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# Regulation of High-Affinity Nitrate Uptake in Roots of *Arabidopsis* Depends Predominantly on Posttranscriptional Control of the NRT2.1/NAR2.1 Transport System<sup>1[W][OA]</sup>

Edith Laugier, Eléonore Bouguyon, Adeline Mauriès, Pascal Tillard, Alain Gojon, and Laurence Lejay\*

Institut de Biologie Intégrative des Plantes, Claude Grignon, Unité Mixte de Recherche 5004, Biochimie et Physiologie Moléculaire des Plantes, Agro-M/Centre National de la Recherche Scientifique/Institut National de la Recherche Agronomique/SupAgro/UM2, F-34060 Montpellier, France

In *Arabidopsis* (*Arabidopsis thaliana*), the *NRT2.1* gene codes for the main component of the root nitrate ( $\text{NO}_3^-$ ) high-affinity transport system (HATS). Due to the strong correlation generally found between high-affinity root  $\text{NO}_3^-$  influx and *NRT2.1* mRNA level, it has been postulated that transcriptional regulation of *NRT2.1* is a key mechanism for modulation of the HATS activity. However, this hypothesis has never been demonstrated, and is challenged by studies suggesting the occurrence of posttranscriptional regulation at the *NRT2.1* protein level. To unambiguously clarify the respective roles of transcriptional and posttranscriptional regulations of *NRT2.1*, we generated transgenic lines expressing a functional *35S::NRT2.1* transgene in an *atnrt2.1* mutant background. Despite a high and constitutive *NRT2.1* transcript accumulation in the roots, the HATS activity was still down-regulated in the *35S::NRT2.1* transformants in response to repressive nitrogen or dark treatments that strongly reduce *NRT2.1* transcription and  $\text{NO}_3^-$  HATS activity in the wild type. In some treatments, this was associated with a decline of *NRT2.1* protein abundance, indicating posttranscriptional regulation of *NRT2.1*. However, in other instances, *NRT2.1* protein level remained constant. Changes in abundance of *NAR2.1*, a partner protein of *NRT2.1*, closely followed those of *NRT2.1*, and thus could not explain the close-to-normal regulation of the HATS in the *35S::NRT2.1* transformants. Even if in certain conditions the transcriptional regulation of *NRT2.1* contributes to a limited extent to the control of the HATS, we conclude from this study that posttranscriptional regulation of *NRT2.1* and/or *NAR2.1* plays a predominant role in the control of the  $\text{NO}_3^-$  HATS in *Arabidopsis*.

The uptake of nitrate ( $\text{NO}_3^-$ ) by roots cells from the soil solution is the main process of nitrogen (N) acquisition in most herbaceous plant species (Marschner, 1995). It relies on the combined activity of both high- and low-affinity transport systems (HATS and LATS, respectively) that together ensure an efficient intake over a wide range of external  $\text{NO}_3^-$  concentrations (Crawford and Glass, 1998; Miller et al., 2007). However, with the exception of well-fertilized agricultural systems,  $\text{NO}_3^-$  concentrations in the soil are often below the millimolar range, making the HATS particularly important for nutrition of the plant (Marschner, 1995; Malagoli et al., 2004). To date, the membrane

carriers associated with root uptake of  $\text{NO}_3^-$  belong to either NRT1 or NRT2 transporter families (Miller et al., 2007; Tsay et al., 2007). In general, NRT1 proteins are low-affinity transports, whereas NRT2 proteins are believed to be active in high-affinity transport only (Miller et al., 2007; Tsay et al., 2007).

In *Arabidopsis* (*Arabidopsis thaliana*), at least three transporters (*NRT1.1*, *NRT2.1*, and *NRT2.2*) have been proposed to contribute to the  $\text{NO}_3^-$  HATS (Li et al., 2007; Tsay et al., 2007), but it is now clear that the HATS activity, in most environmental conditions, is predominantly dependent on the *NRT2.1* protein. Null mutants for *NRT2.1* have lost up to 75% of the HATS activity (Cerezo et al., 2001; Filleur et al., 2001; Li et al., 2007), and consequently cannot grow normally with  $\text{NO}_3^-$  as sole nitrogen (N) source when provided at a low concentration (e.g. <1 mM; Orsel et al., 2004). However, as other NRT2 proteins identified in *Chlamydomonas reinhardtii* and barley (*Hordeum vulgare*; Quesada et al., 1994; Tong et al., 2005), the *Arabidopsis* *NRT2.1* protein needs to interact with a *NAR2*-like partner protein (*NAR2.1/NRT3.1*) to be functional in  $\text{NO}_3^-$  transport (Okamoto et al., 2006; Orsel et al., 2006). Both gene products need to be coexpressed to yield  $\text{NO}_3^-$  uptake in *Xenopus* oocytes (Orsel et al., 2006), and *nar2.1* null mutants lack the *NRT2.1* protein at the plasma mem-

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\* Corresponding author; e-mail lejay@supagro.inra.fr.

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brane (Wirth et al., 2007), and are strongly deficient in  $\text{NO}_3^-$  HATS (Okamoto et al., 2006; Orsel et al., 2006). The precise function of NAR2.1 remains unclear, but it has been recently proposed that the active form of the transporter is in fact a NRT2.1/NAR2.1 heterooligomer (Yong et al., 2010).

A key feature of the  $\text{NO}_3^-$  HATS activity is that it can be rapidly and markedly modulated, to allow a fine coordination of the high-affinity root  $\text{NO}_3^-$  uptake with the N demand of the whole plant as well as its response to signaling mechanisms integrating N and carbon metabolisms (Crawford and Glass, 1998; Forde, 2002; Glass et al., 2002; Miller et al., 2007; Gojon et al., 2009; Nunes-Nesi et al., 2010). Furthermore, many studies have shown that regulation of the HATS is always highly correlated with changes in *NRT2.1* transcript accumulation in the roots. In more detail, both  $\text{NO}_3^-$  HATS activity and *NRT2.1* expression are similarly induced by  $\text{NO}_3^-$  (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999; Zhuo et al., 1999), repressed by high N supply to the plant (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001; Gansel et al., 2001), repressed by darkness (Lejay et al., 1999), and stimulated by sugar supply to the roots (Lejay et al., 2003, 2008). All these changes in *NRT2.1* expression (mRNA accumulation) were found to be associated with parallel changes in the activity of the *NRT2.1* promoter (Girin et al., 2007, 2010). Thus, it has often been hypothesized that the transcriptional control of *NRT2.1* is a major mechanism for regulation of the  $\text{NO}_3^-$  HATS in Arabidopsis (Forde, 2002; Miller et al., 2007; Gojon et al., 2009; Girin et al., 2010). As a consequence, many studies have focused on the identification of regulatory proteins governing *NRT2.1* gene expression (Muños et al., 2004; Krouk et al., 2006; Castaings et al., 2009; Ho et al., 2009; Wang et al., 2009; Girin et al., 2010; Widiez et al., 2011).

However, several reports have pointed out that NRT2 transporters may also be strongly regulated at the posttranscriptional level. First, overexpression of the *NpNRT2.1* gene in *Nicotiana plumbaginifolia* succeeded in stimulating the HATS activity when plants were supplied with  $\text{NO}_3^-$  as sole N source, but not when plants were fed with a mixed ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ) N source (Fraisier et al., 2000), indicating that *NRT2.1* transcript accumulation was not the limiting factor in the latter case. More recently, the abundance of the Arabidopsis NRT2.1 protein in the root plasma membrane was reported to be little affected by treatments that strongly regulate both *NRT2.1* transcript accumulation and  $\text{NO}_3^-$  HATS activity (Wirth et al., 2007). In barley too, changes in HvNRT2.1 protein were not correlated with those of HvNRT2.1 transcript accumulation during induction by  $\text{NO}_3^-$  (Ishikawa et al., 2009). Finally, studies on NRT2 homologs in lower eukaryotes, e.g. NrtA in *Aspergillus nidulans* (Wang et al., 2007) and YNT1 in *Hansenula polymorpha* (Navarro et al., 2008) have also evidenced the occurrence of posttranscriptional regulatory mechanisms for these proteins. Altogether, these data question the role of *NRT2.1* transcription as a major process

for the regulation of root  $\text{NO}_3^-$  uptake, and suggest that posttranscriptional events affecting abundance and/or activity of the NRT2.1 protein may actually have a stronger impact on functionality of the HATS.

