

Identification of Arabidopsis Mutants Impaired in the Systemic Regulation of Root Nitrate Uptake by the Nitrogen Status of the Plant^{1[C][W]}

Thomas Girin^{2,3}, El-Sayed El-Kafafi^{2,4}, Thomas Widiez, Alexander Erban, Hans-Michael Hubberten, Joachim Kopka, Rainer Hoefgen, Alain Gojon, and Marc Lepetit*

Biochimie et Physiologie Moléculaire des Plantes, UMR 5004, INRA-CNRS-Sup Agro-UM2, Institut de Biologie Intégrative des Plantes, F-34060 Montpellier, France (T.G., E.-S.E.-K., T.W., A.G., M.L.); and Max-Planck-Institut für Molekulare Pflanzenphysiologie, 14476 Potsdam-Golm, Germany (A.E., H.-M.H., J.K., R.H.)

Nitrate uptake by the roots is under systemic feedback repression by high nitrogen (N) status of the whole plant. The *NRT2.1* gene, which encodes a NO_3^- transporter involved in high-affinity root uptake, is a major target of this N signaling mechanism. Using transgenic Arabidopsis (*Arabidopsis thaliana*) plants expressing the *pNRT2.1::LUC* reporter gene (NL line), we performed a genetic screen to isolate mutants altered in the *NRT2.1* response to high N provision. Three *hmi* (for *high nitrogen insensitive*) mutants belonging to three genetic loci and related to single and recessive mutations were selected. Compared to NL plants, these mutants display reduced down-regulation of both *NRT2.1* expression and high-affinity NO_3^- influx under repressive conditions. Split-root experiments demonstrated that this is associated with an almost complete suppression of systemic repression of *pNRT2.1* activity by high N status of the whole plant. Other mechanisms related to N and carbon nutrition regulating *NRT2.1* or involved in the control of root SO_4^{2-} uptake by the plant sulfur status are not or are slightly affected. The *hmi* mutations did not lead to significant changes in total N and NO_3^- contents of the tissues, indicating that *hmi* mutants are more likely regulatory mutants rather than assimilatory mutants. Nevertheless, *hmi* mutations induce changes in amino acid, organic acid, and sugars pools, suggesting a possible role of these metabolites in the control of NO_3^- uptake by the plant N status. Altogether, our data indicate that the three *hmi* mutants define a new class of N signaling mutants specifically impaired in the systemic feedback repression of root NO_3^- uptake.

As sessile organisms, plants must constantly adapt to fluctuating environmental conditions that almost always limit their optimal growth and development. This adaptation is made possible by the action of sensing and signaling mechanisms allowing the various organs to modify their physiology and morphology in response to a wide range of external and internal stimuli. For instance, mineral nutrient limitation induces a marked stimulation of nutrient uptake efficiency by the roots, which relies on the

up-regulation of specific high-affinity ion transport systems (Clarkson and Lüttge, 1991; Gojon et al., 2009). Although these responses often differ in nature or timing between nutrients, they are in most cases triggered by two types of signaling pathways: (1) local signaling pathways associated with sensing of nutrient availability in the immediate root environment and (2) systemic signaling pathways informing the roots of the overall nutrient status of the whole plant (Forde, 2002; Schachtman and Shin, 2007; Gojon et al., 2009).

Knowledge of these signaling pathways is restricted concerning nitrate (NO_3^-), the main nitrogen (N) source for nutrition of most herbaceous species. It has been clearly shown that NO_3^- uptake systems are under stringent control by both local NO_3^- signaling and systemic signaling driven by the N status of the whole plant, but very few molecular components of the corresponding regulatory pathways have been identified so far (Forde, 2002; Vidal and Gutierrez, 2008; Gojon et al., 2009; Liu et al., 2009). Nitrate acts as a signal per se, and its effects on plant physiology, development, and whole-genome expression have been particularly well described (Crawford, 1995; Stitt, 1999; Wang et al., 2000, 2003, 2004). For instance, NO_3^- induces the expression of numerous genes involved in its utilization by the plant, including those encoding some of its own transporters and assimilatory en-

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² These authors contributed equally to the article.

