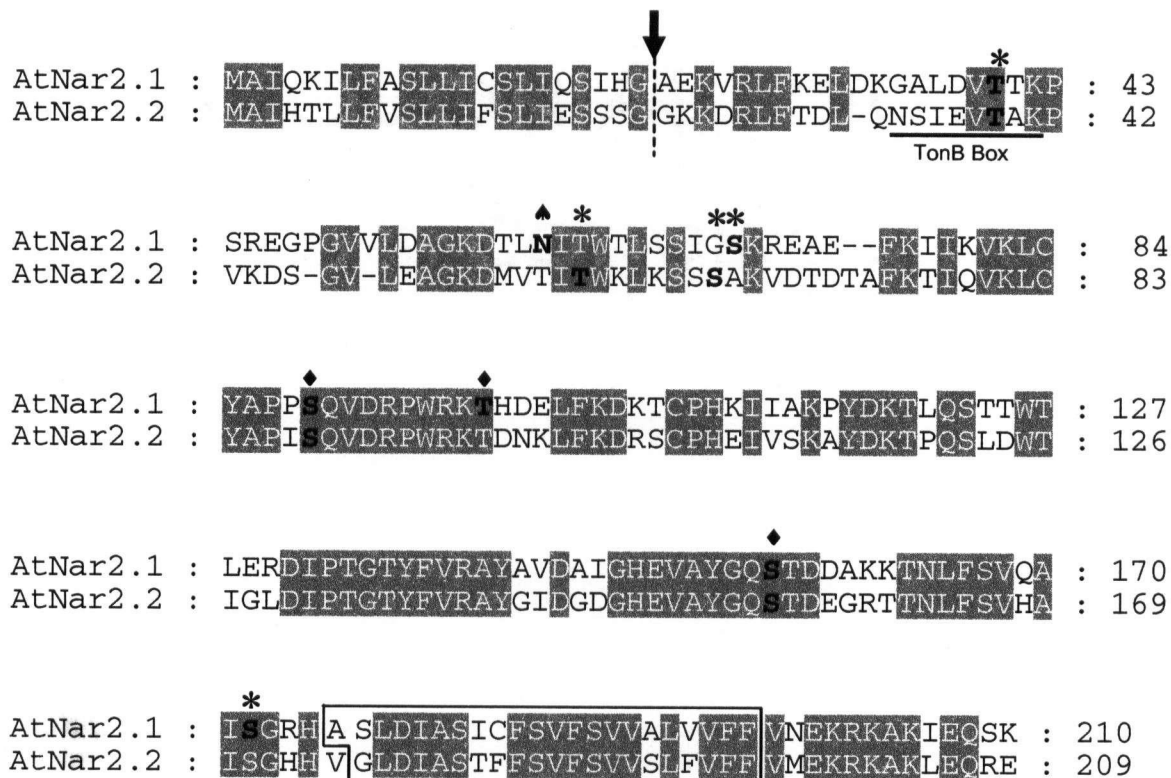


Figure 5-7. Amino acid sequence alignment of AtNar2.1 and AtNar2.2.

Arrow indicates predicted cleavage sites. Transmembrane regions predicted by TMHMM (Sonnhammer et al., 1998) are boxed. Potential protein kinase C and casein kinase 2 phosphorylation, and N-glycosylation sites, specified in bold letters with (\*), (♦), and (♠), respectively, were searched with the ProSite database (<http://www.expasy.ch/prosite/>). The serine-70 of AtNar2.1 is a recognition site for both PKC and CK2. Accession number: AtNar2.1 (BAB09391), AtNar2.2(T05562). TonB-dependent receptor signature (TonB Box) is underlined.

Lundrigan and Kadner, 1986; Schramm et al., 1987). The receptors fail to uptake substrates in the absence of TonB (Raynolds et al., 1980).