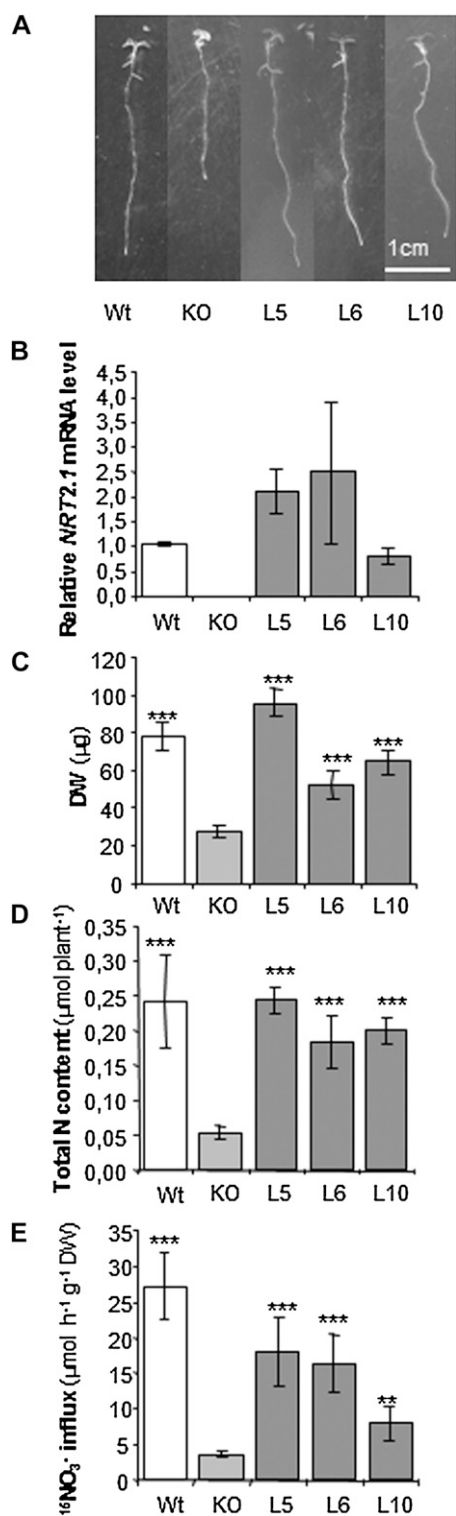
The aim of our work was to clarify the respective importance of transcriptional and posttranscriptional regulations of *NRT2.1* expression in the modulation of the  $\text{NO}_3^-$  HATS activity in Arabidopsis. Therefore, our strategy was to suppress the transcriptional level of control by expressing a *35S::NRT2.1* transgene in a *nrt2.1* null-mutant background, and to determine whether this prevented, or not, regulation of the  $\text{NO}_3^-$  HATS in response to N or light treatments known to affect both *NRT2.1* transcription and root  $\text{NO}_3^-$  acquisition in wild-type plants. The outcome of this study is that, although the transcriptional regulation of *NRT2.1* may account for part of the control, posttranscriptional regulatory mechanisms clearly play a predominant role in the response of the HATS to environmental cues.

## RESULTS

### Functional Complementation of the *atnrt2.1-2* Mutant by a *35S::NRT2.1* Transgene

To prevent transcriptional regulation of *NRT2.1*, we transformed the *atnrt2.1-2* mutant (Little et al., 2005), with a *35S::NRT2.1* construct containing the genomic sequence of *NRT2.1*, fused to the constitutive cauliflower mosaic virus 35S promoter (see "Materials and Methods"). After selection of the transformants, we obtained three independent, homozygous, single-insertion transgenic lines (L5, L6, and L10) that we selected for further investigation on the basis of high *NRT2.1* mRNA level in the roots.

Because of its crucial role in the  $\text{NO}_3^-$  HATS (Cerezo et al., 2001; Filleur et al., 2001; Li et al., 2007), disruption of *NRT2.1* results in a dramatic growth reduction when plants are cultivated on media with low  $\text{NO}_3^-$  (<1 mM) concentration (Orsel et al., 2004; Li et al., 2007). Indeed, when grown for 8 d in vertical petri agar plates containing 0.2 mM  $\text{NO}_3^-$  as a N source, *atnrt2.1-2* plants show severely impaired primary root growth (Fig. 1A), and an average of 3.1- and 4.6-fold decrease in total dry biomass accumulation and total N content as compared to wild-type plants, respectively (Fig. 1, C and D). Root  $^{15}\text{NO}_3^-$  influx assays by short-term labeling at 0.2 mM external  $^{15}\text{NO}_3^-$  concentration confirmed that under these conditions, the mutant has lost up to 85% to 90% of the HATS activity as compared to the wild type (Fig. 1E). Therefore, we used these conditions to determine whether the *35S::NRT2.1* transgene was actually expressed in the transformants, and could functionally complement the *atnrt2.1-2* mutant phenotype in restoring both root  $\text{NO}_3^-$  acquisition and growth at the level of wild-type plants. When assayed at day 8, the three transgenic lines (L5, L6, and L10) displayed a similar or higher level of *NRT2.1* mRNA accumulation in the roots as compared to the wild type (Fig. 1B). The *35S::NRT2.1* construct was able to rescue, either totally (L5) or



**Figure 1.** Characterization of Arabidopsis transgenic lines expressing *NRT2.1* under the control of the 35S promoter. Wild type (WT), *nrt2.1-2* knockout mutant (KO), and three transgenic lines (L5, L6, and L10) were grown in vitro during 8 d on 0.2 mM KNO<sub>3</sub> as N source. A, Primary root growth after 8 d. B, Root *NRT2.1* expression quantified by quantitative (Q)-PCR. Values are means of two biological replicates ± SD. C, Dry weight (DW) of 8-d-old plants. Values are means of six replicates ± SD.

partially (L6 and L10), defects in primary root growth (Fig. 1A), dry biomass accumulation (Fig. 1C), and total N content of the seedlings (Fig. 1D). This shows that the transformants performed an efficient high-affinity NO<sub>3</sub><sup>-</sup> uptake, resulting from the expression of a functional HATS under these conditions. Accordingly, high-affinity root <sup>15</sup>NO<sub>3</sub><sup>-</sup> influx in all three transformants lines was strongly enhanced as compared to the mutant, although it remained lower than that in the wild type (Fig. 1E).

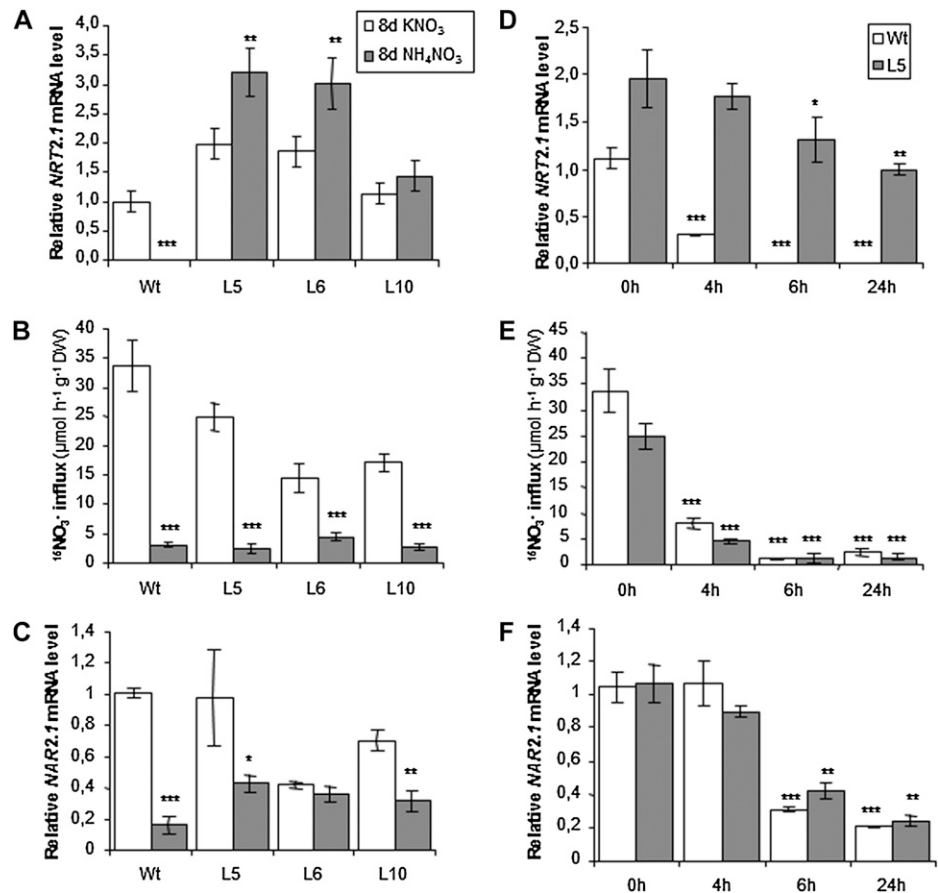
#### Constitutive Expression of *NRT2.1* Does Not Suppress Feedback Repression of the HATS by High N Supply

