Regulation of the High-Affinity NO_3^- Uptake System by NRT1.1-Mediated NO_3^- Demand Signaling in Arabidopsis[W]

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The *NRT2.1* gene of *Arabidopsis thaliana* encodes a major component of the root high-affinity NO_3^- transport system (HATS) that plays a crucial role in NO_3^- uptake by the plant. Although *NRT2.1* was known to be induced by NO_3^- and feedback repressed by reduced nitrogen (N) metabolites, *NRT2.1* is surprisingly up-regulated when NO_3^- concentration decreases to a low level (<0.5 mM) in media containing a high concentration of NH_4^+ or Gln (≥1 mM). The *NRT3.1* gene, encoding another key component of the HATS, displays the same response pattern. This revealed that both *NRT2.1* and *NRT3.1* are coordinately down-regulated by high external NO_3^- availability through a mechanism independent from that involving N metabolites. We show here that repression of both genes by high NO_3^- is specifically mediated by the NRT1.1 NO_3^- transporter. This mechanism warrants that either NRT1.1 or NRT2.1 is active in taking up NO_3^- in the presence of a reduced N source. Under low NO_3^-/high NH_4^+ provision, NRT1.1-mediated repression of *NRT2.1/NRT3.1* is relieved, which allows reactivation of the HATS. Analysis of *atnrt2.1* mutants showed that this constitutes a crucial adaptive response against NH_4^+ toxicity because NO_3^- taken up by the HATS in this situation prevents the detrimental effects of pure NH_4^+ nutrition. It is thus hypothesized that NRT1.1-mediated regulation of *NRT2.1/NRT3.1* is a mechanism aiming to satisfy a specific NO_3^- demand of the plant in relation to the various specific roles that NO_3^- plays, in addition to being a N source. A new model is proposed for regulation of the HATS, involving both feedback repression by N metabolites and NRT1.1-mediated repression by high NO_3^-.

Higher plants acquire mineral nitrogen (N) from the soil mainly in the form of NO_3^-, through the activity of both high-affinity transport systems (HATS) and low-affinity transport systems (LATS), respectively (Crawford and Glass, 1998; von Wirén et al., 2000). Except in agricultural soils after fertilizer application, where NO_3^- concentration can rise up several millimolar (Crawford and Glass, 1998), it is generally assumed that root NO_3^- uptake is mostly determined by the activity of the HATS (Crawford and Glass, 1998; von Wirén et al., 2000; Malagoli et al., 2004). The current model of the NO_3^- HATS is constituted by at least two genetically separate transport systems: (1) a constitutive HATS present even in the absence of NO_3^- (Wang and Crawford, 1996; Crawford and Glass, 1998); and (2) an inducible HATS (iHATS), quantitatively more important than the constitutive HATS, and activated within hours after NO_3^- provision to the plant (Behl et al., 1988; Mackown and McClure, 1988; Crawford and Glass, 1998; Daniel-Vedele et al., 1998; Forde, 2000).

In Arabidopsis (*Arabidopsis thaliana*), two gene families have been found to encode transporter proteins involved in root NO_3^- uptake (Forde, 2000; Williams and Miller, 2001; Orsel et al., 2002; Okamoto et al., 2003): the *NRT2* family (seven members) and the *NRT1* family, belonging to the large PTR family of transporter genes (53 members). Convincing evidence has accumulated that the NO_3^--inducible *NRT2.1* gene encodes a major component of the iHATS (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999; Zhuo et al., 1999). First, *atnrt2.1* mutants display a strong reduction of root NO_3^- influx in the low external NO_3^- concentration range (i.e. below 1 mM; Filleur et al., 2001; Orsel et al., 2004). Second, NO_3^- inducibility of high-affinity root NO_3^- uptake is suppressed by *NRT2.1* deletion (Cerezo et al., 2001). Recently, another key component of the NO_3^- HATS has been identified in the form of the product of the *NAR2*-like Arabidopsis gene *NRT3.1* (Okamoto et al., 2006). It is suspected that interaction between NRT2.1 and NRT3.1 is required for functionality of the iHATS because NRT2 proteins need to be coexpressed with NAR2-like proteins to generate NO_3^- uptake activity in Xenopus oocytes (Quesada and Fernandez, 1994; Zhou et al., 2000; Tong et al., 2005).

Besides induction by NO_3^-, *NRT2.1* expression and NO_3^- HATS activity are also feedback repressed by reduced N metabolites, such as NH_4^+ and amino acids (Lejay et al., 1999; Zhuo et al., 1999; Nazoa et al., 2003). This regulation involves systemic signaling (Gansel

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et al., 2001) and ensures the specific control of root NO_3^- uptake by the N status of the whole plant (Imsande and Touraine, 1994; Forde, 2002). Indeed, N sufficiency triggers the repression exerted by N metabolites and down-regulates the HATS, whereas N deprivation has the opposite effect (Crawford and Glass, 1998; Lejay et al., 1999; Zhuo et al., 1999; Gansel et al., 2001). Again, NRT2.1 plays a key role in these processes because both repression of NO_3^- HATS by N metabolites and its stimulation by N starvation are suppressed in the *atnrt2.1-1* mutant (Cerezo et al., 2001). Another important aspect of NRT2.1 function relates to the recent reports showing that it is not only involved in high-affinity NO_3^- uptake, but also plays a NO_3^--sensing role in the regulation of lateral root initiation (Little et al., 2005; Remans et al., 2006). This latter role is still unclear because, depending on the conditions, NRT2.1 either represses (Little et al., 2005) or, on the contrary, stimulates (Remans et al., 2006) lateral root development.

Recently, investigation of several *chl1* mutants of Arabidopsis unexpectedly revealed that disruption of another NO_3^- transporter gene, *NRT1.1* (formerly *CHL1*), results in a major alteration of the regulation of *NRT2.1* expression by the N status of the plant (Muños et al., 2004). First, feedback repression of *NRT2.1* by either NH_4^+ or Gln supply is suppressed or strongly attenuated in *chl1* mutants compared with wild type, resulting in its overexpression in normally suppressive conditions (e.g. in NH_4NO_3-fed *chl1* plants). Second, expression of *NRT2.1* is no more stimulated by N starvation in *chl1* mutants. These data suggest that mutation of *NRT1.1* blocks both *NRT2.1* expression and NO_3^- HATS activity in some kind of derepressed state, making *chl1* mutants the only known genotypes affected in the regulation of root NO_3^- uptake in higher plants.

NRT1.1 is an unusual dual-affinity NO_3^- transporter (Tsay et al., 1993; Wang et al., 1998), shifting from low to high affinity in response to phosphorylation of the Thr-101 residue (Liu and Tsay, 2003). Although NRT1.1 also contributes to root NO_3^- uptake (Tsay et al., 1993; Huang et al., 1996; Touraine and Glass, 1997), its precise role in the regulation of *NRT2.1* expression remains unclear. A first hypothesis was that NRT1.1 may be directly involved in the signaling pathway responsible for feedback repression of *NRT2.1* by N metabolites. However, this interpretation was questioned by the finding that repression of *NRT2.1* by NH_4^+ could also be alleviated in wild-type plants when the external NO_3^- concentration was decreased down to 0.1 mM in the presence of 1 mM NH_4^+ (Muños et al., 2004). This showed that mutation of *NRT1.1* is not required to suppress the repressive effect of N metabolites and led to a second alternative hypothesis that *NRT2.1* up-regulation in NH_4NO_3-fed *chl1* plants could actually be due to the lowered NO_3^- uptake resulting from *NRT1.1* mutation. Because NH_4NO_3-fed *chl1* plants were not N deficient, this in turn suggested that *NRT2.1* expression is not only repressed by reduced N metabolites, but also by NO_3^- itself (Muños et al., 2004).

This work had three aims: (1) to investigate the occurrence of such repression of *NRT2.1* by NO_3^- in wild-type plants; (2) to determine whether NRT1.1 plays a direct (i.e. specific) or indirect (through modulation of NO_3^- uptake rate) role in triggering this repression; and (3) to determine the physiological role of this regulation. Concerning this last point, we hypothesized that repression of *NRT2.1* by NO_3^- may correspond to a mechanism allowing the HATS to be stimulated by a specific NO_3^- demand of the plant independently of its overall N status (Muños et al., 2004). In particular, we anticipated that this may have a crucial function to avoid that the bulk of N acquisition from mixed NH_4NO_3 medium is made through NH_4^+ uptake, thus protecting the plant against the toxicity generally associated with NH_4^+ nutrition (Givan, 1979; Hageman, 1984; Salsac et al., 1987; Kronzucker et al., 2001).

