Transcript Profiling in the chl1-5 Mutant of Arabidopsis Reveals a Role of the Nitrate Transporter NRT1.1 in the Regulation of Another Nitrate Transporter, NRT2.1

Stéphane Muñoz,1 Céline Cazettes, Cécile Fizames, Frédéric Gaymard, Pascal Tillard, Marc Lepetit, Laurence Lejay, and Alain Gojon2
Biochimie et Physiologie Moléculaire des Plantes, Unité Mixte de Recherche 5004, Agro-M/Centre National de la Recherche Scientifique/Institut National de la Recherche Agronomique/Université Montpellier 2, 34060 Montpellier, Cedex 1, France

Arabidopsis thaliana mutants deficient for the NRT1.1 NO3− transporter display complex phenotypes, including lowered NO3− uptake, altered development of nascent organs, and reduced stomatal opening. To obtain further insight at the molecular level on the multiple physiological functions of NRT1.1, we performed large-scale transcript profiling by serial analysis of gene expression in the roots of the chl1-5 deletion mutant of NRT1.1 and of the Columbia wild type. Several hundred genes were differentially expressed between the two genotypes, when plants were grown on NH4NO3 as N source. Among these genes, the N satiety-repressed NRT2.1 gene, encoding a major component of the root high-affinity NO3− transport system (HATS), was found to be strongly derepressed in the chl1-5 mutant (as well as in other NRT1.1 mutants). This was associated with a marked stimulation of the NO3− HATS activity in the mutant, suggesting adaptive response to a possible N limitation resulting from NRT1.1 mutation. However, derepression of NRT2.1 in NH4NO3-fed chl1-5 plants could not be attributed to lowered production of N metabolites. Rather, the results show that normal regulation of NRT2.1 expression is strongly altered in the chl1-5 mutant, where this gene is more repressible by high N provision to the plant. This indicates that NRT1.1 plays an unexpected but important role in the regulation of both NRT2.1 expression and NO3− HATS activity. Overexpression of NRT2.1 was also found in wild-type plants supplied with 1 mM NH4+ plus 0.1 mM NO3−, a situation where NRT1.1 is likely to mediate very low NO3− transport. Thus, we suggest that it is the lack of NRT1.1 activity, rather than the absence of this transporter, that derepresses NRT2.1 expression in the presence of NH4+. Two hypotheses are discussed to explain these results: (1) NRT2.1 is upregulated by a NO3− demand signaling, indirectly triggered by lack of NRT1.1-mediated uptake, which overrides feedback repression by N metabolites, and (2) NRT1.1 plays a more direct signaling role, and its transport activity generates an unknown signal required for NRT2.1 repression by N metabolites. Both mechanisms would warrant that either NRT1.1 or NRT2.1 ensure significant NO3− uptake in the presence of NH4+ in the external medium, which is crucial to prevent the detrimental effects of pure NH4+ nutrition.

INTRODUCTION

The acquisition of nitrogen by plant roots mostly relies on the activity of NO3− and NH4+ transport systems located at the plasma membrane of root cells. For both NO3− and NH4+, these transport systems are functionally separated in high-affinity transport systems (HATS), mediating N uptake in the low external concentration range (<0.5 mM), and low-affinity transport systems (LATS), predominately active in the high external concentration range (>0.5 mM). To date, the genes encoding NO3− or NH4+ transporters have been found in four different families; namely, NRT1 and NRT2 families for NO3− transporters (Forde, 2000) and AMT1 and AMT2 families for NH4+ transporters (von Wirén et al., 2000). Concerning more specifically NO3− transport in Arabidopsis thaliana, the NRT2 family includes seven genes (Orsel et al., 2002), but the NRT1 family is more difficult to define precisely. It has been restricted to four genes in previous studies (Okamoto et al., 2003), but these genes belong to the large PTR family of transporters, with 51 members. To date, nothing excludes the possibility that NO3− transporters are also encoded by other PTR genes than the four NRT1 initially investigated. Of these 58 putative transporters (seven NRT2 and 51 PTR), only three (NRT1.1, NRT1.2, and NRT2.1) have been functionally characterized in planta and shown to ensure part of the NO3− uptake from the external medium. NRT1.1 (also called CHL1) was the first NO3− transporter identified in plants (Tsay et al., 1993) and was initially believed to be a NO3−-inducible low-affinity transporter (Tsay et al., 1993; Huang et al., 1996; Touraine and Glass, 1997). In the same family as NRT1.1, NRT1.2 was also characterized as a low-affinity NO3− transporter, but with a...
constitutive expression, not dependent on the presence of NO$_3^-$ (Huang et al., 1999). Thus, both NRT1.1 and NRT1.2 were considered to belong to the NO$_3^-$ LATS in *A. thaliana* (Crawford and Glass, 1998). These two transporters do not fulfill similar functions because NRT1.1 mutants appear to be strongly defective in LATS activity only when plants are supplied with a mixed NO$_3^-$ plus NH$_4^+$ source (Touraine and Glass, 1997; Crawford and Glass, 1998), whereas antisense lines of NRT2.2 also display a markedly reduced LATS activity on NO$_3^-$ as sole N source (Huang et al., 1999). On the other hand, the NRT2.1 gene was shown to encode a major component of the NO$_3^-$–HATS (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001; Filleur et al., 2001). It appears to play a crucial role in the control of the high-affinity NO$_3^-$ uptake by the plant because its expression is regulated as the NO$_3^-$–HATS is. For example, it is inducible by NO$_3^-$ itself (Filleur and Daniel Vedele, 1999; Zhuo et al., 1999; Nazoa et al., 2003), repressed by reduced N metabolites (Lejay et al., 1999; Zhuo et al., 1999), and stimulated by photosynthates (Lejay et al., 1999, 2003). Although NRT1.1, NRT1.2, and NRT2.1 do not mediate all NO$_3^-$ transport steps occurring in the plant, these three transporters have a central importance for NO$_3^-$ acquisition in *A. thaliana*. Hence, in addition to the study of some of the other 55 putative transporters (Orsel et al., 2002; Okamoto et al., 2003), further analysis of the overall function of NRT1.1, NRT1.2, and NRT2.1 is still needed. Concerning NRT1.1, the picture becomes more and more complex with recent reports indicating that the role of this transporter is far from being limited to low-affinity NO$_3^-$ uptake in roots. First, NRT1.1 is now considered as a dual affinity transporter, belonging to both HATS and LATS (Wang et al., 1998; Liu et al., 1999). Phosphorylation of NRT1.1, triggered by limited external NO$_3^-$ availability, is responsible for the shift from low to high affinity, thus adapting the functional properties of the transporter to the resource level in the root environment (Liu and Tsay, 2003). Second, NRT1.1 is strongly expressed in nascent organs of both root and shoot (root tip, emerging lateral roots, and nascent leaves) and plays a crucial role in the early phases of development of these young organs (Guo et al., 2001). In particular, several NRT1.1 mutants display altered root architecture in some conditions, with a reduced growth of both primary and secondary roots, sometimes even in the absence of added NO$_3^-$ in the external medium. This suggests an alternative function for NRT1.1, independent of NO$_3^-$ transport (Guo et al., 2001). Finally, it has been reported recently that the mutation of NRT1.1 also leads to a lower sensitivity to drought, related to reduced stomatal opening because of impaired NO$_3^-$ transport in stomatal guard cells (Guo et al., 2003). Clearly, the view that NRT1.1 behaves only as a transporter in charge of the NO$_3^-$ uptake from the external medium is an oversimplification. This protein appears to fulfill a multiplicity of physiological functions, which begin to be unravelled more than 30 years after the identification of the first NRT1.1 mutant (Oostindier-Braaksma and Feenstra, 1973).