To investigate the role of the transcriptional regulation of *NRT2.1* in the control of the HATS activity, we first explored the response of both root *NRT2.1* mRNA accumulation and root <sup>15</sup>NO<sub>3</sub><sup>-</sup> influx in the transformants to the repressive action of high N provision in the medium. Furthermore, root *NAR2.1* mRNA accumulation was also measured, to determine whether putative changes of the HATS activity in the transformants could be accounted for by regulation of *NAR2.1* expression. Since supply of ammonium (NH<sub>4</sub><sup>+</sup>) in addition to NO<sub>3</sub><sup>-</sup> was previously proposed to trigger posttranscriptional regulation of the HATS in *N. plumbaginifolia* (Fraisier et al., 2000), our first series of experiments aimed at comparing L5, L6, and L10 transgenic lines with wild-type plants after 8 d of growth either with 0.2 mM KNO<sub>3</sub> or 10 mM NH<sub>4</sub>NO<sub>3</sub> as a N source. As shown in Figure 2A, *NRT2.1* expression was almost totally suppressed in wild-type plants grown on 10 mM NH<sub>4</sub>NO<sub>3</sub>, while the *NRT2.1* mRNA level in the roots of the 35S::*NRT2.1* lines remained constitutively high under both N regimes. On 10 mM NH<sub>4</sub>NO<sub>3</sub>, due to the strong repression in wild-type plants, this level was 530-, 500-, and 240-fold higher in L5, L6, and L10 than in Columbia-0 (Col-0), respectively. Nevertheless, despite this tremendous difference in *NRT2.1* mRNA accumulation, root <sup>15</sup>NO<sub>3</sub><sup>-</sup> influx was markedly inhibited on 10 mM NH<sub>4</sub>NO<sub>3</sub> as compared with 0.2 mM KNO<sub>3</sub> in all genotypes (Fig. 2B). *NAR2.1* mRNA accumulation in roots was also down-regulated by high N supply (Fig. 2C), but to a much lower extent than root <sup>15</sup>NO<sub>3</sub><sup>-</sup> influx, especially in the transgenic plants. These data demonstrate that transcriptional regulation of *NRT2.1* is not strictly required for feedback repression of HATS activity by high NH<sub>4</sub>NO<sub>3</sub> provision, and that posttranscriptional mechanisms, possibly associated with altered *NAR2.1* expression, are sufficient to inhibit high-affinity NO<sub>3</sub><sup>-</sup> uptake under these conditions.

In the above experiments, we analyzed the HATS regulation in plants subjected to long-term (8 d) repressive treatment on 10 mM NH<sub>4</sub>NO<sub>3</sub>. This prevented unraveling any putative delay in the HATS repression

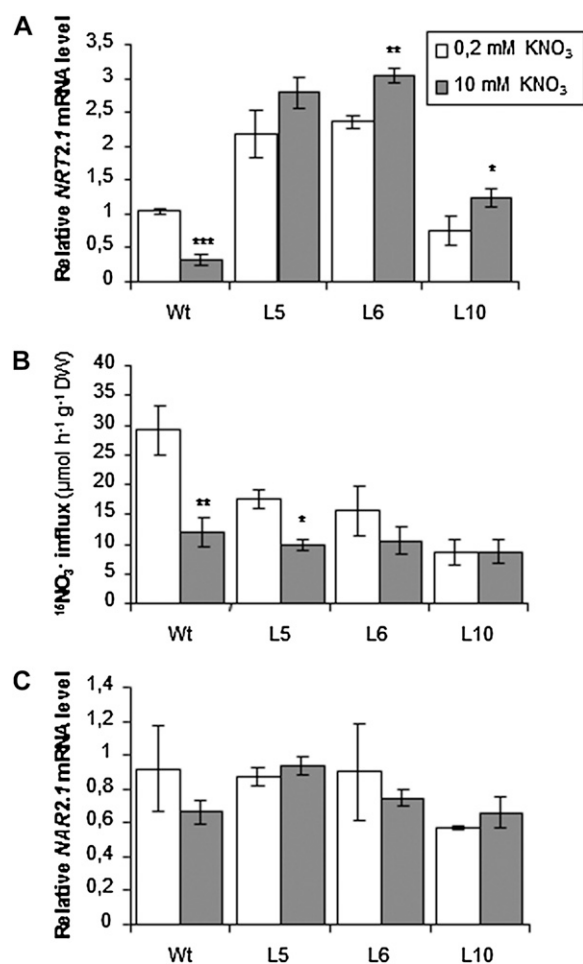
D, Total N contents quantified after 8 d of growth. Values are means of six replicates ± SD. E, Root NO<sub>3</sub><sup>-</sup> influx measured at the external concentration of 0.2 mM <sup>15</sup>NO<sub>3</sub><sup>-</sup>. Values are means of 12 replicates ± SD. Differences between WT/transformants and the KO mutant are significant at \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 (Student's *t* test).

**Figure 2.**  $\text{NO}_3^-$  influx, *NRT2.1*, and *NAR2.1* mRNA level in roots of wild-type (WT) and transgenic plants in response to 10 mM  $\text{NH}_4\text{NO}_3$ . A to C, Plants were grown in vitro during 8 d on 0.2 mM  $\text{KNO}_3$  (white bars) or 10 mM  $\text{NH}_4\text{NO}_3$  (gray bars) as N source. D to F, WT plants (white bars) and the transgenic line L5 (gray bars) were grown in vitro on 0.2 mM  $\text{KNO}_3$  and treated during 4, 6, and 24 h by the addition of 2 mL of a 250 mM  $\text{NH}_4\text{NO}_3$  solution to reach a final concentration of 10 mM  $\text{NH}_4\text{NO}_3$ . A and D, Root *NRT2.1* expression quantified by Q-PCR. Values are means of two biological replicates  $\pm$  SD. B and E, Root  $\text{NO}_3^-$  influx measured at the external concentration of 0.2 mM  $^{15}\text{NO}_3^-$ . Values are means of 12 replicates  $\pm$  SD. C and F, Root *NAR2.1* expression quantified by Q-PCR. Values are means of two biological replicates  $\pm$  SD. Differences between plants on  $\text{KNO}_3$  and  $\text{NH}_4\text{NO}_3$  are significant at \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (Student's *t* test).



that could have been caused by constitutive expression of *NRT2.1*. To rule out this hypothesis, we performed time-course experiments with L5 and wild-type plants supplied with 10 mM  $\text{NH}_4\text{NO}_3$  for up to 24 h. As compared to control plants left on 0.2 mM  $\text{KNO}_3$ , expression of *NRT2.1* in the wild type was already repressed 3.6-fold as soon as 4 h following addition of 10 mM  $\text{NH}_4\text{NO}_3$  in the medium, and decreased further to become barely detectable after 6 and 24 h (Fig. 2D). In L5 plants, the level of *NRT2.1* mRNA remained very high after 10 mM  $\text{NH}_4\text{NO}_3$  supply, although it was slightly reduced after 6 and 24 h of repressive conditions (Fig. 2D). Despite the high and relatively stable level of *NRT2.1* mRNA in L5 plants, root  $^{15}\text{NO}_3^-$  influx was rapidly and similarly down-regulated in wild-type and L5 plants when compared to the controls on 0.2 mM  $\text{KNO}_3$  (Fig. 2E). Unlike *NRT2.1* mRNA in wild-type plants, *NAR2.1* transcript accumulation in both Col-0 and L5 plants started to decrease only after 6 h of  $\text{NH}_4\text{NO}_3$  supply, and remained significant (approximately 20% of the controls) even after 24 h of treatment (Fig. 2F). These conclusions were confirmed with the L10 transgenic line (Supplemental Fig. S1). Thus, these data show that down-regulation of HATS activity by high  $\text{NH}_4\text{NO}_3$  supply occurs as fast in the transgenic lines as in the wild type, and occurs before any visible change in *NAR2.1* mRNA accumulation.

To determine if the repression of HATS activity in *35S::NRT2.1* transformants was specifically due to the presence of  $\text{NH}_4^+$  in the external medium (as suggested by Fraiser et al., 2000), or was the consequence of high N provision, plants were grown for 8 d on either 0.2 or 10 mM  $\text{KNO}_3$  as the sole N source. As compared to 0.2 mM, supply of 10 mM  $\text{KNO}_3$  significantly repressed *NRT2.1* expression in the roots of wild-type plants, although to a lesser extent than 10 mM  $\text{NH}_4\text{NO}_3$  (compare Fig. 3A with 2A), but had little effect on *NRT2.1* mRNA accumulation in transgenic lines (Fig. 3A). As a consequence, the transgenic lines overexpressed *NRT2.1* mRNA 3- to 9-fold when compared to the wild type on 10 mM  $\text{NO}_3^-$ . Interestingly, high  $\text{NO}_3^-$  supply also markedly reduced HATS-mediated  $^{15}\text{NO}_3^-$  influx in wild-type plants (by 60%), but not so much in the transgenic lines where root  $^{15}\text{NO}_3^-$  influx was lowered at most by 30% to 40% as compared to the controls (Fig. 3B). Unlike with 10 mM  $\text{NH}_4\text{NO}_3$ , *NAR2.1* mRNA levels were not significantly affected by 10 mM instead of 0.2 mM  $\text{KNO}_3$  supply, in any of the genotypes (Fig. 3C). Altogether, these data suggest that down-regulation of the HATS activity by high  $\text{NO}_3^-$  (10 mM) supply (1) cannot be suppressed, but can possibly be attenuated by constitutive expression of *NRT2.1*, and (2) cannot be explained by concurrent repression of *NAR2.1* expression.



