Gene Expression of the NO_3^- Transporter NRT1.1 and the Nitrate Reductase NIA1 Is Repressed in Arabidopsis Roots by NO_2^-, the Product of NO_3^- Reduction

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NRT1.1 and *NIA1* genes, which encode a nitrate (NO_3^-) transporter and the minor isoform of NO_3^- reductase (NR), respectively, are overexpressed in roots of NR-deficient mutants of Arabidopsis grown on nutrient solution containing NO_3^- and reduced N. The overexpression is found only in mutants with reduced NIA2 activity, and disruption of the *NIA1* gene alone has no effect on *NRT1.1* expression. Because the up-regulation of *NRT1.1* and *NIA1* is observed in N-sufficient NR mutant plants, it cannot be related to a release of the general feedback repression exerted by the N status of the plant. Our data do not support the hypothesis of overinduction of these genes by an increased concentration of NO_3^- in tissues. Furthermore, although a control by external pH might contribute to the regulation of *NRT1.1*, changes in external pH due to lack of NR activity cannot alone explain the up-regulation of both genes. The stimulation of *NRT1.1* and *NIA1* in NR mutants in these conditions suggests that NR activity is able to repress directly the expression of both genes independently of the availability of reduced N metabolites in wild-type plants. Accordingly, nitrite (NO_2^-) strongly represses *NRT1.1* and *NIA1* transcript accumulation in the roots. This effect is rapid, specific, and reversible. Furthermore, transport studies on plants exposed to NO_2^- show that down-regulation of the *NRT1.1* gene is associated with a decrease in NO_3^- influx. These results indicate that feedback regulation of genes of NO_3^- assimilation relies not only on the repression exerted by reduced N metabolites, such as NH_4^+ or amino acids, but may also involve the action of NO_2^- as a regulatory signal.

Nitrate (NO_3^-), which is the most important source of mineral nitrogen for most crop species, is acquired by higher plants from the soil through the combined activities of high- and low-affinity uptake systems. Subsequently, NO_3^- may be accumulated or reduced in root cells, transported via the xylem vessels to be assimilated or stored in the shoot, or released outside of the root via efflux systems. The reduction of NO_3^- involves two enzymatic steps, reduction of NO_3^- to nitrite (NO_2^-) by NO_3^- reductase (NR), and reduction of NO_2^- to NH_4^+ by NO_2^- reductase (NiR).

Several structural genes encoding transporters of the uptake systems and assimilatory enzymes have been identified in Arabidopsis. To date, the genes encoding NO_3^- transporters belong to two different families (*NRT1* and *NRT2*). Each family is represented by multiple genes that are differentially regulated and may encode transporters with different regulatory or kinetic properties (Forde, 2000; Orsel et al., 2002; Glass et al., 2002). In Arabidopsis, NRT2.1, the major member of the NRT2 family (seven members) has been characterized as a component of the high-affinity and low-capacity NO_3^- transport system (Filleur and Daniel-Vedele, 1999; Filleur et al., 2001; Orsel et al., 2002). The *NRT1* genes belong to a large family (52 genes)—the so-called peptide transporter (PTR) family—that also contains two transporters able to mediate oligopeptide and His uptake in yeast (Frommer et al., 1994; Steiner et al., 1994). Two members of the NRT1 family (NRT1.1 and NRT1.2) were shown to be involved in the low-affinity and high-capacity transport of NO_3^- (Tsay et al., 1993; Huang et al., 1996; Touraine and Glass, 1997). Subsequent studies revealed that NRT1.1 is also involved in high-affinity NO_3^- uptake, thus behaving as a dual affinity transporter (Wang et al., 1998; Liu et al., 1999). The gene is expressed in nascent organs, preferentially in root tips, and is up-regulated in response to exogenous addition of auxin (Tsay et al., 1993; Huang et al., 1996; Guo et al., 2001, 2002). In Arabidopsis, two genes, *NIA1* and *NIA2*, encode the two isoforms of the NR apoprotein with divergent sequences but similar structure (Wilkinson and Crawford, 1993), whereas a single gene encodes the NiR apoprotein (Tanaka et al., 1994). The NIA isoforms do not contribute equally to the NR activity (NRA) of the plant. The G5 mutant, which lacks the *NIA2* gene, retains only 10% of the wild-type (WT) NRA in the leaves (Wilkinson and Crawford, 1991). This activity is reduced to 0.5% in the G′4-3 double mutant, which carries an additional point mutation in *NIA1* (Wilkinson and Crawford, 1993). Despite a common catalytic function, the expression patterns of *NIA1* and *NIA2* are not similar in response to NO_3^- induction, light,

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and cytokinins (Cheng et al., 1992, 1991; Lin and Cheng, 1997; Yu et al., 1998).

The recent advances in the understanding of the regulation of NO_3^- uptake and assimilation (for review, see Crawford and Glass, 1998; Stitt, 1999; Forde, 2002) have shown that these functions are highly integrated at the whole-plant level, through a complex control system involving the action of signals related to metabolism and nutritional status (probably many of them remain unknown). At least three major types of regulation have been identified. The first one is the induction by NO_3^- itself. The second one is the coordination with photosynthesis through the stimulation of various steps of NO_3^- acquisition and metabolism by sugars. The third one is the control of the N status of the whole plant through feedback repression exerted by downstream N metabolites such as NH_4^+ or amino acids. A similar complexity is found at the molecular level. *NRT1.1* and *NRT2.1*, as *NIA1* and *NIA2*, are inducible by NO_3^- and up-regulated by sugars (Cheng et al., 1991; Filleur and Daniel-Vedele, 1999; Lejay et al., 1999; Zhuo et al., 1999). These two characteristics are common features of the expression of most of the Arabidopsis genes involved in NO_3^- uptake and assimilation (Stitt, 1999; Coruzzi and Bush, 2001; Forde, 2002; Glass et al., 2002), with the exception of the expression of *NRT1.2*, that does not depend on the presence of NO_3^- (Huang et al., 1999). Expression of *NRT1.1* and *NRT2.1* has been studied in detail. So far, *NRT2.1* is the only NO_3^- transporter gene known to be under feedback repression by N metabolites (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999; Zhuo et al., 1999). Its expression is strongly increased by N deficiency (Lejay et al., 1999; Gansel et al., 2001), and the gene has been characterized functionally as a major component mediating the response of NO_3^- uptake to variation of the N status of the whole plant (Cerezo et al., 2001). In contrast to *NRT2.1*, the *NRT1.1* gene is neither repressed by reduced N metabolites nor induced by N limitation (Lejay et al., 1999).