³ Present address: Crop Genetics Department, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK.

⁴ Present address: Botany Department, Faculty of Agriculture, Al-Azhar University, Nasr City, Cairo, Egypt.

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

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Marc Lepetit (lepetit@supagro.inra.fr).

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zymes (Wang et al., 2004). In *Arabidopsis thaliana*, only a few regulatory genes have been shown to contribute to these signaling effects of NO_3^- . The first gene identified to play a regulatory role in the regulation of root NO_3^- transporters is *NRT1.1* (formerly *CHL1*), encoding a dual-affinity NO_3^- transporter (Tsay et al., 1993; Liu et al., 1999). Mutation of *NRT1.1* prevents down-regulation of another NO_3^- transporter gene (*NRT2.1*) by high NH_4NO_3 supply to the plant (Muños et al., 2004). This was attributed to the impairment of specific local NO_3^- signaling responsible for repression of *NRT2.1* expression by high NO_3^- , suggesting that *NRT1.1* plays a dual transport/signaling role (Krouk et al., 2006; Ho et al., 2009; Wang et al., 2009). More recently, *CIPK8*, encoding a CBL-interacting kinase, and *NLP7*, encoding a NIN-like transcription factor, were both shown to be required for full stimulation by NO_3^- of several NO_3^- acquisition genes, such as *NRT2.1*, or *NIA1* and *NIA2*, encoding two isoforms of the nitrate reductase apoprotein (Castaings et al., 2009; Hu et al., 2009; Wang et al., 2009).

Even less is known concerning the regulation of root NO_3^- acquisition by systemic signaling driven by the N status of the plant. The occurrence of such a regulation has clearly been demonstrated by split-root experiments in various species, where increased N supply on one side of the root system results in a compensatory down-regulation of root NO_3^- uptake in the untreated part of the root system, which remained under unchanged N provision (Drew and Saker, 1975; Burns, 1991; Gansel et al., 2001; Ruffel et al., 2008). This systemic control is thought to involve specific repression of root NO_3^- uptake systems by long-distance signals triggered by high N status of the plant, which may correspond to organic N metabolites, such as amino acids (Cooper and Clarkson, 1989; Crawford and Glass, 1998; Miller et al., 2008; Vidal and Gutierrez, 2008). Several genes (*GLR1.1*, *NLA*, *CCA1*, *DOF1*, *OSU1*, and *PLD ϵ*) and one microRNA (miR167) have been proposed to be involved in N (not specifically NO_3^-) signaling in *Arabidopsis*. *GLR1.1* encodes a putative Glu receptor modulating both N and carbon (C) metabolism (Kang and Turano, 2003). *NLA* is a RING-type ubiquitin ligase that controls various leaf responses (such as senescence) to N limitation (Peng et al., 2007). *CCA1* is a master clock core gene regulated by organic N metabolites, which in turn regulates gene expression of key enzymes of amino acid metabolism (Gutierrez et al., 2008). *OSU1*, a putative methyltransferase, triggers various responses to N/C nutrient balance (Gao et al., 2008). *PLD ϵ* encodes a phospholipase possibly involved in regulation of root growth and biomass accumulation (Hong et al., 2009). Finally, miR167, with its target *ARF8*, is part of a regulatory circuit modulating lateral root emergence (Gifford et al., 2008). However, with the possible exception of *PLD ϵ* , none of these regulators were shown to play a role in the systemic signaling of plant N status or in the control of root NO_3^- uptake.