**Figure 3.** NO<sub>3</sub><sup>-</sup> influx, *NRT2.1*, and *NAR2.1* mRNA level in roots of wild-type and transgenic plants in response to 10 mM KNO<sub>3</sub>. Plants were grown in vitro during 8 d on 0.2 mM KNO<sub>3</sub> (white bars) or 10 mM KNO<sub>3</sub> (gray bars). A, Root *NRT2.1* expression quantified by Q-PCR. Values are means of two biological replicates ± SD. B, Root NO<sub>3</sub><sup>-</sup> influx measured at the external concentration of 0.2 mM <sup>15</sup>NO<sub>3</sub><sup>-</sup>. Values are means of 12 replicates ± SD. C, Root *NAR2.1* expression quantified by Q-PCR. Values are means of two biological replicates ± SD. Differences between plants grown on 0.2 mM KNO<sub>3</sub> and 10 mM KNO<sub>3</sub> are significant at \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 (Student's *t* test).

### Constitutive Expression of *NRT2.1* Does Not Suppress Feedback Repression of the HATS by Darkness

Sugar production through photosynthesis in the light is another main regulatory factor stimulating *NRT2.1* expression and NO<sub>3</sub><sup>-</sup> HATS activity (Lejay et al., 2003, 2008). As a consequence, *NRT2.1* mRNA accumulation and HATS activity undergo marked diurnal rhythms, with a gradual repression following light/dark transition (Lejay et al., 1999). To determine the functional impact of constitutive *NRT2.1* expression on the response of the HATS to photosynthesis, 8-d-old plants grown in vitro on a medium containing 0.2 mM KNO<sub>3</sub> were transferred to the dark for 4 or 24 h following a period of 10 or 8 h in the light, respectively. In the wild type, as expected, *NRT2.1* expression de-

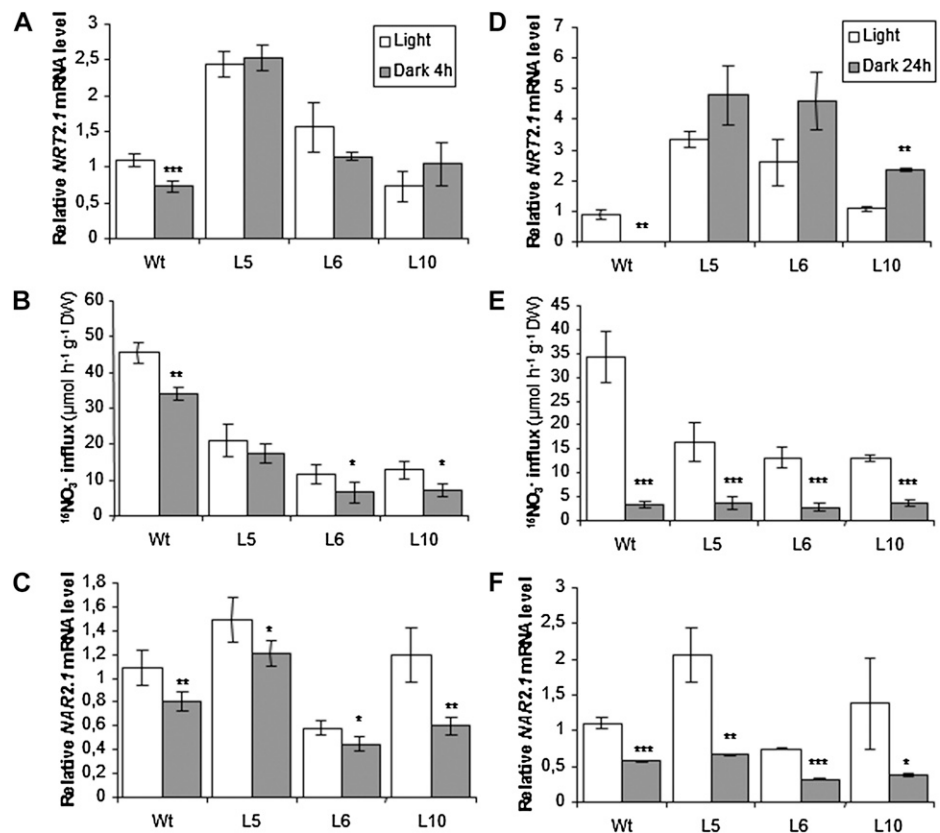
creased by 34% and 98% after 4 and 24 h in darkness, respectively (Fig. 4, A and D). Accordingly, these treatments resulted in a 24% and 90% decrease in root <sup>15</sup>NO<sub>3</sub><sup>-</sup> influx, respectively (Fig. 4, B and E). In the transgenic lines, light/dark transition had no impact on the *NRT2.1* mRNA levels, which remained high even after 4 or 24 h in the dark. After 4 h in darkness, root <sup>15</sup>NO<sub>3</sub><sup>-</sup> influx was only slightly reduced in the L6 and L10 lines, and not affected in the L5 line, as compared to the control plants kept in the light (Fig. 4B), whereas after 24 h in the dark, strong down-regulation of root <sup>15</sup>NO<sub>3</sub><sup>-</sup> influx was observed in all transgenic lines, in spite of the fact that they dramatically overaccumulated *NRT2.1* mRNA as compared to the wild type. The response of *NAR2.1* expression to dark treatments in both wild-type and transgenic lines mirrored that of *NRT2.1* in the wild type (Fig. 4, C and F), however with a much reduced amplitude since *NAR2.1* mRNA level was only decreased by approximately 50% to 70% after 24 h of darkness (against 98% for *NRT2.1* mRNA in wild-type plants). Thus, as it was the case for repression by high N supply, repression of the HATS activity by darkness (1) cannot be suppressed by constitutive expression of *NRT2.1*, and (2) is much stronger than down-regulation of *NAR2.1* expression.

### Regulation of *NRT2.1* and *NAR2.1* Protein Abundance Does Not Always Explain Repression of HATS by High N Supply or Darkness

According to the above results, changes in *NAR2.1* expression only poorly correlate with those of the HATS activity. However, changes in *NAR2.1* mRNA may not reflect those of *NAR2.1* protein. Therefore, a specific polyclonal anti-*NAR2.1* antibody was raised in rabbit, against a peptidic sequence within the N terminus of the protein, to further investigate if changes in *NAR2.1* protein level could explain the regulation of the HATS activity. The affinity-purified anti-*NAR2.1* antibody was tested on western blots with total microsomal membranes purified from roots of hydroponically grown wild-type plants or *nrt2.1-2* and *nar2.1-1* mutants (Supplemental Fig. S2). In the wild-type and *nrt2.1-2* mutant, the anti-*NAR2.1* antibody revealed one band at approximately 25 kD that corresponds to the theoretical *M<sub>r</sub>* of *NAR2.1* at 23.4 kD. This band was specific for *NAR2.1* since it was absent in the microsomal membranes from *nar2.1-1* mutant. It is interesting to note that the level of *NAR2.1* protein was much lower in the *nrt2.1-2* mutant compared to the wild type, suggesting that the lack of *NRT2.1* has an impact on *NAR2.1* accumulation. This link between the level of *NRT2.1* and *NAR2.1* proteins has already been observed in *NAR2.1* KO mutants, in which the lack of *NAR2.1* prevents accumulation of the *NRT2.1* protein at the plasma membrane (Wirth et al., 2007; Yong et al., 2010).

In previous studies the level of *NRT2.1* protein has been shown to be very stable compared to *NRT2.1* mRNA level and HATS activity in response to repressive environmental conditions such as high N or dark-

**Figure 4.**  $\text{NO}_3^-$  influx, *NRT2.1*, and *NAR2.1* mRNA level in roots of wild-type and transgenic plants in response to darkness. A to C, Plants were grown in vitro for 8 d on 0.2 mM  $\text{KNO}_3$  as N source and harvested after 14 h of light (white bars) or 10 h of light + 4 h of darkness (dark bars). D to F, Plants were grown in vitro for 7 d on 0.2 mM  $\text{KNO}_3$  as N source and transferred, after 8 h of light, either in continuous dark (dark bars) or in continuous light (white bars) for 24 h. A and D, Root *NRT2.1* expression quantified by Q-PCR. Values are means of two biological replicates  $\pm$  SD. B and E, Root  $\text{NO}_3^-$  influx measured at the external concentration of 0.2 mM  $^{15}\text{NO}_3^-$ . Values are means of 12 replicates  $\pm$  SD. C and F, Root *NAR2.1* expression quantified by Q-PCR. Values are means of two biological replicates  $\pm$  SD. Differences between plants treated in the light and in the dark are significant at \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (Student's *t* test).