The use of NR-deficient mutants has been a powerful tool to unravel the specific regulatory effects of NO_3^- and of products of its assimilation (Scheible et al., 1997; Krapp et al., 1998; Filleur and Daniel-Vedele, 1999; Zhang et al., 1999). When NRA is strongly reduced compared with WT, mutant plants are under N limitation when supplied with NO_3^- as sole nitrogen source but are N sufficient if a reduced nitrogen form is added together with NO_3^- in the nutrient solution. This allows investigation of the response of genes to feedback repression by N metabolites without modifying the supply of NO_3^-. Using this strategy, we have previously shown that *NRT2.1* is strongly up-regulated in the G′4-3 Arabidopsis NR-deficient mutant compared with WT when both genotypes are grown on NO_3^- as sole nitrogen source (Lejay et al., 1999). However, this up-regulation of *NRT2.1* in the NR-deficient mutant is suppressed as soon as NH_4^+ is added to the nutrient solution. This indicates that *NRT2.1* expression is not directly controlled by NRA, but rather by the availability of downstream N metabolites such as NH_4^+ or amino acids. Accordingly, exogenous supply of amino acid or NH_4^+ results in a strong decrease in *NRT2.1* transcript accumulation (Zhuo et al., 1999). An unexpected outcome of our study was to find the NRT1.1 transcript level markedly increased in the roots of G′4-3 NR-deficient mutant compared with WT (Lejay et al., 1999). However, in contrast to *NRT2.1*, the up-regulation of *NRT1.1* in the G′4-3 mutant is also observed in N-sufficient plants cultivated on NH_4NO_3 and cannot be attributed to the release of feedback repression exerted by NH_4^+ or amino acids. To explain these results, we have proposed the existence of an as yet unknown regulatory mechanism, corresponding to a direct repression of *NRT1.1* expression by NR independently of the N status of the plant.

The aim of the present work was to investigate this hypothesis. To demonstrate the generality of the observations made on the G′4-3 NR mutant, expression of *NRT1.1* has been analyzed in various other mutants impaired either in the NR apoprotein isoform or in the NR molybdenum cofactor (MoCo) biosynthesis. Investigations concerning the effect of the nitrogen source (reduced nitrogen and NO_3^-) and the external pH on the expression of *NRT1.1* in WT and NR-deficient plants are described. Finally, the action of NO_2^-, the direct product of the reaction catalyzed by NR, has been investigated on both *NRT1.1* expression and root NO_3^- influx.

RESULTS

NRT1.1 and NIA1 Are Up-Regulated in Various NR-Deficient Mutants

Several NR-deficient mutants were investigated to determine whether one specific component of NRA (NIA1 or NIA2 apoenzymes, MoCo) is responsible for the repression of *NRT1.1* expression in roots. The G5 mutant has a deletion in the *NIA2* gene encoding the major isoform of Arabidopsis NR apoenzyme (Wilkinson and Crawford, 1991). The G′4-3 double mutant, derived from G5, has an additional point mutation in the *NIA1* gene that encodes the minor isoform of the NR apoenzyme (Wilkinson and Crawford, 1993). This mutation reduces NRA but does not abolish *NIA1* transcript accumulation. The *nia1*::*Ds* mutant carries a Ds nonautonomous transposable element inserted in the coding sequence of the *NIA1* gene (Parinov et al., 1999). The *chl6*, *chl4*, and *chl2* mutants possess intact WT NIA1 and NIA2 apoproteins, but are deficient in MoCo biosynthesis, which leads to altered activities of both enzymes (LaBrie et al., 1992).

In plants grown hydroponically on 1 mM NH_4NO_3 as the sole nitrogen source, the amount of *NRT1.1* transcript in the roots was higher in most NR-deficient mutants than in WT plants (Fig. 1). The only exception was the *nia1*::*Ds* mutant, in which only the *NIA1* gene is disrupted and which displayed unaltered *NRT1.1* transcript accumulation compared with Landsberg *erecta* (L*er*) plants. Thus, comparison between mutants indicates that *NRT1.1* is overexpressed only when NIA2 activity is altered, due to either the absence of the *NIA2* gene (G5 and G′4-3 mutants) or the mutation of MoCo biosynthesis (*chl2*, *chl4*, and *chl6* mutants). This indicates that NIA2 plays a predominant role in the regulation of *NRT1.1* expression. An unexpected result of these studies was that NIA1 transcript was accumulated in parallel to NRT1.1 transcript in roots, indicating that expression of *NIA1* also is probably under the same control as *NRT1.1*. Interestingly, transcripts of *NIA1* and *NIA2* do not display the same behavior in the various NR mutants. Neither the loss of NIA1 isoform (*nia1*::*Ds* mutant) nor the altered NRA resulting from mutations on MoCo biosynthesis (*chl2*, *chl4*, and *chl6* mutants) significantly alters *NIA2* transcript accumulation.