RESULTS

Low NO_3^- Availability in the Presence of NH_4^+ Up-Regulates Both Root *NRT2.1* Expression and NO_3^- HATS Activity

As commonly observed in many experimental conditions, the supply of NH_4^+ together with NO_3^- at equimolar concentration (1 mM for both ions) is associated with very faint expression of *NRT2.1* in the roots (Fig. 1A, lane T0). However, transfer of plants to fresh nutrient medium with lower NO_3^- concentration (0.1 mM), but unmodified NH_4^+ availability (1 mM), rapidly resulted in a marked increase in *NRT2.1* mRNA accumulation, which was detectable after 6 h and leveled off between 3 and 4 d (Fig. 1A).

To identify more precisely which parameter modified by the above treatment (NO_3^- concentration, total NO_3^- plus NH_4^+ concentration, or NH_4^+ to NO_3^- ratio) was responsible for the up-regulation of *NRT2.1* expression, we explored the effects of various combinations of NO_3^- and NH_4^+ concentrations. Figure 1B displays typical results obtained from this series of experiments. They show that the increase of *NRT2.1* transcript accumulation is not due to the lowering of the total external N (NO_3^- + NH_4^+) concentration because it was also observed after transfer to solutions containing 0.1 mM NO_3^- supplemented with 2 mM, or even 5 mM NH_4^+. On the other hand, a high NH_4^+ to NO_3^- ratio in the external medium is also not responsible per se for up-regulation of *NRT2.1* expression because, when compared to 1:0.1 mM NH_4^+:NO_3^-, the 5:0.5 mM NH_4^+:NO_3^- mixture (with the same NH_4^+:NO_3^- ratio of 10) resulted in much less pronounced *NRT2.1* up-regulation (Fig. 1B). Furthermore, *NRT2.1* is also overexpressed by low NO_3^- availability in mixed N medium when Gln (another potent repressor of *NRT2.1* expression) is provided as the reduced N

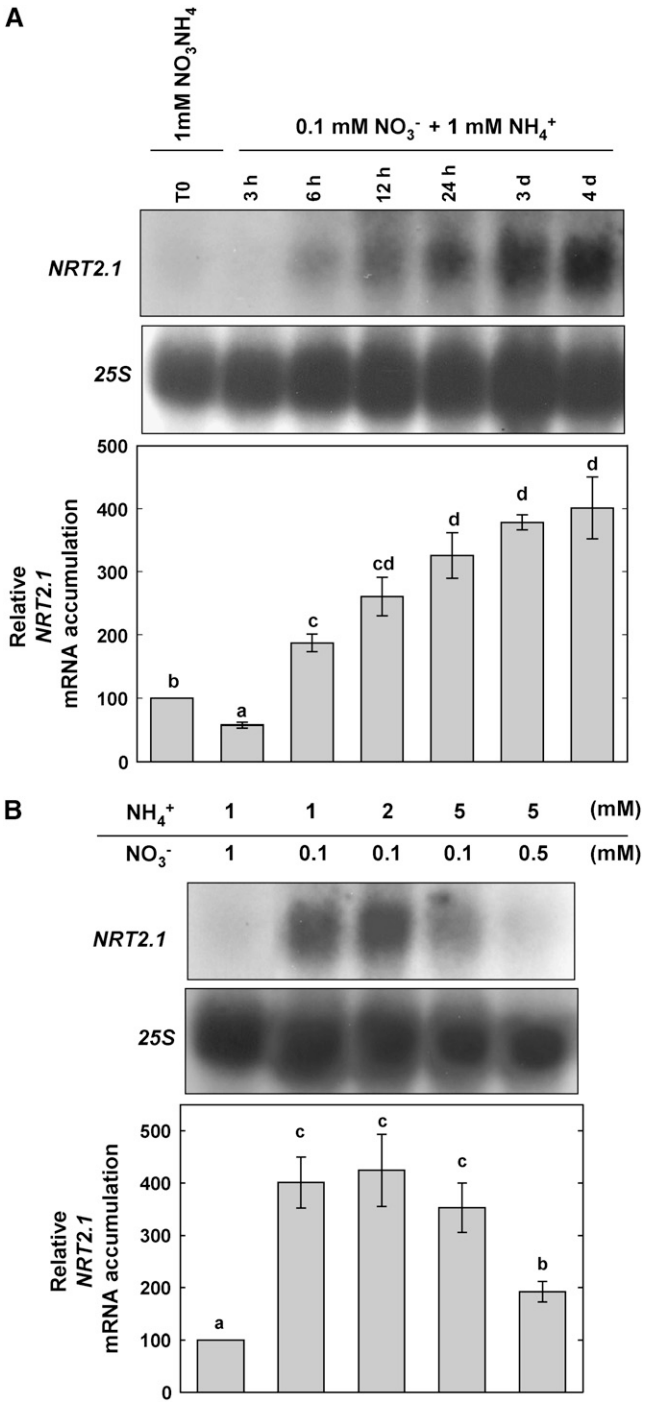

Figure 1. Changes in *NRT2.1* mRNA accumulation in response to various NO_3^-/NH_4^+ mixtures in the external medium. Plants were grown hydroponically for 6 weeks on complete nutrient solution containing 1 mM NH_4NO_3 before transfer to the various media indicated in the figure. A, Time-course response in plants transferred to nutrient solution containing 1 mM NH_4^+ plus 0.1 mM NO_3^-. B, Effect of transfer for 4 d to media containing various combinations of NH_4^+ and NO_3^- concentrations. Transcript accumulation was monitored in roots both by northern-blot analysis (gel-blot images) and real-time RT-PCR (bar graphs) on the same samples. A 25S rRNA signal is presented as a loading control for northern blots. Quantification by real-time RT-PCR was normalized using actin genes as controls. Errors bars represent SE ($n = 3$). Different letters on top of the bars indicate statistically significant differences ($P < 0.05$; *t* test).

source (Supplemental Fig. S1). This shows that *NRT2.1* up-regulation is not specifically due to excess NH_4^+ supply. Altogether, the data obtained suggest that *NRT2.1* expression is responsive to the absolute NO_3^- concentration present in the mixed N solution. Indeed, whatever the nature and concentration of the reduced N source (NH_4^+ or Gln, 1–5 mM), the *NRT2.1* transcript level was always high when the external NO_3^- concentration remained low at 0.1 mM, but strongly decreased when the NO_3^- concentration was raised at 0.5 mM.

One interesting finding related to the increased expression of *NRT2.1* under low NO_3^-/high NH_4^+ availability is that the effect is local and not systemic. Indeed, split-root experiments showed that when only one side of the root system is provided with 0.1 mM NO_3^- + 1 mM NH_4^+, *NRT2.1* is up-regulated in this side only and not in the other one fed with 1 mM NH_4NO_3 (Fig. 2, compare lanes b and c). Furthermore, the *NRT2.1* transcript level in the side of the split-root system supplied with 0.1 mM NO_3^- + 1 mM NH_4^+ (Fig. 2, lanes c and e) was the same, whatever the NO_3^- availability on the other side (1 or 0 mM in Fig. 2, lanes b and f, respectively), and equaled that in plants homogeneously supplied with 0.1 mM NO_3^- + 1 mM NH_4^+ on the whole root system (Fig. 2, lane d).

In wild-type plants, up-regulation of *NRT2.1* expression by low NO_3^- availability in the presence of NH_4^+ resulted in similar stimulation of NO_3^- HATS activity (as measured by root $^{15}NO_3^-$ influx at 0.2 mM), which was not observed in the *atnrt2.1-1* mutant (Fig. 3A). Interestingly, the NH_4^+ uptake system did not display this stimulation because root $^{15}NH_4^+$ influx measured either at 1 mM (Fig. 3B) or 0.2 mM (data not shown) was only marginally increased upon lowering of the external NO_3^- concentration. Total N content of roots and shoots was unaffected between the treatments (data not shown), most probably as a result of both increased NO_3^- HATS activity and sustained NH_4^+ uptake.

Collectively, the above results suggest that low NO_3^- availability alleviates repression of both *NRT2.1* expression and NO_3^- HATS activity triggered by provision of a reduced N source to the plant.