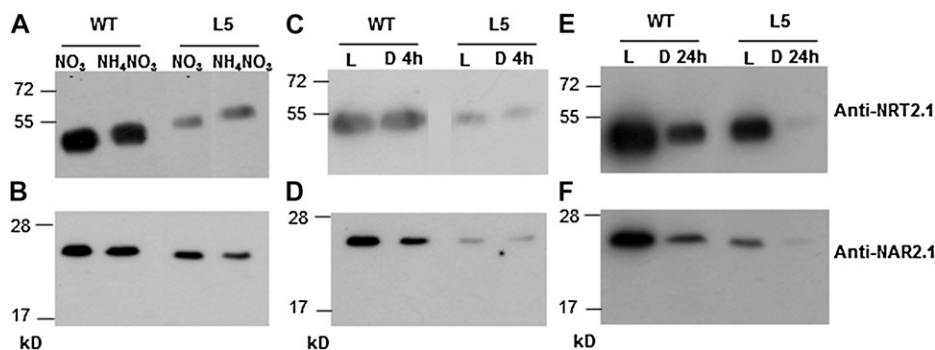
ness (Girin et al., 2007; Wirth et al., 2007). *NRT2.1* protein level was only decreased after long-term treatments with 10 mM  $\text{NH}_4\text{NO}_3$ . To determine if the level of *NAR2.1* protein could explain the repression of HATS activity in conditions where *NRT2.1* level is stable, *NAR2.1* and *NRT2.1* protein levels were measured in response to 4 h of 10 mM  $\text{NH}_4\text{NO}_3$  provision, and to 4 or 24 h of darkness in the wild-type and in L5 plants (Fig. 5). Western blots, using the anti-*NAR2.1* antibody described above and the antibody against *NRT2.1* previously described by Wirth et al. (2007), revealed two different situations for both *NRT2.1* and *NAR2.1*. In response to treatments of 4 h with  $\text{NH}_4\text{NO}_3$  or 4 h of darkness the level of *NRT2.1* and *NAR2.1* proteins were not affected in both wild-type and L5 roots (Fig. 5, A-D), whereas after 24 h of darkness the level of *NAR2.1* protein was reduced along with the level of *NRT2.1* protein (Fig. 5, E and F). This last result was surprising for *NRT2.1* since Wirth et al. (2007) found that the accumulation of *NRT2.1* was not affected after 24 h of darkness in wild-type plants. However, the growth conditions strongly differed between our present study (8 d of growth in vitro) and that of Wirth et al. (2007; 6 weeks of growth in hydroponics). When we performed western blots on wild-type plants grown for 6 weeks in hydroponics, like in Wirth et al. (2007), we also found that in the wild type the levels of both *NRT2.1* and *NAR2.1* proteins were stable after 20 h of darkness (Supplemental Fig. S3). It suggests that, when plants were grown in vitro during 8 d, the levels of

*NRT2.1* and *NAR2.1* proteins respond much faster to darkness than when plants were older and grown in hydroponics. Furthermore, as observed in the *nrt2.1-2* mutant (Supplemental Fig. S2), the level of *NAR2.1* was always lower in L5 plants compared to the wild type along with the level of *NRT2.1* protein. It confirms that the *35S::NRT2.1* construction does not fully complement the *nrt2.1-2* mutant and further illustrates the apparent strong link between the level of *NRT2.1* and *NAR2.1* proteins.

Altogether the results show that, for short treatments of 4 h of 10 mM  $\text{NH}_4\text{NO}_3$  or darkness, the regulation of *NRT2.1* and *NAR2.1* protein levels cannot explain the repression of HATS activity. However, for long treatments with high N or darkness, post-transcriptional mechanisms are involved to decrease the synthesis or increase the degradation of *NRT2.1*, and possibly *NAR2.1*, proteins, and thus participate in the repression of HATS activity.

#### The Quantitative Importance of the Transcriptional Control of *NRT2.1* and Other High-Affinity $\text{NO}_3^-$ Transporters in the HATS Regulation

Altogether, the data presented above clearly indicate that constitutive expression of *NRT2.1* does not eliminate the  $\text{NO}_3^-$  HATS repression by either high N supply or darkness. However, this may not mean that changes in *NRT2.1* transcription play no role at all in this regulation. Indeed, in some instances (see Figs. 3



**Figure 5.** Immunoblot for NRT2.1 (A, C, and E) and NAR2.1 (B, D, and F) using microsomes from roots of wild type (WT) and the transgenic line L5. A and B, Plants were grown in vitro during 8 d on 0.2 mM KNO<sub>3</sub> and treated during 4 h by the addition of 2 mL of a 250 mM NH<sub>4</sub>NO<sub>3</sub> solution to reach a final concentration of 10 mM NH<sub>4</sub>NO<sub>3</sub>. C and D, Plants were grown in vitro for 8 d on 0.2 mM KNO<sub>3</sub> as N source and harvested after 14 h of light or 10 h of light + 4 h of darkness. E and F, Plants were grown in vitro for 7 d on 0.2 mM KNO<sub>3</sub> as N source and transferred, after 8 h of light, either in continuous dark or in continuous light for 24 h. Samples were separated on a 11% SDS-PAGE gel (12 μg protein/lane).

and 4), it appeared that down-regulation of the HATS activity was somehow less pronounced in the transgenic plants as compared to the wild type. To precisely quantify this apparent difference in a more general way, we plotted together all individual values obtained for repression of root <sup>15</sup>NO<sub>3</sub><sup>-</sup> influx in the various genotypes and in response to the various treatments (Fig. 6). This unraveled that in most cases, down-regulation of HATS activity by repressive treatments was attenuated in transgenic plants as compared to the wild type. Thus, although certainly not decisive, transcriptional regulation of *NRT2.1* does seem to contribute to the response of the HATS to the N and light signals investigated here.

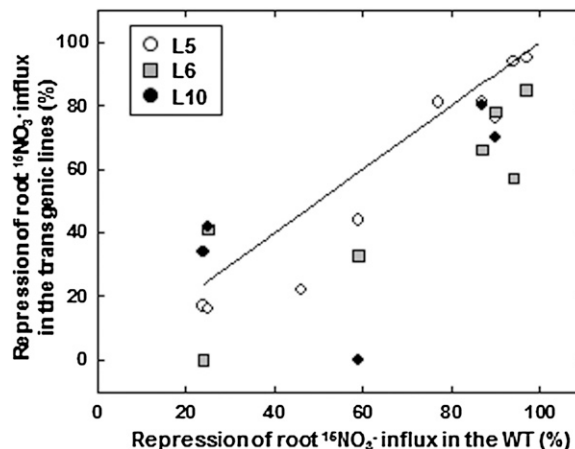
To rule out the possibility that a lower repression of the HATS in the transgenic lines as compared to the wild type could be due to a specific up-regulation in these lines of the other transporters participating in the HATS, root mRNA levels of *NRT2.2* and *NRT1.1* were measured in response to the various treatments in wild-type and L5 plants (Supplemental Fig. S4). As expected *NRT2.2* mRNA was not recorded in L5 plants that have a *nrt2.1-2* mutant background, described as a double mutant for both *NRT2.1* and *NRT2.2* (Li et al., 2007). In no instance *NRT1.1* was found to be overexpressed in L5 plants as compared to wild-type plants, and its regulation was similar in both genotypes.