The ability of mutations affecting the MoCo biosynthesis pathway (*chl2*, *chl4*, and *chl6* mutants) to stimulate expression of both *NRT1.1* and *NIA1* in roots indicates that the repression exerted by NR is not related only to the expression of the NR apoprotein, but requires the activity of the enzyme. Additional experiments were performed to quantify more precisely the correlation between expression of both *NRT1.1* and *NIA1* in the roots of the various mutants and total NRA in these organs (Fig. 2). Mutants display various levels of NR deficiencies in both roots and shoots (Fig. 2A). An inverse correlation was found between *NRT1.1* and *NIA1* transcript levels and total NRA in the roots in most of the mutants (Fig. 2, B and C). Similar inverse correlation was also found with total shoot NRA (data not shown). Together these data suggest that the repression of the two genes depends on the plant capacity to reduce NO_3^-. According to the hypothesis of a direct control exerted by root NRA, loss of NIA2 is expected to have a major effect on the repression of *NRT1.1* and *NIA1*, because *NIA2* encodes the main isoform of NR responsible for most of the catalytic activity of the plant. However, not all of the data agree with this hypothesis. In particular, G′4-3 and G5 plants (Fig. 2A) have markedly different root NRA (9% and 49% of the WT root NRA, respectively; Fig. 2A), whereas *NRT1.1* is expressed at similar levels in both genotypes (Fig. 2B). Moreover, the root NRA found in the G5 mutant (deleted for *NIA2*) is fully attributable to NIA1. This activity is especially high in the mutant because of the overexpression of *NIA1* (Figs. 1 and 2B). This indicates that NIA1-related NRA alone is unable to repress *NRT1.1* and suggests that both

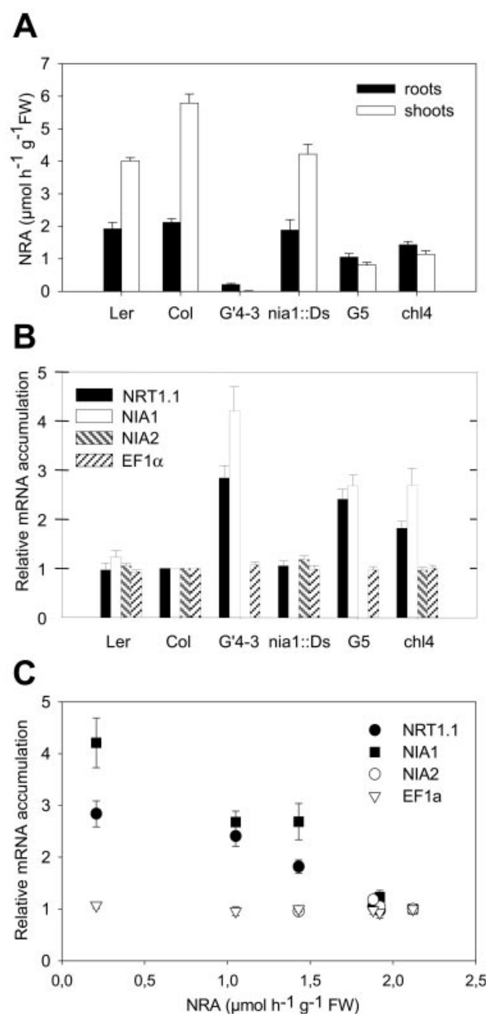

Figure 2. NRA (A) and root transcript accumulation of *NRT1.1*, *NIA1*, *NIA2*, and *EF1α* (B) in NR-deficient mutants compared with WT (Col and L*er*). C, Transcript accumulation was plotted as a function of root NRA. Plants (8 weeks old) were grown hydroponically on nutrient solution containing 1 mM NH_4NO_3 as nitrogen source. The values of NRA are means of six replicates ± SE. The values of relative accumulation of transcript are means of four independent experiments ± SE.

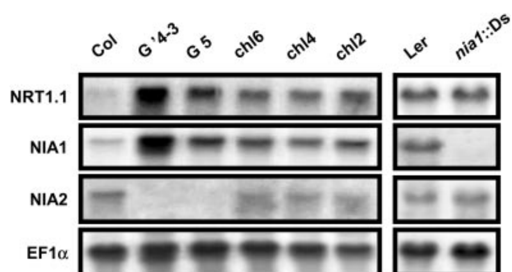

Figure 1. Transcript accumulation of *NRT1.1*, *NIA1*, and *NIA2* in the roots of NR-deficient mutants compared with WT (Col and L*er*). Plants (8 weeks old) were grown hydroponically on nutrient solution containing 1 mM NH_4NO_3 as the nitrogen source. Total RNAs were analyzed by northern blot.

NR isoforms are not equivalent in the regulation of this gene.

Repression of NRT1.1 and NIA1 Expression by NR Depends on NO_3^- Supply

The initial evidence for the up-regulation of *NRT1.1* in response to NR deficiency has been obtained in G′4-3 plants grown on nutrient solution containing 1 mM NH_4NO_3 as the sole nitrogen source. To further investigate the mechanisms involved in the repression of *NRT1.1* and *NIA1* expression by NR, the effects of the nitrogen source were studied in more detail.

First, two sources of reduced nitrogen that can be assimilated by NR-deficient plants were compared (Fig. 3). G′4-3 plants were cultivated hydroponically with a nutrient solution containing 2 mM NO_3^- supplemented either with 1 mM Gln or with 2 mM NH_4^+. In both conditions, higher levels of *NRT1.1* and *NIA1* transcripts were found in the roots of the G′4-3 mutant than in those of the WT. Thus, up-regulation of *NRT1.1* and *NIA1* in NR-deficient mutants cannot be attributed to a specific effect of the exogenous supply of NH_4^+.