Most of the novel and important findings mentioned above concerning NRT1.1 rely on physiological or morphological analyses of mutants. Very few molecular data are associated with these reports, thus resulting in a lack of understanding of the gene networks functionally associated with NRT1.1 in the control of N acquisition, root and shoot development, and water use in the plant. To obtain further insight on this point, we performed large-scale transcript profiling in roots of both the *chl1-5* mutant of NRT1.1 (Tsay et al., 1993) and the related Columbia (Col) wild type. Our transcriptomic approach was based on the serial analysis of gene expression (SAGE) methodology (Velculescu et al., 1995), which involves the generation of a short specific tag (14 bp) for each mRNA in a sample. The sequencing of a large number of SAGE tags in a sample library allows a high-throughput analysis of the frequencies of these tags, which are representative of the relative amounts of the corresponding mRNAs. Thus, the comparison of the tag sequences and copy numbers obtained from two different libraries allows the identification of the genes differentially expressed between the two original samples. SAGE has been mostly employed in cancer research (Boon et al., 2002) but is now increasingly used in plants (Lorenz and Dean, 2002; Matsumura et al., 2003), especially in *A. thaliana* (Jung et al., 2003; Lee and Lee, 2003), in which the full genome sequence provides a unique tool for identifying the genes corresponding to the tags found experimentally (Fizames et al., 2004).

In addition to the finding that many genes show a markedly altered level of expression in the roots of the *chl1-5* mutant as compared with the Col wild type, we report here the observation that NRT2.1 expression is markedly deregulated in the mutant, a response that could not be explained by the known regulation affecting this gene. This suggests either the occurrence of a yet unknown signaling for control of NRT2.1 expression or a role of NRT1.1 in the regulation of other NO$_3^-$ transporters at the gene expression level.

**RESULTS**

**Comparison of SAGE Libraries from Col and chl1-5 Roots**

Because NRT1.1 has been shown to be the major NO$_3^-$ transporter involved in root NO$_3^-$ uptake under mixed N nutrition (NO$_3^-$ plus NH$_4^+$; Touraine and Glass, 1997; Crawford and Glass, 1998), the two SAGE libraries were generated from roots of Col-0 and *chl1-5* plants grown hydroponically on 1 mM NH$_4$NO$_3$. These libraries were sequenced up to 31,354 and 28,451 tags for Col-0 and *chl1-5* roots, respectively (the Col-0 library has already been reported under the name of the NH$_4$NO$_3$ library in Fizames et al., 2004). The 59,805 total tags correspond to 25,230 different sequences, among which 7583 are represented by tags found at least twice and up to 228 times. Because of rare but unavoidable sequencing and PCR errors, the use of single tags is not totally safe for gene identification. Thus, we restricted our analysis to the 7583 different tags found at least twice in the two combined libraries. The identification of the genes represented in our SAGE transcriptomes was performed by matching the list of the 7583 experimental tags to that of the virtual ones obtained by extracting the predicted SAGE tag sequence from each gene annotated in the whole *Arabidopsis* genome (Fizames et al., 2004). Among the 7583 different experimental tags, 1972 had no gene match in the database of virtual SAGE tags, 885 matched several genes and were thus not specific, and 4726 matched one single gene. The whole set of data on the 5611 tags matching one or several genes can be
The statistical analysis of the comparison between Col and chl1-5 libraries (Figure 1) resulted in the identification of 419 tags with different copy numbers in the two libraries at $P < 0.01$ (1194 tags at $P < 0.05$). Among these, 296 tags could be attributed to one single gene at $P < 0.01$ (797 tags at $P < 0.05$). The 296 differentially expressed genes ($P < 0.01$) reveal a large variety of functions affected in the chl1-5 mutant (http://genoplante-info.infobiogen.fr; see Supplemental Table 2 online) but also include genes directly related either to N nutrition or ion transport (Table 1). A few genes encoding enzymes of N metabolism have a strongly altered expression. This is the case of two isoforms of glutamate dehydrogenase (GDH1 and GDH2), which are markedly underexpressed in roots of chl1-5 compared with Col. Several transporter or channel genes also show changes in expression between the two genotypes. Of particular interest are those related to NO$_3^-$ or amino acid transport, such as NRT2.1, At1g32450 (a member of the large PTR multigene family including NRT1.1), At4g38250, and AAP6, which are all strongly overexpressed in chl1-5. On the other hand, genes encoding aquaporins (PIP2;2, PIP1;2, PIP2;1, and PIP1;1) and metal (IRT1 and NRAMP1), SO$_4^{2-}$ (SULTR1;2), or K$^+$ (SKOR) transporters/channels are repressed in the mutant.

To investigate the reliability of the SAGE data, RNA gel blot analysis was performed on eight selected genes, with the same samples as those used for the construction of the SAGE libraries. The genes investigated corresponded to N assimilation-related genes or ion channel or transporter genes, either found to be differentially expressed (Table 1) or not. These genes include the following: NRT2.1 (encoding a high-affinity NO$_3^-$ transporter; Filleur et al., 2001), NIA1 and NIA2 (encoding the two nitrate reductase [NR] isoforms present in A. thaliana; Wilkinson and Crawford, 1993), GS2 (encoding the chloroplastic isoform of Gln synthetase; Peterman and Goodman, 1991), AMT1.1 (encoding a high-affinity NH$_4^+$ transporter; Ninnemann et al., 1994), SKOR (encoding a K$^+$ channel implicated in xylem loading; Gaymard et al., 1998), and IRT1 (encoding an iron transporter; Vert et al., 2002). The absence of the NRT1.1 SAGE tag in the chl1-5 library could not be verified because this tag is not specific and also matches nine other genes, but as expected, NRT1.1 transcript was not detected in the chl1-5 mutant (data not shown). NIA1, NIA2, GS2, and AMT1.1 were not found in the list of differentially expressed genes and did not show any significant difference in their transcript accumulation between Col and chl1-5 roots (Figure 2). The slightly higher mRNA levels apparent in Figure 2 for NIA1 and NIA2 in the mutant are not representative. In four independent experiments, the chl1-5/Col ratio was measured at 0.81 ± 0.25 and 1.37 ± 0.38 for transcript accumulation of NIA1 and NIA2, respectively. At the opposite, NRT2.1 was found by SAGE to be significantly overexpressed in chl1-5 roots as compared with Col roots (Table 1). This marked difference in transcript accumulation was confirmed by RNA gel blot analysis (Figure 2), with approximately the same ratio of six between Col and chl1-5 (mean value for this ratio in five independent experiments: 6.51 ± 1.76). For both SKOR and IRT1, the SAGE data...
suggested a lower expression level in roots of chl1-5 than in those of Col, which was also verified by RNA gel blot analysis (Figure 2).