## DISCUSSION

### Transcriptional versus Posttranscriptional Regulation of NRT2.1 in the HATS Control

As outlined in numerous studies (for review, see Forde, 2002; Glass et al., 2002; Tsay et al., 2007; Gojon et al., 2009), the regulation of the NO<sub>3</sub><sup>-</sup> HATS is of major importance for adaptation of the plants to fluctuating environmental conditions. In particular, its modulation allows a tight coordination between N acquisition and

growth of the plant in response to external or internal factors, such as changes in NO<sub>3</sub><sup>-</sup> availability in the soil or changes in photosynthesis in the shoot. It has been postulated for long that the HATS regulation is predominantly ensured by transcriptional control of *NRT2.1* expression (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001; Gansel et al., 2001; Glass et al., 2002; Nazoa et al., 2003; Girin et al., 2007). However, recent studies at the NRT2 protein level have challenged this conclusion by evidencing a lack of correlation between NRT2 protein abundance on the one hand, and *NRT2*



**Figure 6.** Effect of constitutive expression of *NRT2.1* on the down-regulation of the HATS by repressive N or dark treatments. For all individual experiments performed (those depicted in Figs. 2–4 plus replicate experiments not shown in Figs. 2–4), the percentage of repression of root <sup>15</sup>NO<sub>3</sub><sup>-</sup> influx in response to repressive N or dark treatments was calculated for each genotypes as: % repression = (influx in control conditions – influx in repressive conditions)/influx in control conditions × 100. The line indicates the identity function. Thus, all data points below the line correspond to assays where repression of the NO<sub>3</sub><sup>-</sup> HATS was lower in the *35S::NRT2.1* plants as compared to the wild type. All data points are mean of six replicates.



gene expression and HATS activity, on the other hand (Wirth et al., 2007; Ishikawa et al., 2009). These data support the previous proposal that posttranscriptional regulatory mechanisms participate in the control of the HATS, because expression of a *35S::NpNRT2.1* or *RolD::NpNRT2.1* transgene in *N. plumbaginifolia* did not prevent down-regulation of the HATS activity by high  $\text{NH}_4\text{NO}_3$  supply (Fraisier et al., 2000). However, this work in *N. plumbaginifolia* could not be fully conclusive because constitutive expression of *NpNRT2.1* using 35S or RolD promoters was achieved in a wild-type background, making it not possible to assess the actual functionality of the transgene product, or to rule out any putative compensation by the endogenous gene.

To unambiguously determine if posttranscriptional regulation of *NRT2.1* can impact the HATS activity in *Arabidopsis*, we generated transgenic plants expressing only a constitutive version of the *NRT2.1* gene by complementing the *atnrt2.1-2* mutant, defective for both *NRT2.1* and *NRT2.2*, with a *35S::NRT2.1* transgene. Previous work showed that the constitutive expression of *NRT2.1* fused to GFP had no impact on the localization of the protein in plasma membrane of epidermis and cortex root cells compared to plants expressing *NRT2.1::GFP* under the control of its own promoter (Chopin et al., 2007; Wirth et al., 2007). We succeeded in isolating three independent transgenic lines (L5, L6, and L10) displaying a deregulated expression of *NRT2.1*. In these lines, the level of *NRT2.1* mRNA in the roots was similar or higher than that in the wild type under control conditions (Figs. 1–4), and remained little affected or was even slightly increased by repressive treatments that strongly diminish *NRT2.1* expression in wild-type plants (Figs. 2–4). This shows that *NRT2.1* mRNA accumulation in the roots is predominantly controlled by *NRT2.1* transcription. Furthermore, all three lines displayed full, or almost full, complementation of the mutant defects in growth and total N accumulation (Fig. 1), demonstrating functionality of the transgene. However, although constitutive expression of *NRT2.1* led to a strong stimulation of the HATS activity as compared to the *atnrt2.1-2* mutant, it never succeeded in restoring the root  $\text{NO}_3^-$  influx measured at day 8 in wild-type plants under control conditions (Figs. 1–4). Using *NRT2.1* antibody we were able to show that this lack of full complementation in L5 plants was associated with a lower level of *NRT2.1* proteins compared to the wild type (Fig. 5). The reasons why rescue of the HATS activity and of *NRT2.1* protein level was only partial in *35S::NRT2.1* lines are not known, but it has been recently reported that complementation of a *atnar2.1* mutant by a *35S::NAR2.1-myc* construct restored only 60% to 70% of the high-affinity  $\text{NO}_3^-$  uptake defect of the mutant (Yong et al., 2010). To maximize the putative effect of deregulated *NRT2.1* expression, we measured the HATS activity in response to high N or dark treatments that are known to dramatically repress both  $\text{NO}_3^-$  HATS activity and *NRT2.1* mRNA level in wild-type plants (Lejay et al., 1999; Zhuo et al., 1999; Cerezo

et al., 2001; Girin et al., 2007). To make sure that the response of the HATS to these treatments is predominantly due to the *NRT2.1* transport system, we measured, in the same experiments, the expression, in L5 plants, of *NRT2.2* and *NRT1.1*, two other transporters involved in high-affinity  $\text{NO}_3^-$  uptake (Supplemental Fig. S4). The general outcome of these series of experiments is that high-affinity root  $^{15}\text{NO}_3^-$  influx in the three transgenic lines was in most cases strongly down-regulated in response to the repressive treatments (Figs. 1–4).

This provides a clear demonstration that transcriptional regulation of *NRT2.1* expression is not the major mechanism for control of the HATS in our conditions. However, down-regulation of the HATS appeared in many instances less pronounced in the transgenic lines than in the wild type. For instance, when plants were grown on a media containing 10 mM  $\text{NO}_3^-$  as the sole N source, the  $\text{NO}_3^-$  HATS activity was strongly lowered in the wild type as compared to control conditions (0.2 mM  $\text{NO}_3^-$ ), but not in the *35S::NRT2.1* plants (Fig. 3B), while in response to 10 mM  $\text{NH}_4\text{NO}_3$  repression of  $\text{NO}_3^-$  HATS was strong in both the wild-type and the transgenic lines. These results are in accordance with those of Fraisier et al. (2000), showing that the HATS activity was less repressed by 10 mM  $\text{NO}_3^-$  supply (as compared to 1 mM  $\text{NO}_3^-$ ) in the *RolD::NpNRT2.1* or *35S::NpNRT2.1* transformants than in the wild type while after ammonium addition  $\text{NO}_3^-$  influx was markedly decreased in both the wild-type and transgenic plants. One possible explanation to the discrepancy between the effects of  $\text{NO}_3^-$  alone and of  $\text{NH}_4\text{NO}_3$  on HATS activity, is the membrane depolarization resulting from  $\text{NH}_4^+$  supply, which might have impaired the energization of  $\text{NO}_3^-$  uptake by root cells, despite constitutive *NRT2.1* expression. However, several lines of evidence make this hypothesis very unlikely. First, membrane depolarization due to  $\text{NH}_4^+$  supply is rapid (within minutes) and transient, with the membrane potential gradient spontaneously recovering to the original or to an intermediate value within 30 min (Ullrich et al., 1984; Wang et al., 1994). A more recent study on tomato (*Solanum lycopersicum*) roots showed that growth in presence of  $\text{NH}_4^+$  even led to a hyperpolarization of the plasma membrane (Nieves-Cordones et al., 2008). Thus, membrane depolarization in response to  $\text{NH}_4^+$  supply cannot explain long-term repression of  $\text{NO}_3^-$  HATS activity observed after several hours or 8 d on 10 mM  $\text{NH}_4\text{NO}_3$  (Fig. 2, B and E). Furthermore, repression of  $\text{NO}_3^-$  influx by  $\text{NH}_4^+$  is known to be a very specific effect that can hardly be explained by a general mechanism such as decreased energization of secondary transport systems. Indeed, if  $\text{NH}_4^+$ -induced membrane depolarization was responsible for  $\text{NO}_3^-$  influx repression, the uptake of the other proton cotransported anions should also be affected. However, this is clearly not the case, and several studies showed that a range of  $\text{NH}_4^+$  concentration, which caused a marked decrease in the rate of  $\text{NO}_3^-$  influx, either produced no consis-

tent pattern of effect on phosphate influx or even increased phosphate and sulfate uptake (Cox and Reisenauer, 1973; Lee and Drew, 1989). Similarly, the provision of  $K^+$ , which depolarizes the plasma membrane to an extent similar to that of  $NH_4^+$  does not have a negative effect on root  $NO_3^-$  uptake (Lee and Drew, 1989; Glass and Siddiqi, 1995; Wang et al., 1996). Finally, Krouk et al. (2006) showed that  $NO_3^-$  HATS activity is up-regulated (along with *NRT2.1* expression) when  $NH_4^+$  concentration in the external medium largely exceeds that of  $NO_3^-$ . Altogether, these data confirm that membrane depolarization is not the main mechanism by which  $NH_4^+$  down-regulates root  $NO_3^-$  uptake and cannot explain why *35S::NRT2.1* plants did respond the same way to treatments with 10 mM  $NH_4NO_3$  compared to the wild type.

Considering the whole set of data from all experiments (Figs. 2–4; Supplemental Fig. S1; and replicate experiments not shown in Figs. 2–4) confirmed that constitutive expression of *NRT2.1* often resulted in a lower level of repression of the HATS activity as compared to the wild type (Fig. 6). This shows that, although not predominant under most situations, the transcriptional regulation of *NRT2.1* has a functional impact on the control of the HATS, and may account for a small but significant part of the changes in high-affinity root  $NO_3^-$  uptake recorded in the wild type. This conclusion is consistent with the reports indicating that up-regulation of *NRT2.1* transcript accumulation in mutants or transformants altered in N signaling or metabolism actually leads to a less-than-proportional increase in  $NO_3^-$  uptake by the roots (Muños et al., 2004; Good et al., 2007; Hong et al., 2009; Girin et al., 2010; Widiez et al., 2011).