To identify genes involved in systemic N signaling responsible for repression of root NO_3^- uptake systems by high N status of the plant, we used a genetic approach with an *Arabidopsis* transgenic line expressing the luciferase (*LUC*) reporter gene under the control of the *NRT2.1* promoter. *NRT2.1* encodes a main component of the high-affinity transport system (HATS) for root uptake of NO_3^- (Cerezo et al., 2001; Filleur et al., 2001; Li et al., 2007), which plays a crucial role in N acquisition by crops (Malagoli et al., 2004). In addition to being induced by NO_3^- and stimulated by sugars, *NRT2.1* transcription is a major target of the systemic feedback repression exerted by high N status of the plant (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001; Gansel et al., 2001), *pNRT2.1* promoter activity being strongly responsive to this signaling pathway (Nazoa et al., 2003; Girin et al., 2007). Screening of an ethyl methanesulfonate-mutagenized population of *pNRT2.1::LUC* plants (NL) allowed us to isolate *hni* (for *high nitrogen insensitive*) mutants into which the *pNRT2.1* activity remains high under repressive conditions (supply of 10 mM NH_4NO_3) that normally suppress *NRT2.1* transcription in the wild type. We report here the physiological analysis of three mutants (*hni9-1*, *hni48-1*, and *hni140-1*) impaired in the systemic control of *NRT2.1* expression by the N status of the whole plant.

RESULTS

Identification of *Arabidopsis* Mutants Impaired in the Regulation of *NRT2.1* Gene

Previously, we have shown that the 1,201-bp sequence located upstream the translation initiating codon of *NRT2.1* is able to confer a root-specific N status-dependent transcription to the *GUS* reporter gene (Girin et al., 2007). To monitor the *pNRT2.1* activity in vivo, a transgenic line (NL, for *pNRT2.1::LUC*) expressing the *LUC* reporter gene under the control of this promoter was generated. The NL line carried a single T-DNA insertion at the top of chromosome 1, located 262 bp upstream the initiating codon of At1g06080 that encodes a fatty acid desaturase (Supplemental Fig. S1). The regulation of the *pNRT2.1::LUC* transgene by the N status of the plant was investigated by comparing 7-d-old plants grown on vertical agarose plates with two media named HN (high N) and LN (low N) containing contrasted levels of N (10 mM NH_4NO_3 and 0.3 mM KNO_3 , respectively), using the strategy previously described by Girin et al. (2007). As expected, bioluminescence imaging and enzymatic assays indicated that the *LUC* activity was found exclusively in the roots and was strongly down-regulated in HN plants compared to LN plants (Fig. 1A). Furthermore, repression of *LUC* transcript accumulation by HN treatment (Fig. 1B) was tightly correlated with repression of both *NRT2.1* transcript accumulation (Fig. 1C) and HATS activity (Fig. 1D),

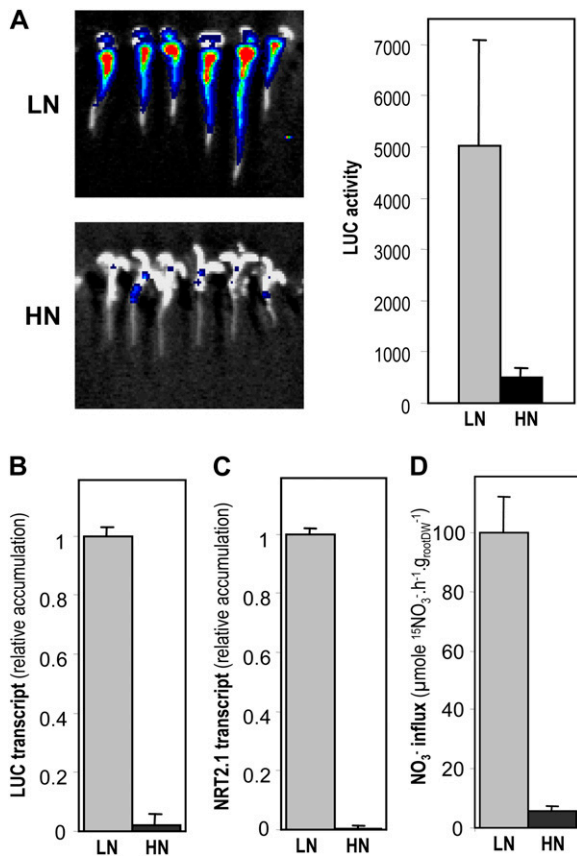


Figure 1. Characterization of NL plants (*pNRT2.1::LUC*). Plants were grown on vertical agarose plates for 7 d on HN or LN medium supplemented with 1% Suc. A, Bioluminescence imaging and quantification of the LUC activity (values are means of six replicates \pm SD). B, Relative accumulation of the LUC transcript (values are means of three replicates \pm SD). C, Relative accumulation of the *NRT2.1* transcript (values are means of three replicates \pm SD). D, High-affinity influx measured in 0.2 mM $^{15}\text{NO}_3^-$ (values are means of 10 replicates \pm SD). DW, Dry weight.

indicating that *pNRT2.1::LUC* is a good marker for *NRT2.1* expression and activity under these conditions. Altogether, these data validated the use of the NL transgenic line for screening mutants impaired in the repression of *NRT2.1* by high N supply.