**Higher Expression of NRT2.1 Is Associated with Upregulation of the High-Affinity NO₃⁻ Uptake System in the chl1-5 Mutant**

The above data reveal complex molecular responses to the mutation affecting chl1-5. However, among all the gene responses identified, the finding that NRT2.1 was markedly overexpressed in the chl1-5 mutant as compared with Col was highly surprising. Indeed, NRT2.1 is a major component of the NO₃⁻ HATS and is strongly repressed when NH₄⁺ is present in the nutrient solution (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001) (i.e., under the conditions of our study). Thus, the totally unexpected observation of a high NRT2.1 expression level in chl1-5 in the presence of NH₄⁺ prompted us to focus further investigation on this intriguing point.

Numerous reports have shown a strong correlation between NRT2.1 transcript accumulation in the roots and the activity of the HATS for NO₃⁻ (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001; Gansel et al., 2001; Okamoto et al., 2003). Thus, we investigated if NRT2.1 overexpression in the chl1-5 mutant also had functional consequences on NO₃⁻ uptake rate by this mutant. To do so, the kinetics of ¹⁵NO₃⁻ influx as a function of external ¹⁵NO₃⁻ concentration was determined in both Col and chl1-5 plants grown for 6 weeks on 1 mM NH₄NO₃ (Figure 3A). In the low NO₃⁻ concentration range (10 to 500 μM), ¹⁵NO₃⁻ influx in chl1-5 was higher than in Col, whereas in the high concentration range (0.5 to 5 mM), the reverse was observed with ¹⁵NO₃⁻ influx in chl1-5 roots lower than in Col. The stimulation of the HATS activity in chl1-5 as compared with Col was most pronounced at 25 to 50 μM external ¹⁵NO₃⁻ (approximately fourfold increase; Figure 3B). This is the exact range of concentration where NRT2.1 was shown to participate predominantly to root NO₃⁻ uptake (Cerezo et al., 2001), indicating that the upregulation of the HATS in chl1-5 plants was most probably attributable to the overexpression of NRT2.1 as compared with Col.

**Characterization of the chl1-5 Deletion and Isolation of T-DNA Insertion Mutants for NRT1.1**

The size and location of the deletion affecting the chl1-5 mutant has not been reported, despite the extensive use of this genotype for functional characterization of the NRT1.1 transporter (Huang et al., 1996; Touraine and Glass, 1997; Wang et al., 1998; Liu et al., 1999; Guo et al., 2001). To determine whether other genes than NRT1.1 are also absent in chl1-5, we used a PCR approach to map the deletion. Successive PCR amplifications were performed on Col and chl1-5 genomic DNA using oligonucleotides designed from the sequence of chromosome 1 (GenBank accession number NC_003070). This revealed that the chl1-5 deletion corresponds to an 18.31-kb DNA fragment, beginning in the last NRT1.1 intron and ending after the At1g12090 gene (Figure 4). These data show that not only NRT1.1 but also two other genes, At1g12090 and At1g12100, are affected in the chl1-5 mutant. These two genes are highly homologous. No EST was found for the At1g12100 gene in all available databases, and its SAGE tag was absent from both Col and chl1-5 libraries. The At1g12090 gene seems to be significantly expressed because many ESTs were found, with some of

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**Table 1. Expression of Genes Related to Nitrogen Metabolism and Ion Transport in Col and chl1-5 Roots**

<table>
<thead>
<tr>
<th>Gene</th>
<th>SAGE Tag</th>
<th>Tag Copy Number in Col</th>
<th>Tag Copy Number in chl1-5</th>
<th>Probability</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>At5g07440</td>
<td>GATCCAGATGCTGGA</td>
<td>26</td>
<td>4</td>
<td>1.27E-05</td>
<td>Glu dehydrogenase (GDH2)</td>
</tr>
<tr>
<td>At5g54810</td>
<td>GATCGGTGTTGACAG</td>
<td>29</td>
<td>7</td>
<td>6.07E-05</td>
<td>Trp synthase TSB1 (β subunit)</td>
</tr>
<tr>
<td>At5g17330</td>
<td>GATCGATATAGAGA</td>
<td>10</td>
<td>0</td>
<td>4.88E-04</td>
<td>Glu dehydrogenase (GDH1)</td>
</tr>
<tr>
<td>At5g18170</td>
<td>GATCTCCGGATGGGC</td>
<td>9</td>
<td>0</td>
<td>9.76E-04</td>
<td>Glu dehydrogenase (GDH1)</td>
</tr>
<tr>
<td>At5g19550</td>
<td>GATCTACCGTTTCT</td>
<td>8</td>
<td>19</td>
<td>8.27E-03</td>
<td>Asp aminotransferase (ASP2)</td>
</tr>
<tr>
<td>At5g11520</td>
<td>GATCGATATAGAGA</td>
<td>7</td>
<td>19</td>
<td>0.015</td>
<td>Asp aminotransferase (ASP3)</td>
</tr>
<tr>
<td>At3g47340</td>
<td>GATCGATATAGAGA</td>
<td>7</td>
<td>19</td>
<td>0.015</td>
<td>Asp aminotransferase (ASP3)</td>
</tr>
<tr>
<td>At2g37170</td>
<td>GATCGATATAGAGA</td>
<td>7</td>
<td>19</td>
<td>0.015</td>
<td>Asp aminotransferase (ASP3)</td>
</tr>
<tr>
<td>At5g11520</td>
<td>GATCGATATAGAGA</td>
<td>7</td>
<td>19</td>
<td>0.015</td>
<td>Asp aminotransferase (ASP3)</td>
</tr>
</tbody>
</table>

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them corresponding to a root specific library. The SAGE tag of At1g12090 was recorded four times in the Col library, but as expected, was not found in the chl1-5 library. As a consequence, it cannot be ruled out that part of the chl1-5 phenotype is a result of the deletion of either the At1g12090 or the At1g12100 gene. To safely attribute specific aspects of this phenotype to the deletion of NRT1.1, we then searched for other NRT1.1-null mutants. Two additional mutants (chl1-10 and chl1-11) were isolated from the T-DNA tagged lines collection of INRA Versailles by a chlorate resistance screen. Both mutants belong to the chl1-5 complementation group (data not shown) and carry a T-DNA insertion in NRT1.1 (determined by DNA gel blot analysis). The chl1-10 mutant has a unique T-DNA insertion, which was located in the beginning of the last exon of NRT1.1 (between nucleotides 3130 and 3132 after the initiation codon). The chl1-11 mutant also has three other T-DNA insertions.

Regulation of NRT2.1 Expression by N Status of the Plant Is Altered in NRT1.1 Mutants

The overexpression of NRT2.1 observed in NH₄NO₃-grown chl1-5 plants was also found in three other NRT1.1 mutants (Figure 5): chl1-10, chl1-11, and the original chl1-1 mutant (formerly called B1; Doddema and Telkamp, 1979). This demonstrates that upregulation of NRT2.1 expression is specifically attributable to the NRT1.1 mutation. Moreover, the comparison of wild-type and mutant plants either grown on NO₃⁻ or NH₄NO₃ showed that NRT2.1 expression was strongly repressed by NH₄⁺ in wild-type plants, but surprisingly not in chl1-1, chl1-10, and chl1-11 plants (Figure 5).