### **Expression Patterns of *AtNAR2.1***

To examine the expression patterns of the *AtNAR2* genes, relative quantitative RT-PCR was carried out. The expression patterns were revealed when PCR cycles reached at 21 and 30 in root and shoots, respectively in *AtNAR2.1*. For unknown reasons, *AtNAR2.2* was undetectable even when cycle numbers were raised to 40 in the roots (data not shown). In roots the time-course expression patterns of *AtNAR2.1* was similar to that of *AtNRT2.1* (Figure 5-8). Linear regression analysis showed a strong correlation between the two gene expression patterns ( $r^2=0.86$ ) (Figure 5-9). *AtNAR2.1* expression patterns in shoots also displayed a similarity with that of *AtNRT2.1* (Figure 5-10).

It is possible that NRT2.1 and perhaps other members of the NRT2 family are unable to function independently i.e., in the absence of NAR2. Rather, they may require other gene product(s) which may act as a receptor, or a part of a signal transduction system in order to activate NRT2.1. The expression of *AtNAR2.1*, a candidate for this function, is highly correlated with *AtNRT2.1* at the transcriptional level. Although many genes are regulated by nitrate (Wang et al., 2000a), and their expression patterns might correlate with that of *NRT2.1*, the evidence from *C. reinhardtii* strongly support this hypothesis. Nevertheless, the function of NAR2 is still unknown.

From the predicted 2-D structure, *AtNAR2* possesses a single transmembrane domain at the C-terminus. Similarly, other proteins with single transmembrane domains are involved in membrane transport activities directly or indirectly. For instance, GmSAT1,

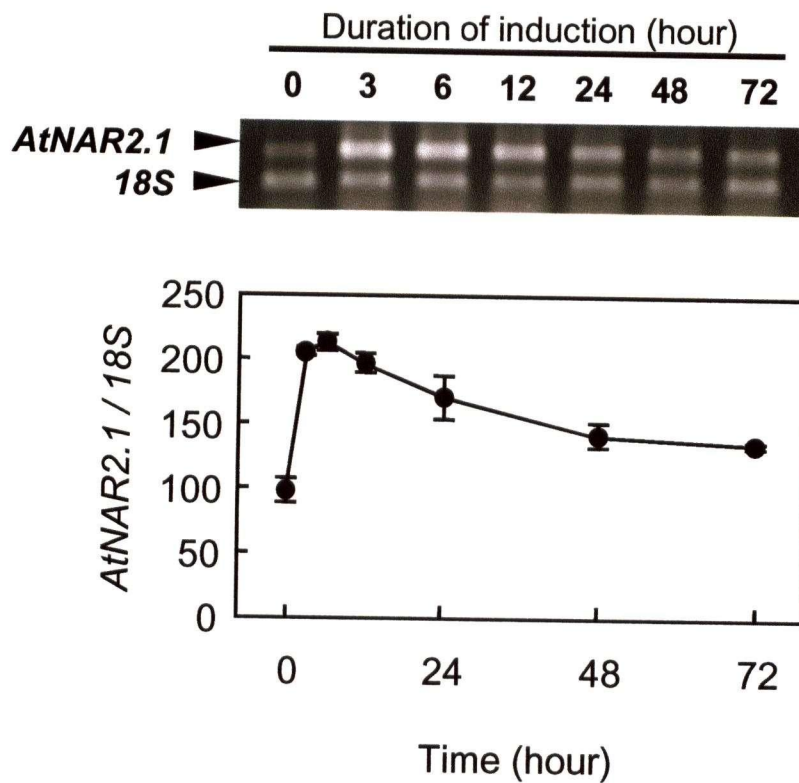


Figure 5-8. Expression patterns of *AtNAR2.1* gene in roots.

RT-PCR products were obtained from 6-week-old *Arabidopsis* plants, which were grown hydroponically for 5 weeks and supplied with 0.5 mM  $\text{NH}_4\text{NO}_3$ . Plants were N deprived for 1 week (0h), and then re-supplied with 0.5 mM  $\text{Ca}(\text{NO}_3)_2$  for 3-72h. Relative values were obtained by the ratio of the gene specific amplicon for the 18S amplicon. The values shown are means of three RT-PCR replicates. Bars indicate SE.