Regulation of *NRT2.1* Expression by External NO_3^- Availability in the Presence of NH_4^+ Is Specifically Mediated by NRT1.1

In previous experiments with *chl1-10* plants, it was observed that increasing NO_3^- concentration from 0.1 to 10 mM in the presence of 1 mM NH_4^+ failed to repress *NRT2.1* expression (Muños et al., 2004), unlike what is found in wild type (Fig. 3A). To determine whether this is due to a direct or an indirect (through lowered total NO_3^- uptake) role of NRT1.1 in regulating *NRT2.1* expression, we checked whether the mutation

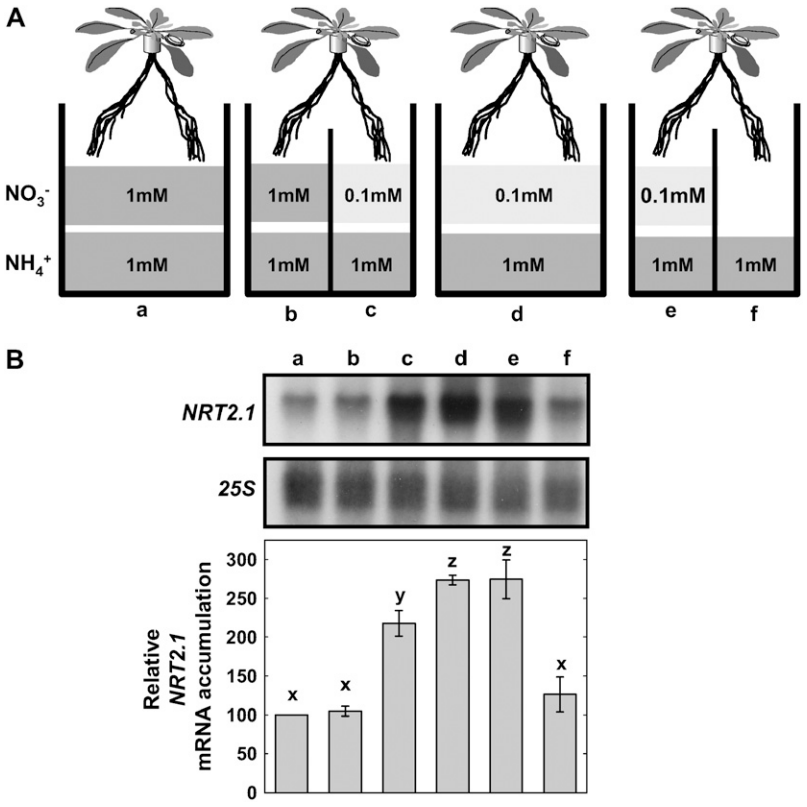

Figure 2. Effect of local changes in the NO_3^-/NH_4^+ external balance on *NRT2.1* mRNA accumulation in split-root plants. A, Representation of the experimental protocol. Plants were grown hydroponically for 5 weeks on complete nutrient solution containing 1 mM NH_4NO_3. After separation of their root system into two approximately equal sides, plants were transferred to specific containers and left undisturbed during 3 d in the 1 mM NH_4NO_3 solution before application of localized supplies with different NO_3^-/NH_4^+ mixtures for 4 d, as indicated in the figure. B, *NRT2.1* mRNA accumulation. Transcript accumulation was monitored in various sides of the split-root systems both by northern-blot analysis (gel-blot images) and real-time RT-PCR (bar graphs) on the same samples. The lane labels relate to the various localized treatments as shown in A. A 25S rRNA signal is presented as a loading control for northern blots. Quantification by real-time RT-PCR was normalized using actin genes as controls. Errors bars represent SE ($n = 3$). Different letters on top of the bars indicate statistically significant differences ($P < 0.05$; *t* test).

of *NRT1.2*, encoding another important component of the NO_3^- low-affinity transport system (Huang et al., 1999), also resulted in *NRT2.1* up-regulation. Therefore, we investigated the quantitative relationship between NO_3^- uptake from NH_4NO_3 medium and *NRT2.1* expression in the roots of three genotypes: the Wassilewskija (Ws) wild type, the *chl1-10* mutant, and the *atnrt1.2-1* mutant, carrying a T-DNA insertion in the *NRT1.2* gene.

Increasing the external NO_3^- concentration from 0.1 to 5 or 10 mM in the presence of 1 mM NH_4^+ resulted in a strong and almost similar down-regulation of *NRT2.1* expression in both Ws and *atnr1.2-1* plants, whereas this down-regulation was absent in *chl1-10* plants (Fig. 4A). Surprisingly, root $^{15}NO_3^-$ influx (measured at the same concentration as that used for treatment of the plants) was little affected by external NO_3^- concentration in Ws plants (Fig. 4B), where a 50-fold decrease of this concentration (from 5–0.1 mM) only resulted in a 30% slowing down of root $^{15}NO_3^-$ influx. This indicates strong homeostasis of root NO_3^- uptake from NH_4NO_3 medium, which probably explains why root NH_4^+ influx was only marginally stimulated by the decrease in external NO_3^- concentration (Fig. 3B). Root $^{15}NO_3^-$ influx was lower in the *atnrt1.2-1* mutant than in the wild type, but not specifically in the high external NO_3^- concentration range. Unexpectedly, root $^{15}NO_3^-$ influx was not reduced in *chl1-10* plants compared with Ws and was even higher in the middle range of external NO_3^- concentration (1–5 mM). These data unambiguously demonstrate that unrepressed *NRT2.1* transcript accumulation in *chl1-10* plants at high external NO_3^- availability cannot be explained by reduced root NO_3^- uptake rate as compared to either Ws or *atnrt1.2-1* plants (Fig. 4C). Thus, whatever the signal responsible for down-regulation of *NRT2.1* expression by high external NO_3^- availability, it requires NRT1.1 to trigger the response.

Additional evidence for NRT1.1-dependent control of the NO_3^- HATS by external NO_3^- availability was provided by expression analysis of *NRT3.1*, which shows the same response pattern as *NRT2.1*, suggesting coregulation of the two genes (Fig. 5). However, the quantitative changes in *NRT3.1* transcript abundance were generally less pronounced than those of *NRT2.1*.

Taken together, the above data strongly support the hypothesis of coordinated regulation of *NRT2.1* and *NRT3.1*, mediated by NRT1.1, which down-regulates the NO_3^- HATS when external NO_3^- availability in mixed N medium is above 0.2 to 0.5 mM. Below this threshold, this repression is alleviated, which enables the plant to reactivate the NO_3^- HATS, despite the strongly repressive conditions related to the ample supply of reduced N source.

Up-Regulation of *NRT2.1* by Low NO_3^- Availability in the Presence of NH_4^+ Prevents Growth Inhibition Associated with NH_4^+ Toxicity

To determine the physiological significance of NRT1.1-dependent control of the NO_3^- HATS by