Such a lack of downregulation of NRT2.1 under repressive conditions (NH₄⁺ supply) in NRT1.1 mutants is at odds with the current knowledge of NRT2.1 regulation. Further work was then...
devoted to investigate, in both wild-type and chl1-5 plants, the main aspects of the control of NRT2.1 expression, namely, repression by external NH₄⁺ or amino acid supply (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001), upregulation in response to N starvation (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001), repression by external NH₄⁺ or amino acid supply (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001), diurnal changes (Lejay et al., 1999).

As previously described (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001), NRT2.1 transcript level was rapidly and markedly lowered in wild-type plants by exogenous NH₄⁺ supply or high external NO₃⁻ concentration (Figure 6). This downregulation of NRT2.1 expression was absent or much less pronounced in chl1-5 plants (Figure 6). Addition of 5 mM Gln (a strong repressor of NRT2.1 expression) to the 1 mM NO₃⁻ medium resulted after 6 h in a nearly 90% decrease of NRT2.1 transcript level in Col roots, whereas this decrease was only of 50% in chl1-5 roots (data not shown). Not only the expression of NRT2.1, but also the activity of the HATS for NO₃⁻ was resistant to the repression exerted by a reduced N source in chl1-5. Root ¹⁵NO₃⁻ influx measured at 0.2 mM external concentration was lowered by ~50% after NH₄⁺ supply in Col plants but was unaffected in chl1-5 plants (Figure 7). Another clear example of altered regulation of NRT2.1 expression in chl1-5 plants relates to the response to N starvation (Figure 8). In Col roots, NRT2.1 transcript level increased 24 and 48 h after transfer of the plants to N-free solution and decreased again thereafter. This transient upregulation has been attributed to the opposite effects of two different regulatory mechanisms (Lejay et al., 1999): relief from repression by N metabolites (initially predominant), on the one hand, and shortage of induction by NO₃⁻ after several days without NO₃⁻ supply (predominant after 2 d), on the other hand. In chl1-5 roots, the initial increase in NRT2.1 expression after removal of the N source was absent, and only the decay of NRT2.1 transcript level because of deinduction was observed (Figure 8). Most importantly, this altered response to N starvation is not found for all genes regulated by N status because AMT1.1, encoding an N starvation induced NH₄⁺ transporter (Gazzarrini et al., 1999; Rawat et al., 1999), displayed a similar upregulation after transfer of the plants to N-free solution in both Col and chl1-5 roots (Figure 8). The two other main regulations identified for the control of NRT2.1 expression, namely induction by NO₃⁻ (Filleur and Daniel-Vedele, 1999) and diurnal changes (Lejay et al., 1999, 2003), are not affected in the chl1-5 mutant as compared with Col (Figure 9).
The chl1-5 Mutant Is Not Affected in NH$_4$ Uptake, Is Not N Deficient When Grown on 1 mM NH$_4$NO$_3$, but Accumulates Less NO$_3$ Than the Wild Type

We investigated two hypotheses that may explain why NRT2.1 is upregulated in NH$_4$NO$_3$-grown chl1-5 plants, namely that the chl1-5 mutant is altered in NH$_4^+$ uptake or that NH$_4$NO$_3$-grown chl1-5 plants suffer from N deficiency as compared with Col. Assay of $^{15}$NH$_4^+$ influx at various concentrations in both Col and chl1-5 plants indicated that chl1-5 is not deficient for both high- and low-affinity NH$_4^+$ uptake systems (Figure 10). Indeed, root $^{15}$NH$_4^+$ influx tended to be slightly higher (5 to 10%) in chl1-5 than in Col, although the difference was never statistically significant. Total N, NO$_3^-$, and amino acid contents of roots and shoots were determined to compare the N status of Col and chl1-5 plants. The hypothesis that chl1-5 plants grown on 1 mM NH$_4$NO$_3$ could be N deficient is contradicted by the fact that total N contents of both roots and shoots were never found to be different between the two genotypes in five independent experiments (data not shown). Furthermore, Gln accumulation in roots of chl1-5 was almost twice that measured in Col (Figure 11). The accumulation of the other amino acids in roots or of all amino acids in shoots did not differ between the two genotypes. On the other hand, in relation with a lower NO$_3^-$ influx at 1 mM NH$_4$NO$_3$ (Figure 3), chl1-5 plants accumulated less NO$_3^-$ than Col plants in both roots and shoots (Figure 12). This reduced accumulation of NO$_3^-$ in the mutant was shown to occur also when NO$_3^-$ was the sole N source provided to the plants (Figure 12).

Overexpression of NRT2.1 in NRT1.1 Mutant Is Dependent on the External NO$_3^-$/NH$_4^+$ Ratio

The above observations may suggest that reduced levels of NO$_3^-$ in tissues of NRT1.1 mutants can be the cause for overexpression of NRT2.1. To investigate this hypothesis, both chl1-10 and Wassilewskija (Ws) plants were grown for 5 weeks on 1 mM NH$_4$NO$_3$, then shifted for 6 d to media containing 1 mM NH$_4^+$, but with 0.1, 1, or 10 mM NO$_3^-$. This was expected to alter NO$_3^-$ accumulation in both genotypes, without resulting in N deficiency (because of the presence of 1 mM NH$_4^+$ in the medium). The modification for 6 d of the external NO$_3^-$ concentration of the medium did not affect NO$_3^-$ levels in the shoots, but resulted in changes in NO$_3^-$ accumulation in the roots (Figure 13). This had no effect on NRT2.1 expression in chl1-10 roots, which remained high in all three conditions. Surprisingly, although low at 1 or 10 mM NO$_3^-$, NRT2.1 transcript level in Ws roots increased dramatically at 0.1 mM NO$_3^-$ despite the presence of 1 mM NH$_4^+$ in the medium (Figure 13). In this last situation, NRT2.1 was not overexpressed any more in chl1-10.
compared with the wild type. These data demonstrate that upregulation of NRT2.1 in NRT1.1 mutant depends on the external NH₄⁺/NO₃⁻ ratio and that high level of NRT2.1 expression in the presence of NH₄⁺ can occur also in the wild type, in situations of excess NH₄⁺ compared with NO₃⁻.

DISCUSSION

Our SAGE data indicate that the expression of a high number of genes is modified in the roots of the chl1-5 mutant as compared with Col. However, despite the extensive use of this mutant to investigate the various aspect of NRT1.1 function (Huang et al., 1996; Touraine and Glass, 1997; Wang et al., 1998; Liu et al., 1999; Guo et al., 2001, 2003), caution is needed in associating all these changes in gene expression to the mutation of NRT1.1. First, transcript profiling was performed in only one series of experiments because of the cost of SAGE. Second, two other genes (At1g12090 and At1g12100) were also found to be deleted in chl1-5. Of these two genes, only At1g12090 seems to be expressed in the roots. Its function is unclear because it encodes a protein sharing similarities with pEARLI, an extensin, a protease inhibitor, and AII1, this latter being possibly involved in the auxin-mediated initiation of lateral roots (Neuteboom et al., 1999). Further work is thus needed, using other chl1 mutants (Figure 5), to determine the individual genes whose expression is specifically altered by NRT1.1 mutation. Nevertheless, some of the molecular responses observed in chl1-5 are correlated with physiological modifications reported in the chl1-1 mutant, suggesting that they result from NRT1.1 deletion. For instance, the fact that various metal/K⁺/SO₄²⁻ transporter/channel genes were found to be downregulated in chl1-5 (Table 1) is consistent with the observation that the chl1-1 mutant is not only altered in NO₃⁻ transport but also in the uptake of several other ions (Scholten and Feenstra, 1986).