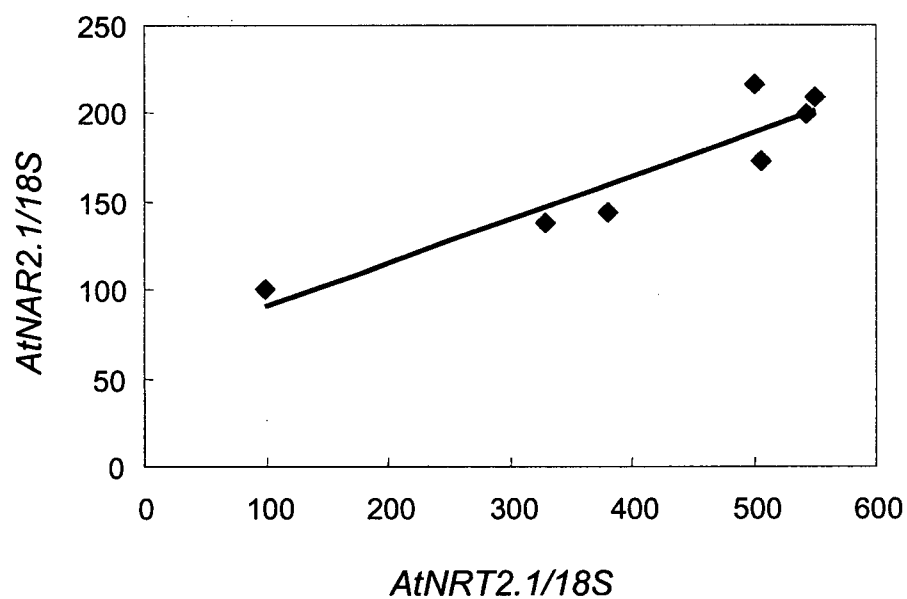


Figure 5-9. Correlation between *AtNRT2.1* and *AtNAR2.1* expression levels in roots. Regression was based on 7 intervals during the time-course experiment (data of *AtNRT2.1* from Figure 3-2B, *AtNAR2.1* from Figure 5-8). Linear regression gives  $r^2$  value 0.86 ( $P < 0.01$ ).