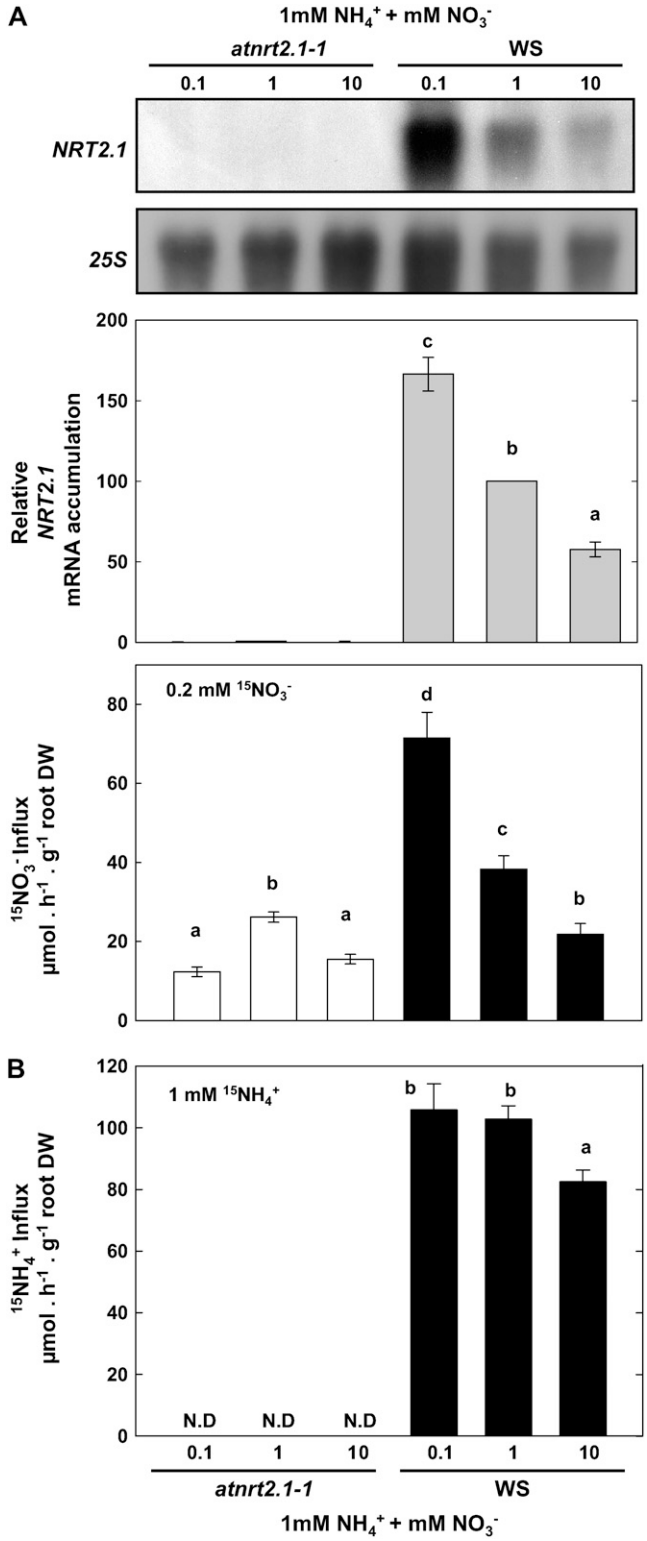

Figure 3. Effect of various NO_3^-/NH_4^+ mixtures on *NRT2.1* mRNA accumulation and root N uptake in Ws wild-type and *atnrt2.1-1* mutant plants. The plants were grown hydroponically for 6 weeks on complete nutrient solution containing 1 mM NH_4NO_3 before transfer for 4 d to various media containing the same NH_4^+ concentration (1 mM), but various concentrations of NO_3^- (0.1, 1, or 10 mM). A, *NRT2.1* mRNA accumulation and root $^{15}NO_3^-$ influx. Transcript accumulation was monitored in roots both by northern-blot analysis (gel-blot images) and real-time RT-PCR (bar graphs) on the same samples. A 25S rRNA signal is presented as a loading control for northern blots. Quantification by real-time RT-PCR was normalized using actin genes as controls. Errors bars represent SE ($n = 3$). Root $^{15}NO_3^-$ influx was assayed by 5-min labeling at 0.2 mM external $^{15}NO_3^-$ concentration to specifically determine activity of the HATS. Each value is the mean of eight to 12 replicates ± SE. B, Root $^{15}NH_4^+$ influx. Root $^{15}NH_4^+$ influx was assayed by 5-min labeling at 1 mM external $^{15}NH_4^+$ concentration to determine the uptake activity at the same concentration as for growth and treatment of the plants. Each value is the mean of eight to 12 replicates ± SE. N.D., Not determined. Different letters on top of the bars indicate statistically significant differences ($P < 0.05$; *t* test).

external NO_3^- availability, we investigated the hypothesis that up-regulation of *NRT2.1* by low NO_3^- concentration in mixed N medium plays a role in preventing NH_4^+ toxicity under situations of excess NH_4^+ over NO_3^- supply. Therefore, we analyzed growth and NO_3^- uptake of wild-type plants and of two independent *NRT2.1* knockout mutants (*atnrt2.1-1* and *atnrt2.1-2*) under pure NH_4^+ or mixed NH_4NO_3 nutrition. Seedlings were first grown for 5 weeks on 1 mM NH_4NO_3 before submitting them for 10 to 14 d to the various N nutrition regimes. During these experiments, wild-type and mutant plants were placed in the same container to make sure that both genotypes experienced the same changes in external pH.

As expected, the supply of 1 mM NH_4^+ as the sole N source led to the appearance of toxicity symptoms in the shoots of both wild-type and *atnrt2.1-1* genotypes (Fig. 6). These symptoms became pronounced between 6 and 10 d after transfer to the NH_4^+ nutrient solution. In particular, NH_4^+-fed plants started to bolt very early and their leaves wilted and yellowed. Interestingly, addition of 0.1 mM NO_3^- in the 1 mM NH_4^+ nutrient solution fully prevented the appearance of toxicity symptoms in wild-type plants, but not in the mutant, which seemed to remain as sensitive as when NH_4^+ is the sole N source supplied (Fig. 6).

These observations were confirmed by shoot growth analysis (Fig. 7A). With 1 mM NH_4^+ as the sole N source, shoots of *atnrt2.1-1* plants grew at the same relative rate as those of wild-type plants, despite slightly lower biomass at the beginning of the experiment. Relative growth rate (RGR) values (determined from the slopes of the linear relationships between ln [fresh weight] and time) were 0.126 g g^{-1} d^{-1} (r^2 = 0.986) and 0.125 g g^{-1} d^{-1} (r^2 = 0.975) for wild-type and *atnrt2.1-1* shoots, respectively. Supply of 0.1 mM NO_3^- together with 1 mM NH_4^+ markedly stimulated shoot growth in wild-type plants (RGR = 0.165 g g^{-1} d^{-1}; r^2 = 0.992), but not in *atnrt2.1-1* plants (RGR = 0.137 g g^{-1} d^{-1}; r^2 = 0.988). In agreement with the fact that *NRT2.1* is up-regulated by low NO_3^- concentration in mixed N medium, and that it encodes a major component of the NO_3^- HATS, the cumulative NO_3^- uptake in wild-type plants transferred on 0.1 mM NO_3^- + 1 mM NH_4^+ was much higher than that in