Concerning NO₃⁻ uptake, our observation of a lowered LATS activity in the chl1-5 mutant compared with Col when the plants are supplied with NH₄NO₃ as an N source (Figure 3) is in very good agreement with previous reports on this mutant (Huang et al., 1996; Touraine and Glass, 1997). However, we found this alteration of the LATS compensated for by a much higher HATS activity in chl1-5 than in Col (Figure 3). These results contrast with previous observations that chl1-5 and other chl1 mutants are defective in both HATS and LATS for NO₃⁻ (Wang et al., 1998; Liu et al., 1999). The reasons for this discrepancy between our results and those of Wang et al. (1998) and Liu et al. (1999) are unclear. However, impaired NO₃⁻ HATS activity in NRT1.1 mutants has always been reported in much younger plants (5 to 12 d old) than those used in our study (6 weeks old). Also, many other specific conditions (in particular carbon sources and NO₃⁻ and NH₄⁺ concentrations) were different between our experiments and those of Wang et al. (1998) and Liu et al. (1999) and may explain these contrasting conclusions.

Although a putative NO₃⁻ transporter gene (At1g32450), uncharacterized to date, is also upregulated in chl1-5, we hypothesize that the stimulation of the NO₃⁻ HATS in the mutant is because of the overexpression of NRT2.1. This gene is believed to play a key role in the N acquisition by the roots. It encodes a major component of the HATS for NO₃⁻ in A. thaliana, and its expression is strongly regulated according to N/C status of the plant (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001; Filleur et al., 2001; Gansel et al., 2001; Lejay et al., 2003). At least three major mechanisms have been proposed to explain the changes in NRT2.1 transcript accumulation in the root: induction by NO₃⁻ (Filleur and Daniel-Vedele, 1999; Zhuo et al., 1999; Nazoa et al., 2003), induction by

Figure 7. Effect of the Presence of NH₄⁺ in the Nutrient Solution on Root ¹⁵NO₃⁻ Influx in Col and chl1-5 Plants.

The plants were grown hydroponically for 5 weeks on complete nutrient solution containing 1 mM NH₄NO₃ as N source and were either kept on this solution or transferred on another one with 1 mM NO₃⁻ as N source 1 week before the harvest. Root ¹⁵NO₃⁻ influx was assayed by 5 min labeling at 0.2 mM external ¹⁵NO₃⁻ concentration. Results are the means of eight replicates ± SE.

The plants were grown hydroponically for 6 weeks on complete nutrient solution containing 1 mM NH₄NO₃ as N source before the transfer to an N-deprived medium.

Figure 8. Gel Blot Analysis of NRT2.1 and AMT1.1 Transcript Accumulation in the Roots of Col and chl1-5 Plants in Response to N Starvation.

The plants were grown hydroponically for 6 weeks on complete nutrient solution containing 1 mM NH₄NO₃ as N source before the transfer to an N-deprived medium.
supply with mixed N sources such as NH₄NO₃. However, is that these mutants suffer from N deficiency even when ing the increase in bar) of the night (09 h, closed bar) or at the end of the light period (17 h, open NRT2.1 bar)

Figure 9. Gel Blot Analysis of NRT2.1 Transcript Accumulation in the Roots of Col and chl1-5 Plants in Response to the Induction by NO₃⁻ and Day/Night Cycle.

The plants were grown hydroponically for 5 (A) or 6 (B) weeks on complete nutrient solution containing 1 mM NH₄NO₃ as N source. The plants used for investigating NRT2.1 induction by NO₃⁻ (A) were transferred for 1 week on an N-deprived medium before the addition of 1 mM NO₃⁻ in the nutrient solution for 6 h. The plants used for investigating the diurnal changes in NRT2.1 expression were harvested either at the end of the night (09 h, closed bar) or at the end of the light period (17 h, open bar) (B). light and sugars (Lejay et al., 1999, 2003), and feedback repression by N metabolites (Lejay et al., 1999; Zhuo et al., 1999; Gansel et al., 2001; Cerezo et al., 2001). Downregulation of NRT2.1 by N metabolites is postulated to involve products of NO₃⁻ assimilation, and more particularly NH₄⁺ and Gln, as negative effectors of the expression of the gene (Lejay et al., 1999; Zhuo et al., 1999; Nazoa et al., 2003). This is expected to ensure the tuning of the HATS activity to the N demand of the whole plant. Accordingly, the deletion of NRT2.1 (together with part of NRT2.2) in the atrnt2 mutant results in both a lowered activity of the HATS and in the loss of the regulation of this uptake system by the N status of the plant (Cerezo et al., 2001; Filleur et al., 2001).

We show here that the regulation of NRT2.1 transcript accumulation by N status of the plant is altered in the chl1-5 mutant (Figures 2, 6, and 8), as well as in other NRT1.1 mutants (Figures 5 and 13). The first hypothesis that can be considered for explaining the increase in NRT2.1 transcript level in the NRT1.1 mutants is that these mutants suffer from N deficiency even when supplied with mixed N sources such as NH₄NO₃. However, several lines of evidence do not support this hypothesis. First, chl1-5 plants are not deficient for NH₄⁺ uptake (Figure 10), and neither the total N influx in roots (NO₃⁻ plus NH₄⁺; Figures 3 and 10), nor the total N content of both roots and shoots (data not shown) markedly differ between chl1-5 and Col plants when grown on 1 mM NH₄NO₃. Second, the observation that the accumulation of free Gln in roots is higher in chl1-5 than in Col (Figure 11) also clearly indicates that chl1-5 plants are N sufficient. Third, the activity of the NH₄⁺ HATS in chl1-5 plants is not derepressed as expected if these plants were N deficient. Root ¹⁵NH₄⁺ influx, measured at 0.2 mM external ¹⁵NH₄⁺, is low (~50 μmol h⁻¹ g⁻¹ root dry weight) and similar in both chl1-5 and Col (Figure 10). In N-limited plants, root ¹⁵NH₄⁺ influx is generally recorded at much higher values (up to 500 μmol h⁻¹ g⁻¹ root dry weight; Gazzarrini et al., 1999; Rawat et al., 1999). Accordingly, the expression of the N starvation-inducible NH₄⁺ transporter gene AMT1.1 (Gazzarrini et al., 1999; Rawat et al., 1999) is low in the chl1-5 mutant on 1 mM NH₄NO₃ and not different than in Col (Figure 8). Thus, the overexpression of NRT2.1 in chl1-5 cannot be explained by general N deficiency. This suggests that normal regulation of NRT2.1 expression by N status of the plant is markedly altered in chl1-5 plants. Indeed, submitting the plants to much more repressive conditions (transfer to 10 mM NO₃⁻, 10 mM NH₄NO₃, or 5 mM Gln) did not result in a strong repression of NRT2.1 expression in chl1-5, whereas these treatments almost completely abolished it in Col (Figure 6). Reciprocally, transfer of the plants to N-deprived solution failed to derepress this gene in chl1-5, whereas the usual transient upregulation was observed in Col (Figure 8). Collectively, these data show that root NRT2.1 expression in NRT1.1 mutants is blocked in a derepressed state and, thus, that NRT1.1 is required for correct regulation of NRT2.1 by N status of the plant. The same conclusion may also be drawn for the activity of the HATS for NO₃⁻, which appears to be unusually insensitive in chl1-5 plants to the repression exerted by the presence of NH₄⁺ in the nutrient solution (Figures 3 and 7). This role of NRT1.1 in controlling both the regulation of the NO₃⁻ HATS activity and NRT2.1 expression seems to be quite specific. First, regulation of AMT1.1 is not affected in chl1-5 plants (Figure 8). Second, the two other known regulations of NRT2.1 expression, namely induction by NO₃⁻...
and regulation by day/night cycle and photosynthates (Lejay et al., 1999, 2003), are also not altered by NRT1.1 mutation (Figure 9).