#### Putative Posttranscriptional Mechanisms Involved in the HATS Control

In Arabidopsis, constitutive expression using a *35S* promoter has already been used to investigate posttranscriptional regulation of several nutrient transporters, such as *AMT1.1* (Yuan et al., 2007), *IRT1* (Connolly et al., 2002), *BOR1* (Takano et al., 2005), *SULTR1.1*, and *SULTR 1.2* (Yoshimoto et al., 2007). The general conclusion from these studies is that, despite a strong regulation at the transcriptional level for all these transporters, various levels of posttranscriptional regulations are also involved in the modulation of their activity. The root-specific and iron-deficiency-inducible expression of Arabidopsis *IRT1*, the major transporter for high-affinity iron uptake, is controlled at both transcription and protein accumulation levels (Connolly et al., 2002). For sulfate acquisition, *SULTR1.1* and *SULTR1.2*, two essential components of the high-affinity sulfate uptake system, are controlled both transcriptionally and posttranscriptionally at the level of protein accumulation in response to changes in environmental sulfur conditions (Yoshimoto et al., 2007). Posttranscriptional regulation of the *AMT1 NH\_4^+* transporters includes both regulation of

transcript stability (Yuan et al., 2007) and posttranslational control through phosphorylation at the C terminus of the protein (Loqué et al., 2007; Neuhäuser et al., 2007; Lanquar et al., 2009). Finally it was also shown that constitutively expressed GFP-tagged *BOR1* transporter proteins in Arabidopsis roots were degraded upon resupply of boron to plants by a mechanism that involved endocytosis from the plasma membrane and subsequent degradation of the transporter protein (Takano et al., 2005). From these studies it appears that posttranscriptional regulation of nutrient transporters is probably a general phenomenon in plant nutrient response, but that the mechanisms involved may be very diverse, depending on the protein.

Concerning *NRT2.1*, posttranscriptional regulation does not seem to involve mechanisms that strongly modulate the transcript stability. In all the experiments we performed, *NRT2.1* transcript level in the transgenic lines was not markedly affected by the treatments, indicating that the *35S* promoter was sufficient on its own to yield almost constitutive *NRT2.1* mRNA accumulation. At the protein level, we previously showed that the abundance of the *NRT2.1* protein in the root plasma membrane of wild-type plants is rather stable and is only affected in response to long-term treatments with high N (Girin et al., 2007; Wirth et al., 2007). Western blots performed in this study confirmed that *NRT2.1* abundance is not affected by short-term N or dark treatments in roots of both the wild-type and L5 plants (Fig. 5, A and C). However, after 24 h of darkness, *NRT2.1* protein level was significantly decreased in the wild-type and L5 plants when grown in vitro for 8 d, compared to plants kept in the light (Fig. 5E). Since this occurred in the absence of any decrease of *NRT2.1* mRNA level in L5 plants (Fig. 4A), this shows that *NRT2.1* is subject to a posttranscriptional regulatory mechanism that lowers its abundance in the membrane in response to long-term repressive treatments. The effects of this mechanism are apparently dependent on the experimental conditions. According to Wirth et al. (2007), the decrease of *NRT2.1* protein level following an extended dark period was not observed when plants were grown in hydroponics for 6 weeks (see also Supplemental Fig. S3A). It suggests that *NRT2.1* protein is more stable in older plants or that sugar depletion is more rapid in young plants due to a lower level of sugar stores than in older plants. Interestingly, similar data were obtained with *NAR2.1* (Fig. 5F; Supplemental Fig. S3B).

A major difference between *NRT2.1* and most other nutrient carriers is that *NRT2.1* protein expression and transport activity requires a functional *NAR2.1* protein (Orsel et al., 2006; Wirth et al., 2007), which interacts with *NRT2.1* to generate a heterooligomer that may be the active form of the transporter (Yong et al., 2010). Several reports have shown that *NAR2.1* expression is regulated as *NRT2.1* expression, although with a much-less-pronounced amplitude in the transcript-level changes (Krouk et al., 2006; Okamoto et al., 2006; Orsel

et al., 2006). Furthermore, at the protein level nothing is known concerning the regulation of NAR2.1 and its possible role in the regulation of HATS activity. Thus, constitutive expression of *NRT2.1* alone may not be sufficient to prevent down-regulation of the HATS if NAR2.1 is still repressed at the mRNA or protein level. Therefore, we investigated the regulation of *NAR2.1* mRNA accumulation and we designed a specific antibody for NAR2.1 to follow its regulation at the protein level. Our data show that, despite a decrease in *NAR2.1* transcript level in response to the most repressive treatments (10 mM  $\text{NH}_4\text{NO}_3$  or 24 h of darkness), these changes were very moderate as compared to the strong down-regulation of root  $^{15}\text{NO}_3^-$  influx in the *35S::NRT2.1* transformants (Figs. 2 and 4). Furthermore, in several instances down-regulation of the HATS could be observed in both wild-type and transgenic plants in the absence of any decay of *NAR2.1* transcript level (Figs. 2 and 3). Similarly, the results obtained at the protein level do not evidence a specific response of NAR2.1 that may explain on its own the regulation of the HATS activity. Like NRT2.1, NAR2.1 protein accumulation was lowered in both the wild-type and L5 plants by long-term dark treatment (Fig. 5F) but not by short-term N or dark treatments (Fig. 5, B and D). Interestingly, this is illustrative of a more general correlation between the levels of NRT2.1 and NAR2.1 proteins. Indeed, in the *nrt2.1-2* mutant lacking NRT2.1, NAR2.1 protein is present at a much lower level as compared to the wild type (Supplemental Fig. S2), although *NAR2.1* mRNA accumulation is not affected (Orsel et al., 2006; data not shown). Conversely, NRT2.1 protein could not be detected in *nar2.1* mutants (Wirth et al., 2007; Yong et al., 2010), despite presence of the transcript (Okamoto et al., 2006; data not shown). Finally, the only partial restoration of NRT2.1 protein expression in L5 plants was associated with a lowered level of NAR2.1 in the membranes as compared to wild-type plants (Fig. 5), although *NAR2.1* mRNA accumulation was similar in both genotypes (Figs. 2–4). These data strongly suggest a tight coregulation of NRT2.1 and NAR2.1 expression at the post-transcriptional level.

Altogether, the above results indicate that the regulation of  $\text{NO}_3^-$  HATS activity, in response to high N and darkness, is a complex mix between three levels of regulation. Both transcriptional regulation of *NRT2.1* and *NAR2.1* and posttranscriptional control of protein abundance may explain long-term regulation (after one or several days) of the root  $\text{NO}_3^-$  HATS. However, the results also collectively show that such mechanisms cannot explain why root  $^{15}\text{NO}_3^-$  influx remains normally repressed in the *35S::NRT2.1* lines in response to short-term N or dark treatments that do not modify NRT2.1 and NAR2.1 protein levels. This suggests that posttranslational modifications of NRT2.1 and/or NAR2.1 proteins also probably play an important role in the control of the HATS activity. Given the complexity of such a two-component system, many hypotheses can be envisaged, such as association/

dissociation of the NRT2.1/NAR2.1 heterooligomer (Yong et al., 2010), partial proteolysis of NRT2.1 (Wirth et al., 2007), or phosphorylation events in NRT2.1 and/or NAR2.1 (Forde, 2000). Furthermore, despite that it is now quite well documented that NRT2.1 is part of a high- $M_r$  complex in the plasma membrane (Wirth et al., 2007; Yong et al., 2010), no evidence is available yet that this complex comprises only NRT2.1 and NAR2.1, leaving the possibility that other unknown regulatory protein(s) may be involved. Whatever the posttranslational mechanisms responsible for down-regulation of the HATS in our experiments, it is quite clear that they are activated by a variety of treatments (high N supply with or without  $\text{NH}_4^+$ , transfer to the dark), making them likely to be of general occurrence.

## MATERIALS AND METHODS

### Plant Material and Culture Conditions

*Arabidopsis* (*Arabidopsis thaliana*) genotypes used in this study were the wild-type Col-0 ecotype, the *atnrt2.1-2* mutant, obtained from the Salk Institute (Salk\_035429; Little et al., 2005), and the *nar2.1-1* mutant (Orsel et al., 2006).