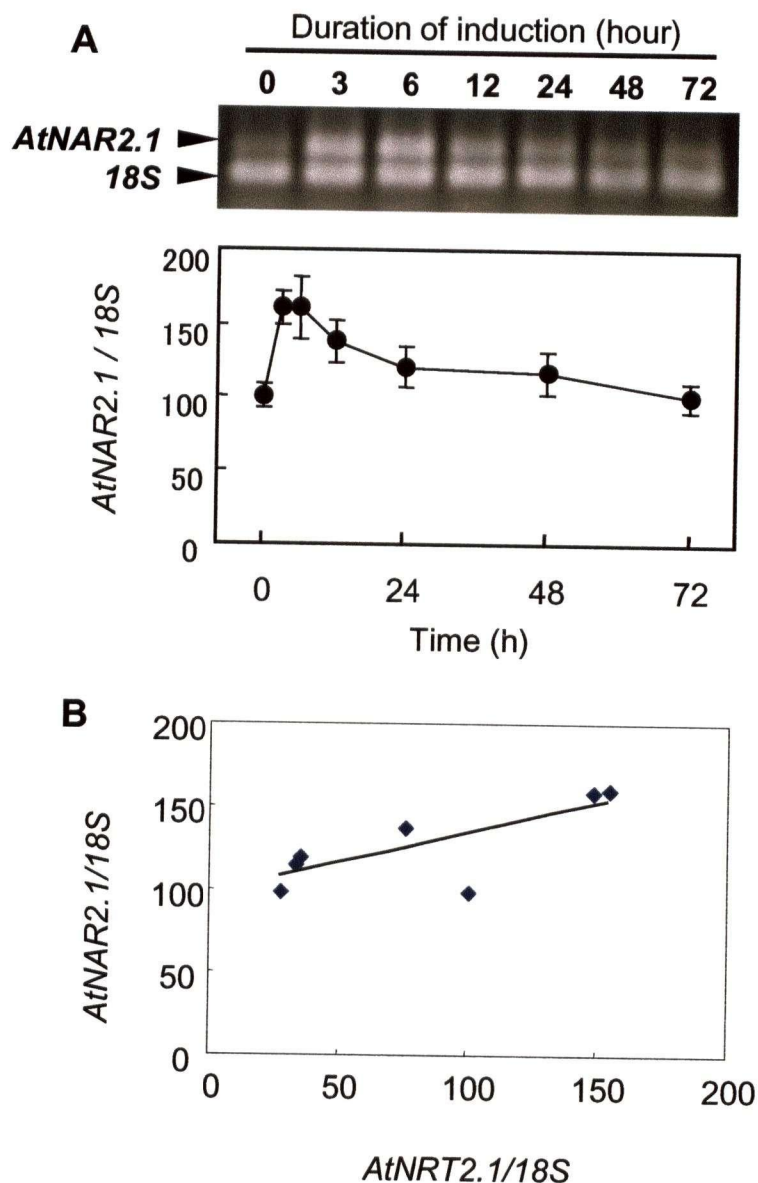


Figure 5-10. Expression patterns of *AtNAR2.1* gene and their correlation with *AtNRT2.1* in shoots.

(A) RT-PCR products were obtained from 6-week-old Arabidopsis plants, which were grown hydroponically for 5 weeks and supplied with 0.5 mM  $\text{NH}_4\text{NO}_3$ . Plants were N deprived for 1 week (0h), and then re-supplied with 0.5 mM  $\text{Ca}(\text{NO}_3)_2$  for 3-72h. Relative values were obtained by the ratio of the gene specific amplicon for the 18S amplicon. The values shown are means of three RT-PCR replicates. Bars indicate SE.

(B) Correlation between *AtNRT2.1* and *AtNAR2.1* expression levels in shoots. Regression was based on 7 intervals during the time-course experiment (data of *AtNRT2.1* from Figure 3-2A, *AtNAR2.1* from (A)). Linear regression gives  $r^2$  value 0.58 ( $P < 0.05$ ).

soybean nodule ammonium transporter, has been proposed as a  $\text{NH}_4^+$  transporter, although it has only single transmembrane region (Kaiser et al., 1998). The receptor-activity-modifying proteins (RAMPs), which have a single transmembrane domain, regulate the transport and ligand specificity of the calcitonin-receptor-like-receptor (CRLR) (McLatchie et al., 1998). AtNAR2 might perform these kinds of functions (i.e., it may transport substrates by itself, or modify and/or traffic other proteins) (Zhou et al., 2000). Furthermore, AtNAR2 also displays TonB-dependent receptor proteins signatures, suggesting that the gene products might also be involved in other protein-protein interactions at the outer surface of the plasma membrane. In other words, the function of the NRT2 nitrate transporters may require more complicated interaction with other proteins, rather than being independently functional membrane proteins. In any event, the function of the NRT2 nitrate transporters is still unclear, and needs to be explored further.

# 6

## Conclusion and Future Prospective

The present study has demonstrated that all putative nitrate transporters of the AtNRT2 and AtNRT1 families, revealed by the *A. thaliana* genome project, could be detected in roots and shoots of this species by relative quantitative RT-PCR. In addition the use of GUS technology has proved information concerning the tissue-specific localization of a limited number of these genes.

It was originally considered that *NRT2* high-affinity nitrate transporter genes are nitrate-inducible (Crawford and Glass, 1998; Forde, 2000). However, it is evident from the forgoing results that not all members of the *NRT2* family of genes can be characterized as nitrate-inducible. Rather, two genes (*AtNRT2.5* and *AtNRT2.6*) showed constitutive expression, and there was one gene even suppressed by nitrate provision (*AtNRT2.7*).

Including classic nitrate-inducible genes (*AtNRT2.1*, 2.2, and 2.4), the *NRT2* family members are perhaps components of the high-affinity nitrate transport systems. Similarly, low-affinity nitrate transporter *AtNRT1* family of genes showed three expression patterns in response to  $\text{NO}_3^-$  provision. Unlike barley or spruce, Arabidopsis possesses inducible LATS. This unique system showed correspondence with a nitrate-inducible low-affinity transporter *AtNRT1.1(CHL1)*.