The mechanism responsible for the role of NRT1.1 in the regulation of NRT2.1 expression is unclear. However, the observation that low NO3\(^-\) availability in presence of 1 mM NH4\(^+\) upregulates NRT2.1 expression in the wild type (Figure 13B) indicates that mutation of NRT1.1 is not strictly required for preventing downregulation of NRT2.1 by N metabolites. NRT1.1 is believed to be the main transporter responsible for NO3\(^-\) uptake from mixed N sources (Huang et al., 1996; Touraine and Glass, 1997; Crawford and Glass, 1998). Thus, the two situations that lead to overexpression of NRT2.1 in the presence of 1 mM NH4\(^+\) (e.g., mutation of NRT1.1 or decrease in external NO3\(^-\) availability) are both expected to result in a reduced NO3\(^-\) uptake rate. Accordingly, these situations are associated with lowered NO3\(^-\) accumulation in tissues (Figures 12 and 13). This strongly suggests that low NO3\(^-\) uptake rate from mixed NH4NO3 nutrient solution is the actual cause for the upregulation of NRT2.1. Because the presence of 1 mM NH4\(^+\) in the medium prevents N deficiency in both wild-type and mutant (see above), this would imply that NRT2.1 expression is specifically repressed by high NO3\(^-\) uptake rate, independently of the products of NO3\(^-\) assimilation. Hence, one hypothesis would be that two distinct signaling pathways have to be considered for mediating repression of NRT2.1 by N status of the plant: (1) the well-known feedback repression by N metabolites, related to a specific reduced N status and mediating the reduced N demand for growth of the plant, and (2) a yet unknown feedback repression by NO3\(^-\) uptake or NO3\(^-\) content of the tissues, related to the NO3\(^-\) status of the plant and mediating a specific NO3\(^-\) demand. The NO3\(^-\) demand signaling would override feedback repression by N metabolites to stimulate NRT2.1 expression in situations where NO3\(^-\) uptake rate is low in presence of NH4\(^+\) (e.g., in the wild type supplied with nutrient solution at high NH4\(^+\)/NO3\(^-\) ratio or in chl1 mutants).

How might NRT2.1 be regulated both by NO3\(^-\) induction (Filleur and Daniel-Vedele, 1999; Zhuo et al., 1999) and by repression by high NO3\(^-\) status remains an unanswered question. Opposite direct signaling roles of NO3\(^-\) (induction/repression) in the regulation of its own uptake systems have already been proposed from physiological studies (Siddiqi et al., 1989; King et al., 1993). However, experiments with NR-deficient mutants or using tungstate, a potent NR inhibitor, provided evidence that on NO3\(^-\) as sole N source, NRT2.1 is predominantly repressed by products of NO3\(^-\) assimilation and not by NO3\(^-\) itself (Krapp et al., 1998; Lejay et al., 1999; Zhuo et al., 1999). On the other hand, it is now well documented that NO3\(^-\) acts both as a positive and a negative signal for the development of the root system, independently of the reduced N status of the plant (Scheible et al., 1997; Stitt, 1999; Zhang et al., 1999). The model proposed for regulation of lateral root development by NO3\(^-\) (Zhang et al., 1999; Zhang and Forde, 2000) is of major interest in our context. It postulates repression of lateral root

![Figure 11](image-url)
Role of NRT1.1 in Regulating NRT2.1

Figure 12. Accumulation of NO\textsubscript{3} in the Roots and Shoot of Col and chl1-5 Plants.

The plants were grown hydroponically for 5 weeks on complete nutrient solution containing 1 mM NH\textsubscript{4}NO\textsubscript{3} as N source and were either kept on this solution or transferred on another one with 1 mM NO\textsubscript{3} as N source 1 week before the harvest. Results are the mean of 12 replicates ± SE.

The emergence (Zhang and Forde, 2000), showing that NO\textsubscript{3} contradicts with the conclusion that was also observed in this situation (Figure 12). However, this is in contradiction with the conclusion that NRT2.1 is predominantly repressed by downstream N metabolites when plants are supplied with NO\textsubscript{3} as the sole N source (see above). Furthermore, lack of derepression of NRT2.1 by N starvation in the chl1-5 mutant (Figure 8) does not fit well with the hypothesis that mutation of NRT1.1 alters NRT2.1 expression through specific regulation by NO\textsubscript{3} status only. In that case, N starvation should still alleviate feedback repression by reduced N status, mediated by N metabolites, and would result in a further stimulation of NRT2.1 expression, which is not observed. Alternatively, the lower NO\textsubscript{3} content in roots of chl1 mutants (Figures 12 and 13A) may result in a faster loss of NRT2.1 induction by NO\textsubscript{3}−, which may then prevent any increase in NRT2.1 transcript level upon transfer of the plants to N-free solution.