For all experiments except those presented in Supplemental Figures S2 and S3, plants were grown in sterile conditions in vertical agar plates (12 × 12 cm) on solid medium (0.8% [w/v] agar type A; Sigma, product A4550) containing 0.5 mM  $\text{CaSO}_4$ , 0.5 mM  $\text{MgCl}_2$ , 1 mM  $\text{KH}_2\text{PO}_4$ , 2.5 mM MES, 50  $\mu\text{M}$  NaFe EDTA, 50  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 12  $\mu\text{M}$   $\text{MnCl}_2$ , 1  $\mu\text{M}$   $\text{CuCl}_2$ , 1  $\mu\text{M}$   $\text{ZnCl}_2$ , and 0.03  $\mu\text{M}$   $\text{NH}_4\text{Mo}$ . The pH was adjusted to 5.7 with KOH. This medium was supplemented with either  $\text{KNO}_3$  or  $\text{NH}_4\text{NO}_3$  as described in the text. After storing for 2 d at 4°C in the dark, plates were transferred in a growth chamber with 16/8 h day/night cycle at 21°C/18°C and 70% relative humidity. Light intensity during the light period was 125  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . All the plants were harvested after 8 d of growth. For  $\text{NH}_4\text{NO}_3$ ,  $\text{NO}_3^-$ , and long dark treatments the light period in the growth chamber started at 6 AM. For short dark treatments the light period in the growth chamber started at midnight.

For Supplemental Figures S2 and S3, plants were grown hydroponically using the experimental set up describes previously (Lejay et al., 1999).

### Generation of Transformant Lines

All constructs were made using Gateway cloning technology (Invitrogen) according to the manufacturer's instructions. The *NRT2.1* DNA sequence from the *bac* clone T6D22 (*Arabidopsis* Biological Resource Center) was amplified using primers pair: GATE NRT2.1 L, 5'-CACCATGGGGTATTCTACTGGT-3'; GATE NRT2.1 R, 5'-TCAAACATTGTGGGTGTGT-3'; and was cloned into the pENTR/D-Topo vector (Invitrogen). One entry clone was fully sequenced before subsequent cloning in the binary Gateway destination vector pGWB2 obtained from Tsuyoshi Nakagawa (Research Institute of Molecular Genetics, Shimane University, Matsue, Japan) by using a Gateway LR clonase enzyme mix (Invitrogen). The pGWB2 vector allows expression of the cloning sequence under the control of the cauliflower mosaic virus 35S promoter. The binary construct was introduced into the *Agrobacterium tumefaciens* strain GV3101, and the resulting bacterial culture was used to transform the *atnrt2.1-2* mutant line by the standard flower-dip method (Clough and Bent, 1998). Transformants (T1) were selected on Murashige and Skoog-2 medium containing hygromycin (30  $\mu\text{g mL}^{-1}$ ). Homozygous lines (T3) were obtained from resistance segregation assays. Integrity of the transgene was checked in transgenic lines by PCR analysis using specific primers.

### RNA Extraction and Gene Expression Analysis

Root samples were frozen in liquid  $\text{N}_2$  in 2-mL tubes containing one steel bead (2.5-mm diameter). Tissues were disrupted for 1 min at 30  $\text{s}^{-1}$  in a Retsch mixer mill MM301 homogenizer (Retsch). Total RNA was extracted from tissues using TRIzol reagent (Invitrogen). Subsequently, 40  $\mu\text{g}$  of RNA were treated with DNase (RNase free DNase kit, Qiagen) and purified (RNeasy MinElute™ cleanup kit, Qiagen) following the manufacturer's instructions.

Reverse transcription was achieved with 4  $\mu\text{g}$  of RNAs in the presence of Moloney murine leukemia virus reverse transcriptase (Promega) after annealing with an anchored oligo(dT)<sub>18</sub> primer as described by Wirth et al. (2007). The quality of the cDNA was verified by PCR using specific primers spanning an intron in the gene *APTR* (At1g27450) forward 5'-CGCTTCTTCGCACTGAG-3'; reverse 5'-CAGGTAGCTTCTGGGCTTC-3'.

Gene expression was determined by quantitative real-time PCR (LightCycler; Roche Diagnostics) with the kit LightCycler FastStart DNA master SYBR green I (Roche Diagnostics) according to the manufacturer's instructions with 1  $\mu\text{L}$  of cDNA in a total volume of 10  $\mu\text{L}$ . The conditions of amplifications were performed as described previously by Wirth et al. (2007). All the results presented were standardized using the housekeeping gene *Clathrin* (At4g24550). Gene-specific primer sequences were: NRT2.1 forward, 5'-AA-CAAGGGCTAACGTGGATG-3'; NRT2.1 reverse, 5'-CTGCTTCTCTGCTC-ATCC-3'; NAR2.1 forward, 5'-GGCCATGAAGTTGCCATAG-3'; NAR2.1 reverse, 5'-TCTTGGCCTTCTCTTCTCA-3'; NRT2.2 forward, 5'-CAGGTG-GAAACAGAGCTGCCATGG-3'; NRT2.2 reverse, 5'-GGACCATAGATA-CACGGCAGTGACGAG-3'; NRT1.1 forward, 5'-GCACATTGGCATTAGGC-TT-3'; NRT1.1 reverse, 5'-CTCAATCCCACCTCAGCTA-3'; *Clathrin* forward, 5'-AGCATACTGCGTGCAAAG-3'; *Clathrin* reverse, 5'-TCGCTT-GTGTCACATATCTC-3'.

### NO<sub>3</sub><sup>-</sup> Influx Studies

NO<sub>3</sub><sup>-</sup> influxes were determined by <sup>15</sup>N labeling as described by Remans et al. (2006). Liquid media for influx studies contained basic N-free medium supplemented with 0.2 mM K<sup>15</sup>NO<sub>3</sub> (atom % <sup>15</sup>N: 99%). Briefly, four plants were transferred to a 5-cm-diameter petri dish containing 0.1 mM CaSO<sub>4</sub>, with the roots in the solution and the aerial parts outside. This solution was replaced after 1 min with the 0.2 mM <sup>15</sup>NO<sub>3</sub><sup>-</sup> solution for 5 min. Plants were then rinsed again for 1 min in 0.1 mM CaSO<sub>4</sub> before being harvested and dried at 70°C for 48 h. After determination of their dry weight, the samples were analyzed for total N and atom % <sup>15</sup>N using a continuous-flow isotope ratio mass spectrometer (IsoPrime mass spectrometer; GV instruments) coupled to a carbon/N elemental analyzer (EuroVector S.p.A.) as described in Clarkson et al. (1996). Each influx value is the mean of six to 12 replicates.

### NAR2.1 and NRT2.1 Immunodetection and Membrane Purification

For plants grown in vitro, microsomes were purified as followed. All procedures were carried out at 4°C. Harvested roots were homogenized with a roller grinder (C. Fauvel, Institut National de la Recherche Agronomique) and 0.25 g/mL of homogenization buffer (50 mM Tris, 500 mM Suc, 10% glycerol, 20 mM EDTA, 20 mM EGTA, 50 mM NaF, 5 mM  $\beta$ -glycerophosphate, 1 mM phenantrolone, 0.6% polyvinylpyrrolidone, 10 mM ascorbic acid adjusted to pH 8 with MES 1 M, 1  $\mu\text{M}$  leupeptine, 5 mM dithiothreitol, 1 mM Na<sub>2</sub> vanadate, 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 2,000 rpm (Eppendorf 5810 R) for 2 min to remove the debris. The supernatant was then centrifuged at 9,000<sub>g</sub> for 12 min and the resulting supernatant was centrifuged again at 50,000<sub>g</sub> to recover the microsomal fraction. To obtain microsomes the pellet was resuspended in a minimal volume of conservation buffer (10 mM Tris, 10 mM borate, 300 mM Suc, 9 mM KCl, 5 mM EDTA, 5 mM EGTA, 50 mM NaF pH 8.3, 4.2  $\mu\text{M}$  leupeptine, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol) and frozen at -80°C. For plants grown in hydroponic, microsomes were purified as described by Giannini et al. (1987).

For western blots proteins were separated on denaturing SDS-PAGE followed by an electrotransfer at 4°C onto a polyvinylidene difluoride membrane (0.2  $\mu\text{M}$ , Immobilon, Millipore) according to manufacturer instructions. NAR2.1 was detected using an anti-NAR2.1 antiserum produced by Eurogentec against the synthetic peptide DVTTKPSREGPGVVL. The polyclonal antiserum was affinity purified by Eurogentec. NRT2.1 was detected using the antibody NRT2.1 20 described by Wirth et al. (2007). The immunodetection for both NRT2.1 and NAR2.1 was performed with a chemiluminescent detection system kit (SuperSignal, Pierce).

### Supplemental Data

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

**Supplemental Figure S1.** NO<sub>3</sub><sup>-</sup> influx, *NRT2.1*, and *NAR2.1* mRNA level in roots of wild type and the L10 transgenic plants in response to 10 mM NH<sub>4</sub>NO<sub>3</sub>.

**Supplemental Figure S2.** Immunoblot for NAR2.1 using microsomes from roots of wild-type and knockout mutants for *NRT2.1* and *NAR2.1*.

**Supplemental Figure S3.** Immunoblot for NRT2.1 and NAR2.1 using microsomes from roots of wild-type plants grown hydroponically.

**Supplemental Figure S4.** *NRT2.2* and *NRT1.1* mRNA level in roots of wild-type (WT) and the transgenic line L5.

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