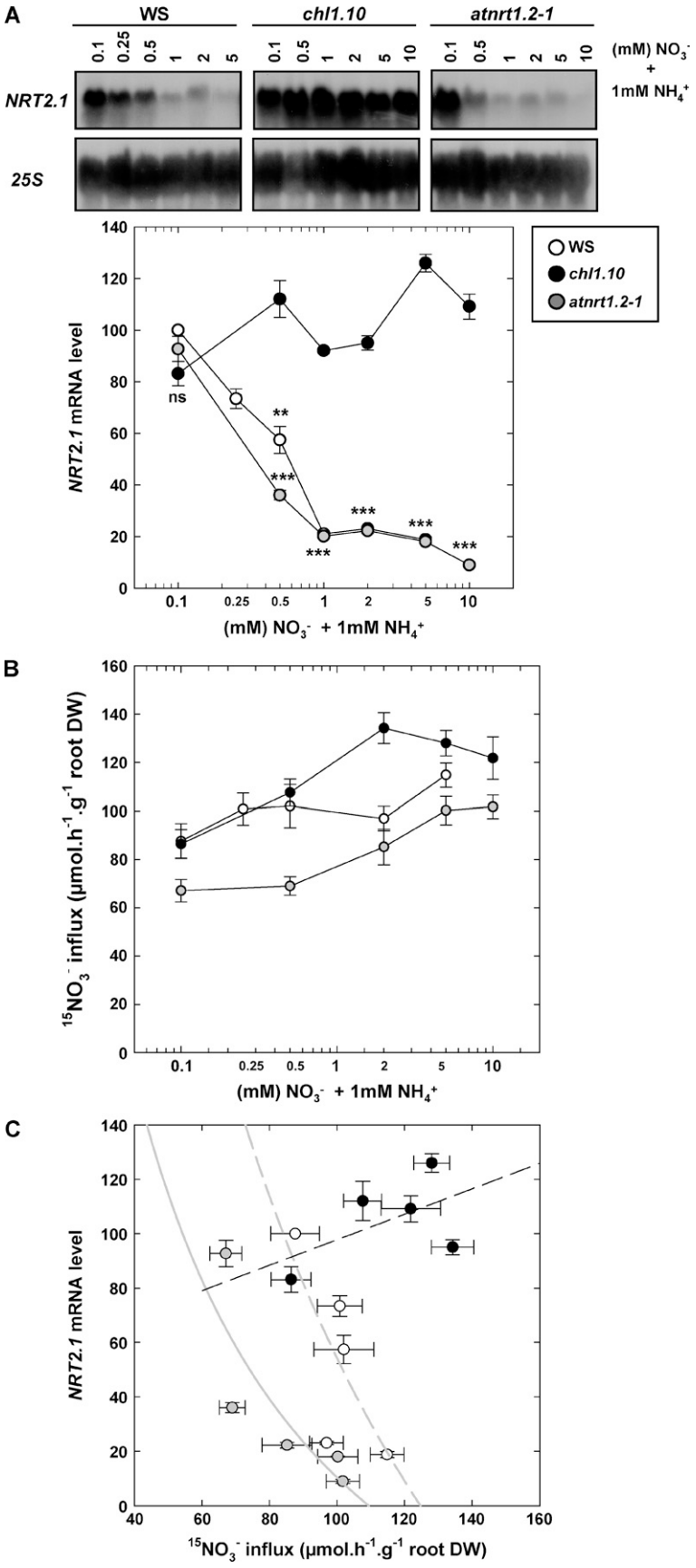

Figure 4. Relationship between *NRT2.1* mRNA accumulation and root NO_3^- influx as a function of NO_3^- concentration in the presence of NH_4^+ in the external medium. Plants of Ws wild-type, *atnrt1.2-1* mutant, and *chl1-10* mutant were grown for 6 weeks on complete nutrient solution containing 1 mM NH_4NO_3, before transfer for 4 d to the nutrient solutions containing 1 mM NH_4^+ and NO_3^- at various concentrations, as indicated in the figure. A, *NRT2.1* mRNA accumulation. Transcript accumulation was monitored in roots both by northern-blot analysis (gel-blot images) and real-time RT-PCR (graph) on the same samples. A 25S rRNA signal is presented as a loading control for northern blots. Quantification by real-time RT-PCR was normalized using actin genes as controls. Errors bars represent SE ($n = 3$). Differences between *chl1-10* and the other genotypes are statistically significant at $^{**}P < 0.01$ and $^{***}P < 0.001$ (*t* test). B, Root $^{15}NO_3^-$ influx. Root $^{15}NO_3^-$ influx was assayed by 5-min labeling at the external $^{15}NO_3^-$ concentration corresponding to that supplied to the plants during the 4-d treatment. Each value is the mean of six to 12 replicates ± SE. C, Plot of *NRT2.1* mRNA accumulation (A) against root $^{15}NO_3^-$ influx (B).

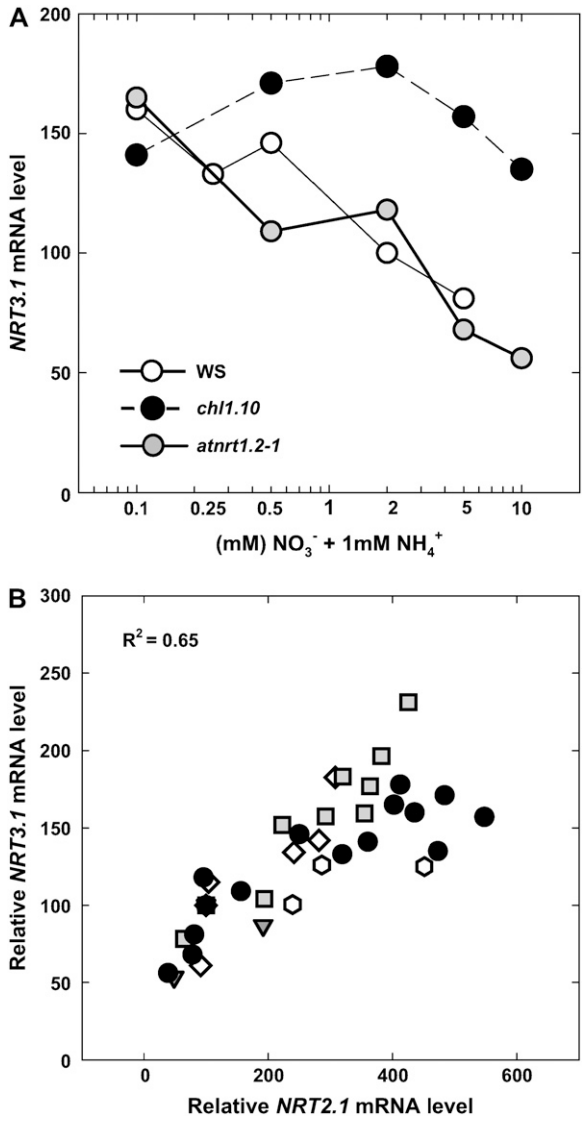

Figure 5. *NRT2.1* and *NRT3.1* coregulation. A, Response of *NRT3.1* mRNA accumulation in Ws, *atnrt1.2-1*, and *chl1-10* plants to the variation of NO_3^- concentration in the presence of 1 mM NH_4^+ in the external medium. The experiment is the same as in Figure 5. B, Correlation between changes in *NRT2.1* and *NRT3.1* mRNA accumulation. All RNA samples used to determine changes in *NRT2.1* expression presented in Figures 1 to 4 and Supplemental Figure S1 were analyzed for *NRT3.1* mRNA accumulation. The figure presents the relative changes in transcript level for both *NRT2.1* and *NRT3.1* normalized to the control (Ws plants left on 1 mM NO_3NH_4) of each experiment. Gray squares, Experiment of Figure 1; white diamonds, experiment of Figure 2; gray triangles, experiment of Figure 3; white hexagons, experiment of Supplemental Figure S1; black circles, experiment of Figure 4.

atnrt2.1-1 plants (Fig. 7B). All the above observations were confirmed with the *atnrt2.1-2* mutant allele (Supplemental Fig. S2). On 1 mM NH_4NO_3, however, a situation where NRT2.1 has a low contribution to total NO_3^- uptake (Cerezo et al., 2001), no significant difference was recorded for both shoot biomass and cumulative NO_3^- uptake between wild-type and *atnrt2.1-1* plants (Supplemental Fig. S3). Thus, the decrease in shoot growth observed in *atnrt2* mutants as compared to wild types correlated with the reduction of NO_3^- uptake resulting from the *NRT2.1* mutation. On the other hand, root biomass was not affected by *NRT2.1* mutation in all media investigated (Supplemental Figs. S2 and S3).

Taken together, the above data demonstrate that, even at a low concentration of 0.1 mM, the presence of NO_3^- in mixed N solution is able to alleviate the detrimental effects of pure NH_4^+ nutrition on shoot growth in wild-type plants, but not in *atnrt2.1* mutants. This indicates that the protective action of 0.1 mM NO_3^- against NH_4^+ toxicity is dependent on *NRT2.1* expression.

DISCUSSION

A Novel Regulation of *NRT2.1* Expression Involving NRT1.1-Mediated Repression by High NO_3^-

Knowledge concerning the control of *NRT2.1* expression by N is that this gene is under two main regulations, namely (1) induction by NO_3^-; and (2) repression by high N status of the whole plant (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999; Zhuo et al., 1999; Forde, 2000; Gansel et al., 2001; Orsel et al., 2002; Nazoa et al., 2003; Okamoto et al., 2003). The molecular mechanisms underlying these regulations are unknown, but there is some consensus about the nature of the signal molecules involved. In particular, the NO_3^- ion itself is believed to be the inducer (Crawford and Glass, 1998; Forde, 2000), and NH_4^+ and Gln are thought to be the main signal molecules involved in the feedback repression exerted by the N status of the plant (Lejay et al., 1999; Zhuo et al., 1999; Nazoa et al., 2003).