Without ruling out the NO\textsubscript{3}− demand signaling as described above, these considerations suggest that other hypotheses could also be envisaged to account for all effects of NRT1.1 mutation on NRT2.1 expression. In particular, the hypothesis that NRT1.1 plays a direct regulatory role and that its activity generates a signal required for feedback repression of NRT2.1 by N metabolites also has the potential to explain our results. According to this, mutation of NRT1.1 in the chl1 mutants would then prevent feedback repression of NRT2.1 by N metabolites in any situation, thus explaining all NRT2.1 expression data obtained in these mutants, even when NO\textsubscript{3}− is the sole N source (Figure 6). Furthermore, if not repressed in N replete plants, NRT2.1 obviously cannot be derepressed by N starvation, which then provides a hypothesis for the lack of increase in NRT2.1 transcript level after transfer of chl1-5 plants to N-free medium (Figure 8). Finally, direct repression of NRT2.1 expression by NRT1.1 activity may also account for the fact that NRT2.1 transcript level is high in wild-type plants supplied with 0.1 mM NO\textsubscript{3}− plus 1 mM NH\textsubscript{4}+, whereas it is low when NO\textsubscript{3}− concentration is increased up to 1 or 10 mM, without modifying that of NH\textsubscript{4}+ (Figure 13B). Indeed, NRT1.1 is dephosphorylated and functions as a low-affinity transporter in plants under high N provision, whereas it is phosphorylated and has a high affinity for NO\textsubscript{3}− in N-limited plants (Liu and Tsay, 2003). Although the phosphorylation status of NRT1.1 has not been investigated under our specific experimental conditions, we can hypothesize that the supply of 1 mM NH\textsubscript{4}+ was sufficient to warrant high N provision to the plants and that, accordingly, NRT1.1 was predominantly in the low-affinity form. If this hypothesis is valid, 0.1 mM NO\textsubscript{3}− in the external medium would have been too low to allow any significant transport activity of NRT1.1, thus preventing generation of the repressive signal for NRT2.1 expression. By contrast, at 1 or 10 mM external NO\textsubscript{3}−, the low-affinity form of NRT1.1 is significantly or fully active, which then leads to repression of NRT2.1. Additional evidence further suggests a signaling role of NRT1.1 in NRT2.1 regulation. Indeed, one puzzling aspect of our results is that NRT2.1 expression was dramatically stimulated in the wild type by the increase in external NH\textsubscript{4}/NO\textsubscript{3}− ratio, with only a small decrease in NO\textsubscript{3}− concentration in roots, and almost no change of this concentration in shoot (Figure 13). Although we cannot exclude a stringent control of NRT2.1 expression by the NO\textsubscript{3}− demand signaling below a threshold level of NO\textsubscript{3}− accumulation, this may indicate that it is the sensing of the external NO\textsubscript{3}− concentration or of the NO\textsubscript{3}− influx, rather than that of NO\textsubscript{3}− content of the tissues, which is important
for the regulation of NRT2.1 in the presence of NH$_4^+$ in the medium. Because this regulation is strongly altered in chl1 mutants, NRT1.1 is thus a good candidate for a NO$_3^-$ flux and/or a NO$_3^-$ concentration sensor. It is now firmly established in both yeast and plants that specific membrane proteins have a dual transport and signaling role (Lalonde et al., 1999). In yeast, various aspects of N signaling are related to the sensing activity of such proteins. For instance, the permease homolog SSY1 is involved in the regulation of the expression of amino acid and peptide transporters by the external N source (Didion et al., 1998; Iraqui et al., 1999), and the high-affinity NH$_4^+$ transporter MEP2 triggers pseudohyphal growth in conditions of N limitation (Lorenz and Heitman, 1998). Most interestingly, both NRT1.1 and NRT2.1 have been recently proposed to trigger morphological changes in the root system of A. thaliana, which are not explained by the purely nutritional role of these proteins (Guo et al., 2001; Rao et al., 2003). Although our data provide additional circumstantial evidence for a sensing function of NRT1.1, much more direct clues are needed for a definite conclusion on this point. Thus, it is not possible yet to exclude any of the two main hypotheses proposed for explaining the surprising role of NRT1.1 in NRT2.1 regulation. Further analysis of the phenotype of chl1 mutants is being performed to answer this question.

Whatever mechanism is responsible for the upregulation of NRT2.1 by low NO$_3^-$ uptake in the presence of ample NH$_4^+$ supply (NO$_3^-$ demand signaling or lack of NRT1.1-mediated repression), these hypotheses have a strong physiological significance because the ability of the plant to take up NO$_3^-$ in presence of NH$_4^+$ in the external medium prevents the detrimental effects of pure NH$_4^+$ nutrition (Salsac et al., 1987; Volk et al., 1992; von Wirén et al., 2000). Indeed, most herbaceous species achieve highest growth rates on a mixed NH$_4$NO$_3$ N source, whereas supply of NH$_4^+$ alone generally results in poor growth and various metabolic disorders (Mehrer and Mohr, 1989; Walch-Liu et al., 2000). Despite its importance, no regulatory mechanism was known to specifically promote NO$_3^-$ uptake from mixed N source. We suggest here that regulation of NRT2.1 by either NO$_3^-$ demand or NRT1.1-dependent signaling corresponds to such a mechanism. Thus, in addition to being involved in satisfying the plant’s N requirements for growth when NO$_3^-$ is the only N source (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999; Cerezo et al., 2001; Gansel et al., 2001), NRT2.1 would also play a key role under mixed nutrition in contributing to maintain a healthy balance between NO$_3^-$ and NH$_4^+$ uptake.

Figure 13. Accumulation of NO$_3^-$ in the Roots and Shoot of Ws and chl1-10 Plants and Gel Blot Analysis of NRT2.1 Transcript Accumulation in the Roots of These Plants as a Function of the External NH$_4^+$/NO$_3^-$ Ratio.

(A) Accumulation of NO$_3^-$ in the roots and shoot of Ws and chl1-10 plants.

(B) Gel blot analysis of NRT2.1 transcript accumulation in the roots of these plants.

The plants were grown hydroponically for 5 weeks on complete nutrient solution containing 1 mM NH$_4$NO$_3$ as N source and transferred for 6 d on media containing 1 mM NH$_4$Cl plus either 0.1, 1, or 10 mM KNO$_3$. NO$_3^-$ accumulation results are the means of 12 replicates ± SE.
METHODS

Plant Material and Treatments

All genotypes (Col-0, Ws, Landsberg erecta, chl1-1, chl1-5, chl1-10, and chl1-11) used in this study were grown hydroponically using the experimental setup described previously (Lejay et al., 1999). Briefly, seeds were sown directly on the surface of wet sand in modified 1.5-mL microcentrifuge tubes, with the bottom replaced by a metal screen. The tubes supporting the seeds were placed on polystyrene floating rafts, on the surface of a 10-liter tank filled with tap water. The culture was then performed in a controlled growth chamber with 8 hr/16-h day/night cycle at 24°C/20°C. Light intensity during the light period was about 250 μmol m−2 s−1. The basal nutrient solutions supplied to the plants are those described by Gansel et al. (2001) and contained either 1 mM NO3− or 1 mM NH4NO3 as N source. For specific experiments involving response to N deprivation or to various N sources, the NH4NO3 solution was used, in which NH4NO3 was either omitted or replaced by other N sources indicated in the figures. One week after sowing, the tap water was replaced by diluted (1/10) basal medium. After one additional week, the plants were supplied with undiluted nutrient solution until the age of 5 weeks when experiments generally began. The nutrient solution was replaced every week during this period. During the experiments, nutrient solutions were renewed daily and adjusted at pH 5.8. Except when the effect of day/night cycle was investigated, all harvests and measurements were done 5 h into the light period.

Characterization of the Genomic Deletion in chl1-5

Three consecutive steps of PCR were performed on Col-0 or chl1-5 genomic DNA to map the deletion, using 15 primer pairs designed from T28K15 and F12F1 BAC sequences. At the end of this process, the right and left borders of the deletion were mapped with 1-kb accuracy each. Then, two oligonucleotides, delF (5′-9TATCCTTCACACACATgCATg-3′) and delR (5′-9AATgCAgTCATgCAgTTTATgCC-3′), with their related genomic sequences separated by 19.4 kb on chromosome 1, were used to amplify the corresponding region in the genomic DNA, but a 1.1-kb fragment was amplified with Pfu polymerase (Promega, Madison, WI). The 1.1-kb fragment obtained was cloned using pCR blunt plasmid (Invitrogen, Carlsbad, CA) and DH5α competent cells. DNA was then sequenced by Genome express (Grenoble, France).