Second, the effect of the level of NO_3^- supply on *NRT1.1* and *NIA1* expression was analyzed in both Columbia (Col) and G′4-3 plants. The two genotypes were cultivated on nutrient solution containing 2 mM NH_4^+ (to ensure N-sufficiency), supplemented with NO_3^- at various concentrations (0.25, 0.5, 2, and 5 mM). The increase of NO_3^- concentration in the nutrient solution resulted in a strong decrease of both *NRT1.1* and *NIA1* transcript accumulations in the roots of WT plants, but had no effect on the expression of these genes in roots of G′4-3 plants (Fig. 4). This indicates that high levels of NO_3^- promote down-regulation of *NRT1.1* and *NIA1* through a mechanism dependent on NO_3^- reduction. This confirms the inverse relationship between the reduction of NO_3^- and the repression of *NRT1.1* and *NIA1* (Fig. 2C). Moreover, repression of *NRT1.1* in WT plants by high availability of NO_3^- suggests that the rate of NO_3^- reduction present in roots rather than total reduction capacity is probably involved in the repression of *NRT1.1* and *NIA1*.

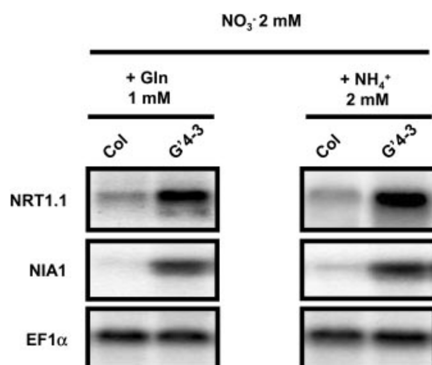

Figure 3. Transcript accumulation of *NRT1.1* and *NIA1* in roots of WT (Col) and G′4-3 plants grown on nutrient solution containing 2 mM KNO_3 with either 1 mM Gln or 2 mM NH_4Cl. Plants (4 weeks old) were grown hydroponically under sterile conditions. Total RNAs were analyzed by northern blot.

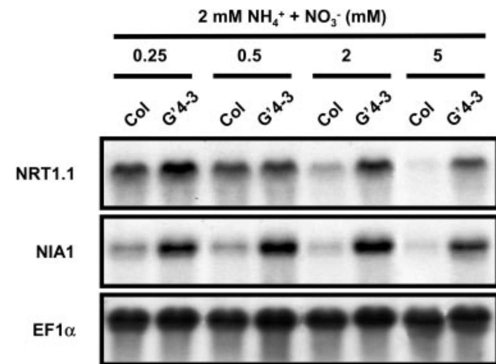

Figure 4. Transcript accumulation of *NRT1.1* and *NIA1* in roots of WT (Col) and G′4-3 plants grown on nutrient solution containing 2 mM NH_4Cl and various concentrations of KNO_3 as a nitrogen source. Plants were grown hydroponically under sterile conditions on 2 mM NH_4NO_3 for 3 weeks and transferred to the various conditions of NH_4^+ and NO_3^- supply 1 week before harvest. Total RNAs were analyzed by northern blot.

Expression of NRT1.1 and NIA1 Is Repressed by NO_2^-

The ability of NO_3^- reduction to trigger the repression of *NRT1.1* and *NIA1* in the presence of NH_4^+ or Gln (Figs. 3 and 4) suggests that products of NO_3^- reduction upstream of NH_4^+ are involved in this down-regulation. NR deficiency may reduce cellular levels of NO_2^-. Because the reduction of NO_3^- generates OH^-, which is generally excreted by the plant, NR deficiency may also promote a decrease of both internal and external pH.

The pH hypothesis was examined in detail because expression of *NRT1.1* is known to be up-regulated by acidification of the nutrient medium (Tsay et al., 1993). In our standard hydroponic conditions, no significant differences of acidification of the external medium were observed between G′4-3 and WT plants. However, it cannot be ruled out that subtle variations might trigger the up-regulation observed in NR-deficient mutants. To investigate the role of the external pH, WT and G′4-3 plants were cultivated in the same container, and the pH of the nutrient solution was maintained at pH 5 or 6 in the presence of organic buffers. In both pH conditions, *NIA1* and *NRT1.1* were up-regulated in the mutant when compared with the WT (Fig. 5). Acidification of the external solution from pH 6 (initial pH of the standard nutrient solution) to pH 5 is correlated with large variations in all genes studied including *NRT1.1* (increased expression) and *NIA1* (reduced expression). These divergent responses of *NIA1* and *NRT1.1* upon pH changes make it highly unlikely that a common

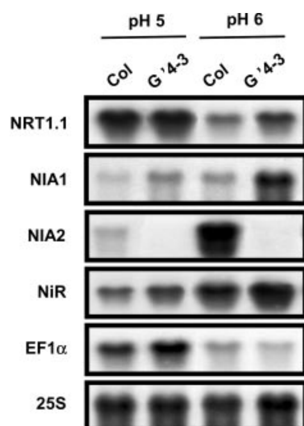

Figure 5. Effect of the external pH on the transcript accumulation of *NRT1.1*, *NIA1*, *NIA2*, and *NiR* in roots of WT (Col) and G′4-3 plants. Plants were grown hydroponically on standard nutrient solution containing 1 mM NH_4NO_3 as nitrogen source for 7 weeks and transferred for 1 week to solutions buffered with 4.4 mM MES (pH 6 or pH 5 with Tris). Total RNAs were analyzed by northern blot.

up-regulation of both genes in the G′4-3 mutant results from acidification related to NR deficiency. However, because the difference of *NRT1.1* expression between the G′4-3 mutant and the wild type is reduced when pH is maintained by organic buffer particularly at pH5 when *NRT1.1* expression is high, a possible involvement of external pH in the up-regulation of *NRT1.1* in G′4-3 cannot be totally ruled out.