Tissue-specific expression patterns of four *NRT2* genes were revealed in the present study *AtNRT2.1*, 2.2, and 2.4, a highly homologous trio, and also categorized as nitrate-inducible genes, showed some similarities in their tissue-specific localization (Chapter 4). Adding *AtNRT1.2* by Huang et al (Huang et al., 1999), localizations of six nitrate transporter genes have been confirmed, showing that those genes were expressed at least in the roots. This could support the belief that *NRT* genes are involved in  $\text{NO}_3^-$  uptake, although it may be too early to conclude their functions only from these localization patterns. In the *GUS* reporter gene analysis, *AtNRT2.3*, 2.5, and 2.7 failed to show expression patterns. The explanations for this might include: 1. The expression levels are too low to detect with current protocols. 2. The promoter regions chosen did not contain sufficient information to initiate transcriptions (Sieburth and Meyerowitz, 1997). 3. Technical difficulties. If the problem were caused by *GUS* reporter gene system itself, other systems such as green fluorescent protein (*GFP*) reporter DNA, *in situ* hybridization, *in situ* RT-PCR (Koltai and Bird, 2000), or immunolocalization analysis could be useful. In any cases, the localization of the rest of members of *NRT* genes probably will certainly be explored soon.



The current study showed both similarities and uniqueness among members of the *NRT* gene families. Surprisingly, genes showed identical expression patterns. Nevertheless, it may not be so simple to conclude that highly homologous multi-gene families are genetically redundant. Rather, each *NRT* gene may have unique functions such as specific localization, specific  $K_m$  and  $V_{max}$  values, or some roles other than  $\text{NO}_3^-$  uptake as shown in *AtNRT1.1(CHL1)* (Guo et al., 2001).

Screening mutants or generating mutant phenotypes is a well-established way to characterize the function of each nitrate transporter. Despite 11 nitrate transporters, only two mutants are identified (i.e., *chl1*; Tsay et al., 1993; and *atnrt2*; Cerezo et al., 2000; Filleur et al., 2001). Protocols for mutant screening are improving steadily. The techniques include well established T-DNA insertion mutagenesis, or gene silencing by over expression of RNAi. In addition to large populations of T-DNA lines, a recent method using degenerate primers enhances the screening efficiency dramatically, and this method is suitable for multi-gene families such like *AtNRT* (Young et al., 2001). Advanced technique of anti-sense recently emerged, in which the transforming gene contains sense and anti-sense cDNA fused with introns under an ectopic promoter, such as the cauliflower mosaic virus 35S promoter. The transgenic lines produced with this technique silence mRNA more efficiently than ordinal anti-sense lines (Chuang and Meyerowitz, 2000; Smith et al., 2000).

The function of the *NRT* genes might also be investigated by heterologous expression system. This was most effectively employed in the original study by Tsay et al., (1993) to investigate *AtNRT1.1(CHL1)* function. Since expressing *AtNRT2.1* alone failed to restore nitrate transport in a double mutant of *Aspergillus nidulans* incapable of nitrate

uptake, a co-expression study with *AtNar2.1* has been undertaken. It will be interesting to see whether *Nar2* is required in Arabidopsis nitrate transport.

The *Arabidopsis* genome project unveiled 25,000 genes encoding proteins from 11,000 gene families, including 600 membrane transporters (The Arabidopsis genome initiative, 2000). Although microarray technique allows us to identify thousands of genes at once and to provide us with rudimentary ideas of gene regulation, follow up analysis is still necessary to arrive at more detail conclusions. It is encouraging that relative quantitative RT-PCR could identify and show the differential expression patterns of highly homologous gene copies as described in this study. It is also notable that the same primer sets, used in the quantitative RT-PCR analysis, are applicable for use in *in situ* RT-PCR for tissue-specific gene expression studies (Koltai and Bird, 2000).

In summary, thanks to the *Arabidopsis* genome project, we now know the absolute number of gene copies in the *NRT* families, and I was able to characterize the gene expression patterns of all members. The results allowed to predict the physiological roles of the *NRT* transporters. Proving these hypothetical functions could be challenging. However, it may be feasible if we combine functional genomics and physiology.

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