This model now appears to be incomplete because *NRT2.1* overexpression in wild-type plants under low NO_3^-/high NH_4^+ availability (Figs. 1–4) cannot be explained by the above mechanisms (i.e. induction by NO_3^- and repression by NH_4^+). A striking illustration of this is the observation that transfer of the plants from 1 mM NH_4NO_3 to 5 mM NH_4^+ + 0.1 mM NO_3^- led to 4-fold stimulation of *NRT2.1* expression (Fig. 1B), although this corresponded to a 10-fold decrease in the concentration of the inducer (NO_3^-) and a 5-fold increase in the concentration of the repressor (NH_4^+). Furthermore, as already shown for up-regulation of *NRT2.1* in NH_4NO_3-fed *chl1* mutants (Muños et al., 2004), up-regulation of *NRT2.1* by low NO_3^- in the presence of NH_4^+ cannot be mistaken with relief of the feedback repression exerted by the overall N status of the plant: (1) plants subjected to low NO_3^-/high NH_4^+ availability have a high total N content of the tissues and thus have high N status; (2) root NH_4^+ influx is not up-regulated in these plants (Fig. 3B), whereas it is also under negative feedback control by the N status of the plant (Gazzarrini et al., 1999; Rawat et al., 1999; von

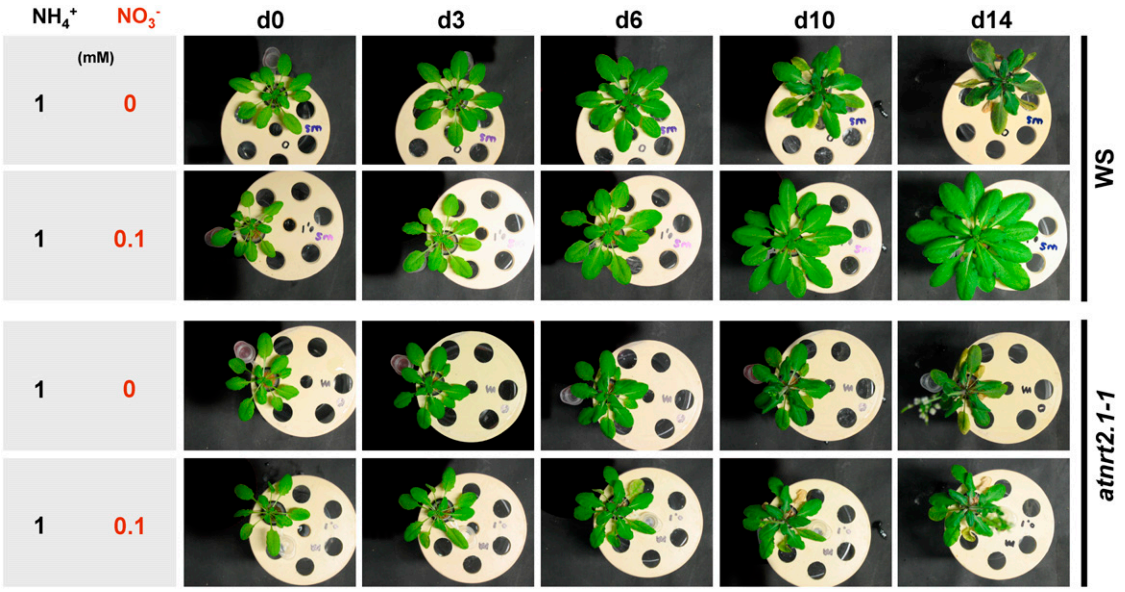

Figure 6. Effect of *NRT2.1* mutation on the protective effect of NO_3^- against NH_4^+ toxicity. Images show the appearance of toxicity symptoms in shoots of Ws and *atnrt2.1-1* mutant plants as a function of the N source supplied in the external medium (either 1 mM NH_4^+ alone or in combination with 0.1 mM NO_3^-).

Wirén et al., 2000); and (3) stimulation of *NRT2.1* expression by low NO_3^-/high NH_4^+ availability is under purely local control (Fig. 2), whereas *NRT2.1* regulation by the N status of the plant involves systemic signaling (Imsande and Touraine, 1994; Gansel et al., 2001; Forde, 2002).

Altogether, our data provide evidence that *NRT2.1* expression is also modulated by a third important regulatory mechanism, triggering repression of this gene by high external NO_3^- availability, which superimposes on repression exerted by NH_4^+ or Gln. Indeed, in the presence of either reduced N source, *NRT2.1* expression in wild-type roots was consistently found to be primarily determined by external NO_3^- concentration, with strong down-regulation as soon as this concentration exceeded the 0.2 to 0.5 mM range. Although surprising at first glance, the hypothesis that NO_3^- may have opposite regulatory effects (induction and repression) is already well documented for its role in the control of lateral root growth (local stimulation of lateral root elongation and systemic repression of lateral root emergence; Zhang et al., 1999). Furthermore, down-regulation of root NO_3^- uptake by NO_3^- itself has already been postulated, in particular from barley (*Hordeum vulgare*) experiments where root NO_3^- uptake was found to be negatively correlated with root NO_3^- concentration, but only when root NO_3^- concentration exceeded a certain threshold level (Siddiqi et al., 1989; Crawford and Glass, 1998). Although these physiological studies provided circumstantial evidence for repression of root NO_3^- uptake by high NO_3^-, our results bring insight to these aspects because they highlight *NRT2.1* and *NRT3.1* as molecular targets of this regulation. Recently, *NRT3.1* expression was also shown to be induced by NO_3^- (Okamoto et al., 2006) and repressed by high N status of the plant (Remans et al., 2006). Thus, *NRT3.1* appears to be, at least partially, controlled by the same regulatory network as *NRT2.1*, suggesting coordinated regulation of these two components of the HATS. Most importantly, our data also reveal specific involvement of NRT1.1 in triggering repression by high NO_3^- (Figs. 4 and 5). Indeed, down-regulation of *NRT2.1*/*NRT3.1* expression by high NO_3^- availability in the presence of NH_4^+ is fully suppressed in the *chl1-10* mutant (and not in a *nrt1.2* mutant), whereas root NO_3^- uptake is not reduced in *chl1-10* compared to wild type (whereas it is reduced in *atnrt1.2-1*). This clearly invalidates one of our initial hypotheses that this phenotype of *chl1* mutants is simply a compensatory response to a general defect in NO_3^- acquisition and thus an indirect consequence of *NRT1.1* mutation. Our proposal for the regulatory role of NRT1.1 in wild-type plants is that the increase in external NO_3^- concentration results in an increasing activity of this transporter, which in turn generates an increasing repressive signal for *NRT2.1* expression (Fig. 4A). Whether this indicates a direct signaling function for NRT1.1 (in analogy with the role of the NO_3^- sensor recently proposed for NRT2.1) and calls for the specific involvement of one isoform of NRT1.1 (high or low affinity) are open questions that deserve further investigation.

To account for our observations, we propose a model for N regulation of *NRT2.1* expression (Fig. 8). In addition to the positive regulation corresponding to the induction by NO_3^-, this model postulates dual negative regulation involving both feedback repression by reduced N metabolites and NRT1.1-mediated repression by high external NO_3^-. An important point is that the absence of NRT1.1-mediated repression (due to

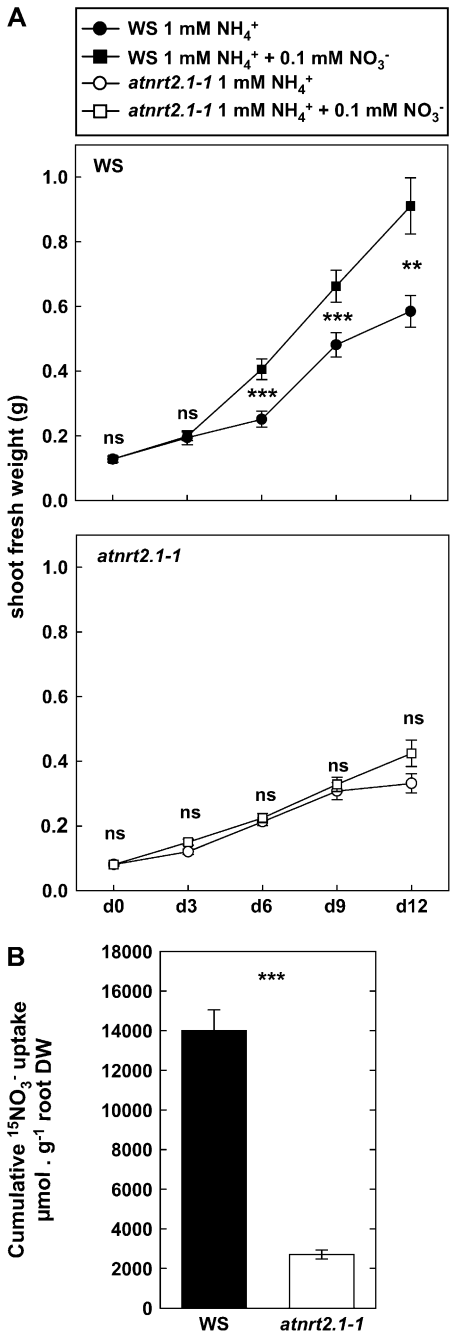

Figure 7. Shoot growth and root NO_3^- uptake of Ws and *atnrt2.1-1* mutant plants supplied with 1 mM NH_4^+ with or without 0.1 mM NO_3^-. Plants were grown hydroponically for 5 weeks on complete nutrient solution containing 1 mM NH_4NO_3 before transfer for 12 d to media containing either 1 mM NH_4^+ alone or 1 mM NH_4^+ plus 0.1 mM $^{15}NO_3^-$. A, Shoot fresh weight. Values are the means of 10 to 18 replicates ± SE. B, Cumulative $^{15}NO_3^-$ uptake from the 1 mM NH_4^+ plus 0.1 mM $^{15}NO_3^-$ solution during the 12-d period determined by total ^{15}N analysis in both root and shoots at the end of the period. Values are the mean of 10 to 12 replicates ± SE. Differences between wild type and mutant are statistically significant at $^{**}P < 0.01$, $^{***}P < 0.001$ (*t* test). Ns, Not significant ($P > 0.05$).