To examine the hypothesis of a NO_2^--mediated regulation, the effect of exogenous supply of NO_2^- was investigated. In these experiments, NO_2^- was added as 1 mM KNO_2 to the nutrient solution containing 1 mM NH_4NO_3 as the sole nitrogen source. Net NO_2^- uptake rate was measured at 24.0 ± 6.5 μmol g^{-1} root dry weight h^{-1} (±SE, n = 7) on G′4-3 plants exposed for 5 h to 1 mM $K^{15}NO_2$. No visible symptoms of toxicity were noticed in response to the exogenous supply of NO_2^-, at least during the first 48 h. This treatment had no effect on the pH of the bulk solution and did not modify NO_2^- accumulation in the shoots (data not shown). However, root NO_2^- content increased in both WT and G′4-3 plants during the first 6 h after addition of NO_2^-, and remained almost stable thereafter until 24 h (Fig. 6A). After 6 h of treatment, *NRT1.1* and *NIA1* transcript levels were markedly reduced in both genotypes as compared with control plants left on 1 mM NH_4NO_3 without KNO_2 (Fig. 6, B and C). A similar decrease could be observed already after 3 h of exposure to NO_2^- (data not shown). Repression was dependent on the concentration of NO_2^- present in the nutrient solution (Fig. 7A). Addition of 0.1 mM or 0.5 mM KNO_2 was able to trigger a significant reduction of the expression of *NIA1* and *NRT1.1*, respectively. The rapid and strong inhibition of *NRT1.1* and *NIA1* expression by exogenous NO_2^- supply was not part of a general response. First, transcript levels of *EFIα*,

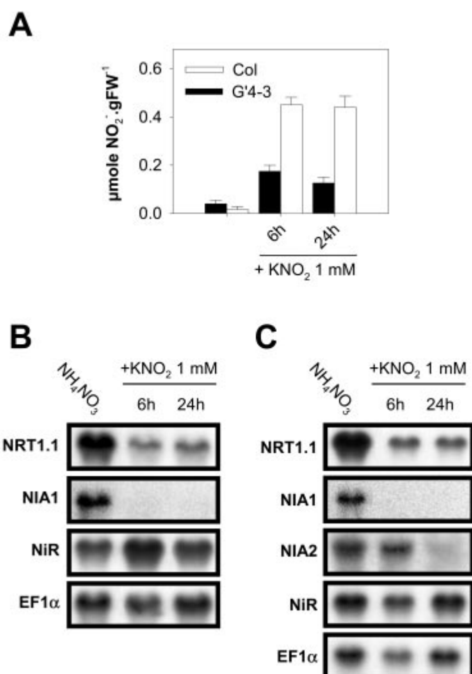

Figure 6. Effect of exogenous supply of 1 mM KNO_2 on NO_2^- accumulation (A) and *NRT1.1*, *NIA1*, and *NiR* transcript accumulation in roots of G′4-3 (B) and WT (Col; C) plants. Plants (8 weeks old) were grown hydroponically on a nutrient solution containing 1 mM NH_4NO_3. KNO_2 was added to the medium for 6 or 24 h before harvest (middle of the light period). The values of NO_2^- content are the mean of 10 replicates ± SE. Total RNAs were analyzed by northern blot.

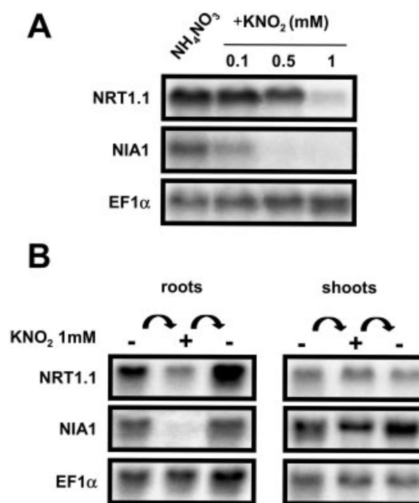

Figure 7. Dose-response and reversibility of the repression of *NRT1.1* and *NIA1* by exogenous supply of KNO_2 in G′4-3 mutants. Plants were grown hydroponically for 8 weeks on a nutrient solution containing 1 mM NH_4NO_3. Total RNAs were analyzed by northern blot. A, Plants were grown during 24 h on nutrient solution containing 0.1, 0.5, or 1 mM KNO_2 before harvest. Plants grown without exogenous supply of KNO_2 were used as a control. B, Plants were grown during 24 h on a nutrient solution containing 1 mM KNO_2, and roots were washed 5 min in $CaSO_4$ 0.1 mM and returned for 24 h to standard nutrient solution before harvest.

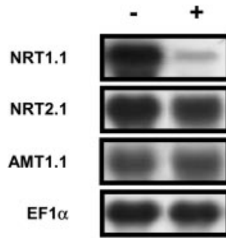

Figure 8. Effect of exogenous supply of KNO_2 on *NRT1.1*, *NRT2.1*, and *AMT1.1* on transcript accumulation in roots of G′4–3 plants. Plants (8 weeks old) were grown hydroponically on a nutrient solution containing 2mM NH_4NO_3; (+) 2 mM KNO_2 was added to the medium for 24 h before harvest (middle of the light period); (-) control plants were left on the same nutrient solution. Total RNAs were analyzed by Northern blot.

NiR, *AMT1.1* (encoding an ammonium transporter; Ninnemann et al., 1994), or *NRT2.1* were not decreased by the treatment in either genotype (Fig. 6, B and C; Fig. 8). The expression level of *NIA2* was also inhibited by NO_2^- in WT, but after a significant delay and to a lesser extent (Fig. 6C). Second, repression of *NRT1.1* and *NIA1* expression by exogenous NO_2^- supply was found only in the roots and was fully reversible (Fig. 7B). The addition of NO_2^- in the nutrient solution also resulted in a repression of NO_3^- uptake in G′4-3 plants (Fig. 9). Root influx was measured at 0.2 and 5 mM external $^{15}NO_3^-$ concentrations to differentiate between the activities of high- and low-affinity transport systems. Supply of 1 mM NO_2^- for 6 h resulted in a 61% inhibition of $^{15}NO_3^-$ influx at 0.2 mM and a 44% inhibition at 5 mM. Interestingly, NO_2^- exposure did not affect $^{15}NH_4^+$ influx.