Isolation of chl1-10 and chl1-11 Chlorate-Resistant Mutants

The chl1-10 and chl1-11 chlorate resistant mutants were isolated from the INRA collection of T-DNA insertion lines of A. thaliana (ecotype Ws, Versailles, France). The screen was done on 6-d-old seedlings germinated on soil. Chlorate treatment was performed by subirrigating plants every 2 d during 15 d with a nutrient solution containing 2 mM KClO3 and 2 mM NH4NO3 as sole nitrogen source. DNA gel blots performed using probes for right and left borders of the T-DNA suggested that chl1-10 and chl1-11 carry one and four insertions, respectively. An allelism test performed with the chl1-5 mutant indicated that two mutants, named chl1-10 and chl1-11, belong to the chl1 complementation group. DNA gel blots performed using a specific probe for NRT1.1 showed that the structure of this gene was disturbed in both chl1-10 and chl1-11 mutants. The disruption of the NRT1.1 gene in the chl1-10 mutant was characterized by PCR amplification and sequencing of the T-DNA flanking sequences using primers specific to both left and right T-DNA borders (5′-9GTCGAGTTTT-3′ and 5′-9CCACAGGCCC-GTCGAGTTTT-3′, respectively) and NRT1.1 flanking genomic sequence (5′-9GACGTAGAAGACTGCCATGTGGAAGC3′ and 5′-9TTTGTGATCGCATGTGGAAGC3′, respectively).

SAGE Protocol

The root samples harvested from Col-0 and chl1-5 NH4NO3-grown plants were stored at −80°C before total RNA extraction. The SAGE libraries were obtained from 100 μg of total RNA, using SAGE protocol described by Vignal et al. (1999), with the difference that the anchoring enzyme was MboI (New England Biolabs, Beverly, MA) instead of Sau3AI. Poly(A) RNAs were isolated from 100 μg of total RNA using Dynabeads mRNA direct kit. Hybridizations were performed on cDNA linked covalently to magnetic beads. cDNA were synthesized directly on the beads, and all enzymatic steps needed before digestion by BamFI were performed on cDNA linked to the beads. All oligonucleotides, with sequences and modifications identical to Vignal et al. (1999), were from Eurobio (Les Ulis, France).

Final concemters were cloned in pBluescript II KS− from Stratagene (La Jolla, CA), digested by EcoRV, dephosphorylated, and purified on agarose gel. Ligation was performed overnight at 16°C and ElectromAX DH10B Escherichia coli cells (Life Technologies, Cleveland, OH) were then used for transformation by electroporation. Sequencing was performed as described previously (Fizames et al., 2004) in the Department Genome et Développement des Plantes (University of Perpignan, France) and Genome Express (Grenoble, France). Altogether, 1176 runs of sequencing were obtained before obtaining 28,952 tags of the chl1-5 root SAGE library and 1335 runs for the 31,354 tags of the Col-0 root SAGE library.

SAGE Data Analysis

The whole procedure developed to obtain transcript profiles from concatenated sequences is described in Fizames et al. (2004). Briefly, experimental tag sequences were extracted from the concatemer sequences using DIGITAG software (Piquemal et al., 2002). Tag to gene assignment was then performed by matching the sequences of the experimental tags with those of virtual tags extracted from 26,620 annotated genes of the A. thaliana genome (ftp://ftpmpis.gsf.de/cress/abricanda/abrici_genomic_sequenceplus500_v111102.gz), taking into account the coding sequence plus 400 bp 5′ and 3′ extensions. We have previously determined (Fizames et al., 2004) that this procedure allows the identification of the transcripts corresponding to ~60% of the tags found experimentally, with a specificity of 85% (only 15% of the experimental tags match more than one gene), and a reliability of 88% (only 12% of the experimental tags are assigned to wrong genes). The statistical analysis of SAGE data for identification of genes differentially expressed between roots of Col-0 and chl1-5 plants was performed as described in Piquemal et al. (2002).

RNA Extraction and RNA Gel Blot Analysis

Total RNA extraction was performed on roots as described previously (Lobreaux et al., 1992). For RNA gel blot analysis, total RNA (15 μg) was separated by electrophoresis on 3−(N-morpholino)-propanesulfonic acid formaldehyde agarose gel and blotted on nylon membrane (Hybond N+; Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were prehybridized for 2 h at 65°C in Church buffer (0.5 M NaHPO4, 1% BSA, and 7% SDS, pH 7.2, with H3PO4). Hybridizations were performed overnight at 65°C after addition of a randomly primed 32P-labeled cDNA probe in the prehybridization buffer. Membranes were washed twice at room temperature for 2 min and twice at 65°C for 15 min with 0.5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS. DNA probes used in this study correspond to full-length cDNAs. A 25S rRNA probe was used as reference for quantification achieved using a PhosphorImager (Storm; Molecular Dynamics, Sunnyvale, CA).

15NO3− and 15NH4+ Uptake

Root 15NO3− or 15NH4+ influxes were assayed as described by Delhon et al. (1995) for NO3− and by Gazzarrini et al. (1989) for NH4+. Briefly, the
plants were sequentially transferred to 0.1 mM CaSO₄ for 1 min, to complete nutrient solution, pH 5.8, containing either ¹⁵NO₃⁻ or ¹⁵NH₄⁺ (99% atom excess ¹⁵N) for 5 min at the concentrations indicated in the figures, and finally to 0.1 mM CaSO₄ for 1 min. Roots were then separated from shoots, and the organs dried at 70°C for 48 h. After determination of their dry weight, the samples were analyzed for total N and atom percentage ¹⁵N using a continuous-flow isotope ratio mass spectrometer coupled with a C/²⁵N elemental analyzer (model ANCA-MS; PDZ Europa, Crewe, UK) as described in Clarkson et al. (1996). Each influx value is the mean of 8 to 12 replicates.

Amino Acid and NO₃⁻ Analysis

After harvest of the plants, roots and shoot were separated and stored either at -20°C for amino acid analysis or dried for 48 h at 70°C for NO₃⁻ analysis. Free amino acids were extracted from 0.5 g of frozen tissue by grinding in 2 mL of EtOH. The extracts were then left for 1 h at 4°C. Supernatants were sequentially transferred to 0.1 mM CaSO₄ for 1 min, to 2446 The Plant Cell

SP4270 integrator piloted by SP-LABNET software [Spectra Physics, Mountain View, CA], fluorimeter 821-FP [Jasco, Easton, MD], and for amino acid quantification by HPLC (gradient pump SP8800 [Spectra Physics]).

The permease homologue Sy1p controls the expression of amino acid and peptide transporter genes in Saccharomyces cerevisiae. Mol. Microbiol. 27, 643–650.


Role of NRT1.1 in Regulating NRT2.1

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Transcript Profiling in the chl1-5 Mutant of Arabidopsis Reveals a Role of the Nitrate Transporter NRT1.1 in the Regulation of Another Nitrate Transporter, NRT2.1

Stéphane Muños, Céline Cazettes, Cécile Fizames, Frédéric Gaymard, Pascal Tillard, Marc Lepetit, Laurence Lejay and Alain Gojon

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