mutation of *NRT1.1* or to low external NO_3^- availability) overrides the negative feedback exerted by reduced N metabolites to yield a high *NRT2.1* expression level even in the presence of ample NH_4^+ supply to the plant (e.g. 5 mM; see Fig. 1B). Conversely, there is also evidence that lack of negative feedback regulation by reduced N metabolites overrides the repressive effect of high external NO_3^- availability. This is shown by the high *NRT2.1* expression level in nitrate reductase-deficient plants supplied with NO_3^- as a sole N source (Lejay et al., 1999; Zhuo et al., 1999). Taken together, these observations suggest that *NRT2.1* expression is suppressed only when both negative regulations by reduced N metabolites and by high NO_3^- are effective (as illustrated in Fig. 8). Whether this means that the two respective signaling pathways directly interfere at some common crucial node or, alternatively, that they are independently strong enough to overcome each other is not known. However, because *NRT1.1* mutation prevents *NRT2.1* repression by high NO_3^-, but does not alter its reinduction by NO_3^- after a period of N starvation (Muños et al., 2004), it is concluded that these opposite actions of NO_3^- most probably involve independent signaling pathways.

NRT1.1-Mediated Regulation of *NRT2.1* Allows an Adaptive Response of the Plant to NH_4^+ Toxicity

A key issue concerning NRT1.1-mediated regulation of the HATS by high NO_3^- is to determine what physiological role such a mechanism may play. In analogy with the well-accepted postulate that repression of root NO_3^- (or NH_4^+) uptake systems by reduced N metabolites corresponds to a regulation by the N demand of the plant (Imsande and Touraine, 1994; vonWirén et al., 2000; Forde, 2002), we hypothesize that repression of *NRT2.1* by high NO_3^- corresponds to regulation by a NO_3^- demand of the plant. Accordingly, relief of this NRT1.1-mediated repression due either to decreased NO_3^- availability in the presence of NH_4^+, or to *NRT1.1* mutation activated the NO_3^- HATS but not the NH_4^+ uptake system (Fig. 3; Muños et al., 2004). This shows that root NO_3^- uptake,

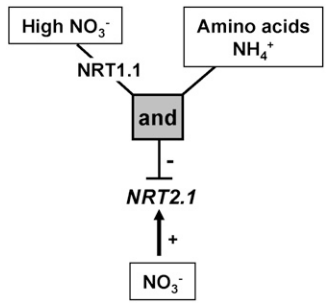

Figure 8. Model for N regulation of *NRT2.1* expression in Arabidopsis roots. The model postulates that, in addition to the induction by NO_3^-, *NRT2.1* is also under dual control by feedback repression by reduced N metabolites and NRT1.1-mediated repression by high external NO_3^- availability, which are both required to suppress *NRT2.1* expression.

and not total root N uptake, is the specific target of this mechanism. Clearly, what the plant perceives under these situations is a lack of NO_3^- and not an overall nutritional N deficiency.

In this context, the significance of the model depicted in Figure 8 is that repression of *NRT2.1* by reduced N metabolites in N-sufficient plants is allowed only when NRT1.1 is active in transporting NO_3^-. This warrants that a significant NO_3^- uptake rate is always ensured in any situation, either by NRT1.1 or by NRT2.1, even when NO_3^- accounts for only a minor fraction of the total N available in the external medium (Fig. 4B). One of the most obvious interests of such a mechanism is to protect the plant against NH_4^+ toxicity. It is known for decades that pure NH_4^+ nutrition is toxic for many plant species (Givan, 1979; Hageman, 1984; Salsac et al., 1987; Kronzucker et al., 2001). In particular, growth of herbaceous dicotyledons such as tomato (*Lycopersicon esculentum*), French bean (*Phaseolus vulgaris*), spinach (*Spinacia oleracea*), and here Arabidopsis, is generally strongly hampered by pure NH_4^+ nutrition, with a decrease in yield of up to 60% as compared with supply of NO_3^- as the sole N source (Salsac et al., 1987). A general observation is, however, that NH_4^+ toxicity is fully prevented by supply of NO_3^- as a N source together with NH_4^+ (Cox and Reisenauer, 1973; Kronzucker et al., 1999; Rahayu et al., 2005). Actually, highest growth rates are generally achieved with mixed $NH_4^+ + NO_3^-$ supplies (Cox and Reisenauer, 1973; Heberer and Below, 1989; Adriaanse and Human, 1993; Cao and Tibbitts, 1993).

Despite this firmly established role of NO_3^- in preventing the detrimental effects of NH_4^+ nutrition, a strong paradox remained unresolved. On the one hand, NO_3^- uptake by the plant was shown to ensure full protection against NH_4^+ toxicity and, on the other hand, NO_3^- uptake systems were shown to be strongly repressed by the supply of high NH_4^+ concentration to the plant. To date, no mechanism was known to stimulate NO_3^- uptake in the presence of potentially toxic concentrations of NH_4^+ in the external medium. We propose that the NRT1.1-mediated regulation of *NRT2.1/NRT3.1* corresponds to such a mechanism because it relieves repression of the HATS under low NO_3^-/high NH_4^+ availability (Figs. 3A and 4). Furthermore, the phenotype of both *atnrt2.1* mutants demonstrates that up-regulation of the HATS under this condition constitutes an essential adaptive response of the plant to avoid NH_4^+ toxicity (actually the only one documented at the molecular level; Figs. 6 and 7; Supplemental Figs. S2 and S3).

A surprising aspect of the HATS repression by high NO_3^- is that it seems to rely on purely local signaling because only the portions of the root system subjected to low NO_3^-/high NH_4^+ availability react in up-regulating *NRT2.1* expression (Fig. 2). Furthermore, high NO_3^- supply on one portion of the root system does not prevent the adaptive response of *NRT2.1* in other portions fed with excess NH_4^+ over NO_3^- (see Fig. 3, lanes b and c). This suggests that the NO_3^- demand governing *NRT2.1* expression is not sensed at the whole-plant level and that the adaptive response of *NRT2.1* aims at stimulating NO_3^- uptake specifically in the root cells experiencing high external NH_4^+ availability. This is in full agreement with the results from split-root experiments on maize (*Zea mays*) and soybean (*Glycine max*), indicating that NO_3^- plays its protective role against NH_4^+ toxicity only when it is locally supplied together with NH_4^+, and not when the two N sources are separately provided to only one half on the root system (Schortemeyer et al., 1993; Saravitz et al., 1994).

Our data thus show that, besides its key role in ensuring the bulk of N acquisition by the plant in many various environmental conditions, NRT2.1 also plays a critical function in maintaining a healthy balance between NO_3^- and NH_4^+ uptake. It is highlighted that, in this latter case, NRT2.1 activity is not required to supply an N source for amino acid synthesis, but to allow the plant to benefit from a specific role of NO_3^- that NH_4^+ cannot fulfill. This illustrates very well why *NRT2.1* cannot be regulated only by feedback repression by N metabolites because this regulation aims at adjusting N uptake to amino acid utilization and is not specific for NO_3^- uptake systems (vonWirén et al., 2000). Interestingly, up-regulation of *NRT2.1* expression by low NO_3^- availability was also observed in the presence of Gln (Supplemental Fig. S1), suggesting that NO_3^- demand signaling may be operative under other circumstances than those associated with NH_4^+ toxicity. It is thus tempting to postulate a more general role for this signaling in regulating NO_3^- acquisition by the plant. Nitrate is not only a nutrient, but also a key signaling compound governing crucial aspects of plant metabolism and development (Crawford, 1995; Stitt, 1999). In particular, NO_3^- regulates many genes related to N or C metabolism (Crawford, 1995; Stitt, 1999), triggers several adaptive responses of root and shoot growth (Forde, 2002; Walch-Liu et al., 2005), and modulates cytokinin signaling (Sakakibara, 2003). Thus, plants may have evolved specific regulatory mechanisms to tightly control these important signaling effects of NO_3^-. At the uptake level, this would require a regulatory mechanism specific for NO_3^- transporters and independent from the feedback regulation by reduced N metabolites, which aims at ensuring efficient use of this ion (as well as of NH_4^+) as a nutrient. In this context, the model of Figure 8 corresponds to an elegant mechanism for integrating both requirements for NO_3^- as a nutrient and as a signal in the regulation of root NO_3^- uptake. Therefore, the question of whether NRT1.1-mediated regulation of *NRT2.1* reported in this work has additional functions other than just protecting the plant from NH_4^+ toxicity deserves further investigation. In particular, given the role of NRT1.1 in regulating *NRT2.1* expression, and the role of NRT2.1 in controlling lateral root initiation, it will be of interest to investigate whether putative NO_3^- signaling mediated by NRT1.1 is also involved in

modulating root branching as a function of external NO_3^- availability.