DISCUSSION

Both NRT1.1 and NIA1 Are under Feedback Repression by NO_3^- Reduction Independent of the N Status of the Plant

Our results confirm, with a large set of genotypes, the overexpression of *NRT1.1* in the roots of NR-deficient plants as initially suggested by studies with the G′4-3 mutant (Lejay et al., 1999). The overexpression is not triggered by low N status of the plant because it is observed with N sources allowing proper growth of NR-deficient mutants (NH_4NO_3 or NO_3^- plus Gln) and because previous studies have shown that *NRT1.1* is not under the control of the feedback repression by downstream N metabolites (Lejay et al., 1999). Our results suggest that *NRT1.1* is under a different type of feedback regulation, exerted directly at the level of NO_3^- reduction. One unexpected outcome of our study is the finding that the pattern of expression of *NIA1* closely parallels that of *NRT1.1*, indicating that *NIA1* is also repressed by NO_3^- reduction. Thus, in addition to a likely regulation of NO_3^- uptake (regulation of *NRT1.1* expression), this feedback repression may also correspond to an auto-regulation of NR gene expression. The compensation for the absence of NIA2 by the overexpression of the *NIA1* gene in the roots of the G5 mutant (deletion of *NIA2*) restores a level of NRA corresponding to 49% of the WT NRA. This regulation may contribute significantly to the control of NRA in Arabidopsis roots. Overaccumulation of *NIA* mRNA in mutants expressing a defective NR apoenzyme and in mutants impaired in MoCo biosynthesis has already been described in leaves of various species, including wild tobacco (*Nicotiana plumbaginifolia*; Pouteau et al., 1989), cultivated tobacco (*Nicotiana tabacum*; Vaucheret et al., 1990), and Arabidopsis (LaBrie et al., 1992; Wilkinson and Crawford, 1993). Similar stimulation of *NIA* transcript accumulation in leaves was found in plants exposed to tungstate, an inhibitor of NRA (Deng et al., 1989). Up-regulation is not restricted to NR. The *NiR* gene and other genes involved in nitrogen, organic acid, and carbon metabolism are also overexpressed in NR-deficient genotypes of tobacco (Scheible et al., 1997). However, in most of these studies, it is unclear whether this up-regulation results from N limitation of the plants or not, because the N source supplied to the plants did not always include reduced N. In the present study, we show that the overexpression of *NIA1* in NR-deficient plants is also found in the roots. In our case, the up-regulation can be unambiguously attributed to a mechanism directly related to NO_3^- reduction and not to the general control exerted by the N status of the plant.

Our results concerning the respective role of the two NR isoforms suggest that catalytic activity of NIA2 has a major role in the repression exerted by NO_3^- reduction. It is unclear whether NIA2 has a predominant action on *NRT1.1* and *NIA1* because it catalyzes the major part of total NRA or because NIA2-related activity has a specific role in the regulation of these two genes. In the absence of investigations describing NIA1- and NIA2-specific activities and distributions across the plant, we can only spec-

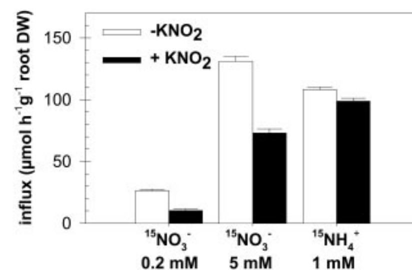

Figure 9. Effect of exogenous supply of 1 mM KNO_2 on $^{15}NO_3^-$ and $^{15}NH_4^+$ influxes in roots of the G′4-3 mutant. Plants (8 weeks old) were grown hydroponically on a nutrient solution containing 1 mM NH_4NO_3. KNO_2 was added to the medium 6 h before influx measurements. Plants grown without exogenous supply of KNO_2 were used as a control. Root influx was measured with complete nutrient solution containing 5 mM $^{15}NO_3^-$, 0.2 mM $^{15}NO_3^-$, or 1 mM $^{15}NH_4^+$ as tracers. The values are the means of 10 replicates ± SE.

ulate about the possible mode of action of NIA2. It has been shown recently that expression of *NRT1.1* is restricted to nascent organs, mainly in root tips (Guo et al., 2001). One hypothesis might be that *NIA1*, *NIA2*, and *NRT1.1* are not expressed in the same root tissues. For instance, if NIA1 and NRT1.1 are present in two distinct cell types, whereas NIA2 is present in both, this may explain why NIA1 has apparently little action on *NRT1.1* expression, while NIA2 governs both *NRT1.1* and *NIA1* transcription. Another hypothesis might be that the two NIA isoforms display subtle differences in their functional properties in vivo, which are not revealed by our in vitro measurements. Finally, because of the inverse correlation between shoot NRA and expression of *NRT1.1* and *NIA1* in roots, a negative control exerted by shoots, mediated by a long-distance signal, has also to be considered as an alternative hypothesis.

The Cause of NRT1.1 and NIA1 Overexpression in NR-Deficient Plants: Decreased NO_3^- Reduction or Increased NO_3^- Accumulation?

Up-regulation of gene expression in NR-deficient plants has been proposed to result from "overinduction" by NO_3^-, which accumulates at very high levels in the absence of active NR (Scheible et al., 1997; Forde, 2000). This may hold true for *NRT1.1* and *NIA1*, known to be inducible by NO_3^-. However, several lines of evidence do not support this hypothesis. First, variation of the accumulation of NO_3^- in NR-deficient or -overexpressing plants mostly occurs in the shoot, not in the roots (Quilleré et al., 1994; Gojon et al., 1998; Lejay et al., 1999). Second, increasing the level of NO_3^- supply does not stimulate expression of *NRT1.1* and *NIA1* either in WT or in the G′4-3 double mutant. The increase in external NO_3^- concentration results in an amplification of the overexpression of *NRT1.1* and *NIA1* in the G′4-3 NR-deficient mutant compared with WT (Fig. 4). However, this amplification is not due to the increased accumulation of *NRT1.1* and *NIA1* transcripts in the roots of G′4-3 plants but to a decrease in the level of these transcripts in the WT. These results are fully consistent with the hypothesis of a feedback repression exerted by NO_3^- reduction in the WT, because in this genotype, NO_3^- reduction rate is expected to go up with increasing NO_3^- supply.

Feedback Repression by NO_2^-

Because expression of *NRT1.1* is not down-regulated by NH_4^+, the effect of two other direct products of NO_3^- reduction, namely NO_2^- and OH^-, has been considered. Stimulation of *NRT1.1* expression by the acidification of the external medium has been described previously (Tsay et al., 1993). Our results do not support the hypothesis that a lowered OH^- production in NR-deficient plants may be the cause for overexpression of *NRT1.1* and *NIA1* in NR-deficient plants. First, even when WT and G′4-3 plants are maintained at the same external pH, *NRT1.1* and *NIA1* transcripts still accumulate at higher levels in the roots of the mutant. Second, acidification of the external medium from pH 6 to 5 triggers opposite responses of *NRT1.1* and *NIA1*, indicating that the common up-regulation of both genes in NR mutants is unlikely to be explained by a pH effect. However, the effect of external pH on *NRT1.1* expression indicates that pH may interact with specific NR-dependent factors to repress the gene in wild type. On the other hand, these data do not rule out a possible role of changes of cytoplasmic pH. Such investigations to test this hypothesis will require direct measurements in root cells expressing *NRT1.1* and *NIA1* with H^+-specific microelectrodes (Walker et al., 1996) or ^{31}P-NMR techniques (Bligny and Douce, 2001).

The strong reduction of both *NIA1* and *NRT1.1* root transcript levels in response to the addition of NO_2^- in the nutrient solution in absence of N limitation supports the hypothesis of NO_2^- acting as a repressor of the expression of both genes. NO_2^- has been shown to inhibit NO_3^- uptake in barley (*Hordeum vulgare*; King et al., 1993), but the explanation usually provided to account for this effect is the competition between the two anions for the activity of the uptake systems. In our experiments, the decrease of *NRT1.1* gene expression is correlated with a reduction of NO_3^- influx in both the high- and low-affinity ranges. This is consistent with the fact that NRT1.1 plays a major role in both high- and low-affinity NO_3^- uptake under conditions similar to those used in our study, in particular in presence of NH_4NO_3 as a N source (Touraine and Glass, 1997; Wang et al., 1998; Liu et al., 1999). In addition to the absence of symptoms on treated plants, the possibility that this down-regulation of both *NRT1.1* and *NIA1* expression results from a toxic effect of NO_2^- is contradicted by at least six arguments: (a) The repression is rapid (less than 3 h); (b) the effect is fully reversible; (c) the treatments promote relatively modest increases of root NO_2^- content (2 orders of magnitude less than NO_3^-), most probably because of the high affinity of the NiR enzyme for its substrate (Beevers and Hageman, 1980); (d) no changes are observed in shoots of plants exposed to NO_2^-; (e) other genes such as *EF1α*, *NiR*, *NRT2.1*, and *AMT1* are not affected; and (f) unlike NO_3^- uptake, NH_4^+ uptake is not affected. The hypothesis of NO_2^- being a repressor of *NIA1* gene is somehow in contradiction with the fact that leaves of NiR antisense tobacco transformants cultivated on NO_3^- as the sole nitrogen source display an increase of NO_2^- accumulation as well as NR transcript level (Vaucheret et al., 1992). Because these tobacco plants were expected to be seriously N limited, the feedback repression exerted by downstream N metabolites was most probably released,

and therefore it cannot be excluded that a repression by NO_2^- might have been overcome by the stimulation of gene expression triggered by the N limitation. Also, whether shoots and roots display similar or different responses upon an increasing NO_2^- accumulation remains to be investigated.

To our knowledge, this work is the first report pointing out the ability of NO_2^- to repress genes involved in N acquisition in higher plants. Such a role is unexpected for NO_2^-, which is believed to be toxic and present at very low levels within the cell, because the activity of the NiR enzyme measured in vitro is in large excess. However, recent results obtained on transgenic Arabidopsis plants overexpressing a spinach (*Spinacia oleracea*) NiR cDNA suggest that reduction of NO_2^- may be a rate-limiting step (Takahashi et al., 2001). This is compatible with the hypothesis of NO_2^- acting as a regulatory signal. Such hypothesis has already been proposed in the unicellular algae *Chlamydomonas reinhardtii*. In this organism, NO_2^- represses genes encoding NR and NO_3^- transporters (Loppes et al., 1999). The mode of action of NO_2^- remains unknown. It may act directly, or it may act indirectly through the involvement of a related metabolite such as NO. NO is now considered to be a regulatory signal involved various in plant responses to environmental stimuli (Beligni and Lamattina, 2001; Wendehenne et al., 2001; Desikan et al., 2002; Murgia et al., 2002). NO might be produced chemically by decomposition of HNO_2 or enzymatically by NR from NO_2^- as a substrate when it accumulates in tissues (Dean and Harper, 1988; Yamasaki and Sakihama, 2000; Rockel et al., 2002). Interestingly, NO has been proposed to inhibit root NRA in lettuce (*Lactuca sativa*) plants (Hufton et al., 1996).

All together, our data support a model postulating that the NO_2^- (or NO) produced by NR represses *NRT1.1* and *NIA1* expression in the roots. This regulation, which appears to be independent of the nitrogen status of the plant, corresponds to a mechanism for coordinating NO_3^- uptake and assimilation. It has the particularity to be specific for NO_3^- nutrition, as opposed to feedback repression by reduced N metabolites (NH_4^+ and/or amino acids), that targets NO_3^- as well as NH_4^+ acquisition. This regulation is not common to all genes involved in NO_3^- assimilation. Although the *NiR* gene is up-regulated in NR-deficient mutants, it is not repressed upon NO_2^- addition, indicating that *NiR* is probably not under the same control as *NRT1.1* and *NIA1*.