MATERIALS AND METHODS

Plant Material and Treatments

The Arabidopsis (*Arabidopsis thaliana*) genotypes used in this study were the wild-type Ws and Columbia-0 ecotypes; the *atnrt2.1-1* mutant in the Ws background (formerly *atnrt2a*), obtained from the collection of Institut National de la Recherche Agronomique, Versailles, and deleted for the *NRT2.1* (At1g08090) and *NRT2.2* (At1g08100) genes (Filleur et al., 2001); the *atnrt2.1-2* mutant in the Columbia-0 background, obtained from the Salk Institute (SALK_035429), and carrying a T-DNA insertion in the first intron of *NRT2.1* (these two mutants were renamed according to the nomenclature proposed by Little et al., 2005); and the *atnrt1.2-1* mutant in the Ws background, obtained from the collection of the Institut National de la Recherche Agronomique, and carrying a T-DNA insertion in the third intron of *NRT1.2*. *NRT1.2* mRNA was not detected by reverse transcription (RT)-PCR in the roots of this mutant (data not shown).

Plants were grown for 6 weeks in hydroponics under nonsterile conditions, as previously described by Lejay et al. (1999). The growth chamber was set with the following environmental conditions: 8-h light/16-h dark 22°C/20°C temperature, 250 μmol m^{-2} s^{-1} irradiance, and 70% hygrometry. Briefly, seeds were sown directly on sand contained by a cut 1.5-mL Eppendorf tube closed at the bottom by a stainless grid. Tubes were supported by PVC discs (six Eppendorf/disc) placed on a floating polystyrene raft (12 discs/raft). These systems were disposed on top of 10-L tanks filled with tap water for the first week, and then with nutrient solution for 4 to 5 additional weeks (during this period, nutrient solutions were renewed weekly). The basal nutrient solution common to all experiments included 1 mM KH_2PO_4, 1 mM $MgSO_4$, 0.25 mM K_2SO_4, 0.25 mM $CaCl_2$, 0.1 mM FeNa-EDTA, 50 μM KCl, 30 μM H_3BO_3, 5 μM $MnSO_4$, 1 μm ZnSO4, 1 μM $CuSO_4$, and 0.1 μM $(NH_4)_6Mo_7O_{24}$. For growth of the plants, 1 mM NH_4NO_3 was added to the basal medium as the N source. Depending on the experiments, 1 mM NH_4NO_3 was replaced as a N source by either KNO_3 or NH_4Cl, or various mixtures of these salts, as indicated in the text and figures. The pH of all solutions was adjusted to 5.8, and the solutions were renewed every other day during the experiments to prevent nutrient depletion. For experiments with media at low NO_3^- concentration (0.1 mM), nutrient solutions were renewed daily, which allowed maintenance of the external NO_3^- concentration above 0.06 to 0.07 mM. For treatments with Gln, 25 mg L^{-1} chloramphenicol and 50 mg L^{-1} penicillin were added to solutions to prevent microbial development. For time-course studies, various treatments were initiated at different times to allow harvest of all plants at the same time of the day (7–8 h into the light period) to prevent any diurnal effect on *NRT2.1* expression (Lejay et al., 2003).

For split-root experiments, the protocol was reported previously (Gansel et al., 2001). At the age of 2 weeks, seedlings were cleared to leave only one plant per tube. After gentle separation of the root system into two approximately equal portions, 5-week-old plants were transferred to specific containers and allowed to adapt 3 d to split-root conditions, with the two parts of the root system supplied with the 1 mM NH_4NO_3 solution. The various treatments were then initiated at the end of this period.

For growth analysis, roots and shoot were separated after harvest and their fresh weight determined. Fresh weight data are the means of 10 to 18 replicates.

RNA Extraction and RNA Gel-Blot Analysis

RNA extraction was performed as previously described (Lobreaux et al., 1992) from eight to 12 plants per treatment (except for six plants in the split-root experiment). Ten micrograms of total RNA were then separated by electrophoresis on MOPS-formaldehyde agarose gel and blotted on nylon membrane (Hybond N^+; Amersham-Pharmacia Biotech). Membranes were prehybridized for 2 h at 60°C in church buffer: 0.5 M $NaHPO_4$, 1% bovine serum albumin, and 7% SDS (pH 7.2 with H_3PO_4). Hybridization was performed overnight at 60°C after addition of a randomly primed ^{32}P-labeled cDNA probe in the hybridization buffer. Membranes were washed twice at root temperature for 2 min and twice at 60°C with 0.5× SSC, 0.1% SDS. The probe used in this study corresponds to the full-length of *AtNRT2.1* cDNA (Filleur and Daniel-Vedele, 1999). A 25S rRNA probe was used to normalize quantifications achieved using a phosphor imager (BAS-5000; Fujifilm).

Quantitative RT-PCR

Ten to 15 μg of total RNA were digested by RQ-DNase (Promega). After phenol-chloroform purification and isopropanol precipitation, RNA was reverse transcribed to one-strand cDNA using Moloney murine leukemia virus reverse transcriptase (Promega) and dT(18) V primers, according to the manufacturer's protocol. Gene expression was determined by quantitative real-time PCR (LightCycler; Roche Diagnostics) using gene-specific primers: *NRT2.1* (forward, 5′-aacaagggctaacgtggatg-3′ and reverse, 5′-ctgcttctcctgctcattcc-3′); *NRT3.1* (forward, 5′-ggccatgaagttgcctatg-3′ and reverse, 5′-tcttggccttcctcttctca3-′); *ACT2/8* (forward, 5′-ggtaacattgtgctcagrggtgg-3′ and reverse, 5′-aacgaccttaatcttcatgctgc-3′), and LightCycler FastStart DNA Master SYBR Green (Roche Diagnostics). Expression levels of tested genes were normalized to expression levels of the *ACT2/8* genes (Charrier et al., 2002).

$^{15}NO_3^-$ and $^{15}NH_4^+$ Uptake

Influx of either $^{15}NO_3^-$ or $^{15}NH_4^+$ into the roots was assayed as described previously (Lejay et al., 1999) by 5-min labeling in basal nutrient medium (pH 5.8) supplemented with appropriate concentrations of $K^{15}NO_3$ or $^{15}NH_4Cl$ (atom % ^{15}N excess: 99%). For specific determination of the activity of the HATS, $^{15}NO_3^-$ or $^{15}NH_4^+$ were at 0.2 mM in the labeling solution. Cumulative NO_3^- uptake during long-term growth studies (10–12 d) was assayed by supplying the plants with nutrient solution containing $^{15}NO_3^-$ (atom % ^{15}N excess: 1%) for the whole experimental period and by measuring total ^{15}N accumulation in roots and shoots at the end of this period. Each influx or cumulative NO_3^- uptake value is the mean of eight to 12 replicates.

The total N content and atomic percentage ^{15}N abundance of the samples were determined by continuous-flow mass spectrometry, as described previously (Clarkson et al., 1996), using a Euro-EA Eurovector elemental analyzer coupled with an IsoPrime mass spectrometer (GV Instruments).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Up-regulation of *NRT2.1* in the presence of Gln.

Supplemental Figure S2. NH_4^+ toxicity in the *atnrt2.1-2* mutant.

Supplemental Figure S3. NH_4^+ toxicity in the *atnrt2.1-1* mutant.

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