Further studies are required to understand the physiological significance of this regulation. One hypothesis may be related to the adaptation to root anoxia, from which plants suffer during flooding periods. The ability of roots to accumulate and to excrete NO_2^- under hypoxia has been extensively used to assay NRA in vivo (Radin, 1974). Under anoxic conditions, NO_2^- can be produced in the roots following uptake and reduction of NO_3^-, whereas NiR activity is strongly lowered by the shortage of reduced ferredoxin availability. It can then be speculated that repression of both NO_3^- uptake and assimilatory systems by NO_2^- might provide a mechanism to prevent toxic accumulation of NO_2^- by limiting its production in hypoxic cells.

MATERIALS AND METHODS

Plant Material and Culture Conditions

Five genotypes of Arabidopsis ecotype Col were used: G′4-3 and G5 (Wilkinson and Crawford, 1991, 1993), *chl2*, *chl4*, and *chl6* (Braaksma and Feenstra, 1982; LaBrie et al., 1992). The *nia1*::*Ds* mutant (Parinov et al., 1999) is of the ecotype L*er*. All mutants were provided by Arabidopsis Biological Resource Center (Ohio State University, Columbus) and Nottingham Arabidopsis Stock Center (Nottingham, UK). Plants at the vegetative stage were grown hydroponically under standard or sterile conditions. Basal nutrient solution without nitrogen contained 1 mM KH_2PO_4, 1 mM $MgSO_4$, 250 μM $CaCl_2$, 0.1 mM Na-Fe-EDTA, 50 μM KCl, 50 μM H_3BO_3, 5 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$, and 0.1 μM $(NH_4)_6Mo_7O_{24}$, pH adjusted to 6 with KOH. Under non-sterile conditions, plants were cultivated in a 10-L tank as previously described (Lejay et al., 1999) with the following environmental parameters: light/dark cycle, 8 h/16 h; light intensity, 300 μmol s^{-1} m^{-2} photosynthetically active radiation; temperature, 22°C/20°C; and 70% hygrometry. The nutrient solution was renewed twice a week during the first part of the culture and daily the last week before the experiment. Under sterile conditions, plants were grown in the same basal medium in presence of 10 g L^{-1} Suc and 2 mM MES-Tris, pH 6, according to Touraine and Glass (1997). Plants were held by a nylon grid (480-μm gauge) on the top of a membrane raft (three to four plants per box) floating on 60 mL of nutrient solution and were renewed twice during the 1st week of culture and daily thereafter. For all experiments, plants of the same age cultivated in various conditions were harvested at the same time in the middle of the light period. When necessary, treatments were initiated for various times before the harvest.

^{15}N Uptake and Assimilation Studies

Root influxes of NO_3^- and NH_4^+ were assayed according to Delhon et al. (1995) and Gazzarrini et al. (1999), respectively. Plants were sequentially transferred to 0.1 mM $CaSO_4$ for 1 min and to the complete nutrient solution containing either NO_3^- or NH_4^+ (99% atom excess ^{15}N) for 5 min. At the end of the labeling, roots were washed for 1 min in 0.1 mM $CaSO_4$ and were separated from shoots. The organs were dried at 70°C for 48 h, weighed, and analyzed for total ^{15}N content using a continuous-flow isotope ratio mass spectrometer coupled with a carbon nitrogen elemental analyzer (ANCA-MS, PDZ Europa, Crewe, UK) as described by Clarkson et al. (1996). Preliminary influx studies were performed using a labeling solution containing 1 mM $^{15}NO_3^-$ and various mixtures of 1 mM NO_2^- or 1 mM NH_4^+. No significant differences were observed between the results measured with these various solutions, indicating that in our conditions, the presence of NH_4^+ and NO_2^- in the labeling solution has a negligible effect on NO_3^- influx (data not shown). NO_2^- was extracted by boiling fresh tissue for 15 min in water. Total NRA and NO_2^- were assayed according to Robin (1979).

Northern Blot

Total RNAs were isolated by phenol-guanidine extraction followed by lithium chloride precipitation according to Lobreaux et al. (1992). RNAs (20 μg per lane) were resolved by electrophoresis on MOPS-formaldehyde agarose gels, blotted on to Biotrans (+) nylon membranes, and covalently linked to the filter by UV cross-linking (Ausubel et al., 1988). Hybridization to a randomly primed radiolabeled probe was done at 42°C in 50% (v/v) formamide, 1% (w/v) sarkosyl, 5× SSC (0.75 M NaCl, and 0.075 M Na_3 citrate, pH 7), and 100 μg mL^{-1} salmon sperm DNA. Membranes were washed twice at 42°C in 0.1% (w/v) SDS and 2× SSC for 15 min and then twice in 0.1% (w/v) SDS and 0.1× SSC for 15 min. Quantification of

radioactive signals were achieved using a PhosphoImager (Storm, Molecular Dynamics, Sunnyvale, CA). Blots were stripped in 0.01× SSC for 5 min at 100°C. Gene-specific probes used in this work corresponded to the full-length cDNA of NRT1.1 (Tsay et al., 1993), the 1.6-kb *Eco*RI-*Hin*dIII fragment of the pAtc46 plasmid carrying a partial sequence of the NIA2 cDNA (Crawford et al., 1988), the expressed sequence tag (EST) 134 J7/T7 carrying a partial sequence of the NIA1 cDNA, and the EST 177N14 carrying a partial sequence of the NiR cDNA (ESTs were provided by the Arabidopsis Biological Resource Center stock center). Two probes were systematically used as controls: the full-length cDNA of the elongation factor EF1αA3 as a gene nondirectly related to N metabolism (Axelos et al., 1989), and a 25S rDNA fragment (Choumane and Heizman, 1988) to monitor the equal loading of blots.

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