NL seeds were mutagenized by an ethyl methane-sulfonate treatment, M1 plants were self-fertilized, and M2 seeds were collected. A total of 22,320 M2 plants (corresponding to 11,160 M1 plants) were screened by bioluminescence imaging of LUC activity in individual plants. Variants displaying a higher level of LUC activity on HN medium than the nonmutagenized NL line (*luc*⁺ phenotype) were selected for further analysis. To determine the heritability of the *luc*⁺ phenotype, variants were systematically self-fertilized and screened again at the M3 generation (yielding 42 *luc*⁺ M3 lines). Putative mutants were then analyzed for expression of *NRT2.1* endogenous gene by quantitative real-time PCR in the roots of M3 plants grown under screening (HN) conditions to confirm the effect

of the mutations on the endogenous promoter (data not shown). Finally, three putative mutants displaying higher levels of both LUC activity and *NRT2.1* transcript accumulation than NL plants on HN medium were selected and named *hni9-1*, *hni48-1*, and *hni140-1*. They were backcrossed with the NL parental line, and F1 hybrids were self-fertilized. Segregations of F2 progenies (Supplemental Table S1) show that the *luc*⁺ phenotype of the three mutants was the result of single recessive mutations. Two additional series of backcrosses were then performed to remove most of the mutations unlinked to the *luc*⁺ phenotype before performing further phenotype characterization of the mutants. DNA sequencing revealed that no mutation was found in the *NRT2.1* promoter sequence of the *LUC* transgene in the mutants.

To localize the mutations on the Arabidopsis genetic map, mutants (Columbia background) were outcrossed with Landsberg *erecta* (*hni9-1* and *hni48-1*) or Wassilewskija (*hni140-1*). F1 hybrids were self-fertilized, and the *luc* phenotype of F2 plants was characterized by bioluminescence imaging in plants grown under screening conditions (HN). Genomic DNA was prepared from individual *luc*⁺ plant of the F2 populations. The three *hni* mutations were mapped using PCR-derived polymorphic markers. As in these outcrosses the *pNRT2.1::LUC* transgene was only present in the mutant parental lines, a strong linkage to the *luc*⁺ trait was observed for the three mutants at the site of the transgene insertion on the top of chromosome 1. Additional linkage regions were also observed corresponding to the three mutations. All of them were located on chromosome 1 (Supplemental Table S2). The *hni9-1* mutation was mapped in the middle of the chromosome closely linked to the CER458867/6 marker (located approximately 3 centimorgans [cM] from the mutation). The *hni48-1* and *hni140-1* mutations were mapped in the lower arm of the chromosome: *hni48-1* was linked to the nga280 marker (located approximately 6 cM from the mutation), and *hni140-1* was linked to the nga111 marker (located approximately 11 cM from the mutation). Crosses between *hni48-1* and *hni140-1* plants confirmed that these two mutations were not allelic (Supplemental Table S3).

hni Mutants Are Impaired in the Systemic Regulation of *NRT2.1* Expression by the N Status of the Plant

The regulation of both LUC activity and *NRT2.1* endogenous gene expression was investigated in roots of NL, *hni9-1*, *hni48-1*, and *hni140-1* plants grown on vertical agarose plates. The three mutants displayed much higher levels (15- to 35-fold) of LUC activity than the NL line on HN medium, indicating a stronger activity of the *NRT2.1* promoter in these mutants when cultivated under repressive conditions (Fig. 2A). This was confirmed at the level of *NRT2.1* transcript accumulation, although the increases in the mutants compared to NL (6- to 14-fold) were not as strong as for LUC activities (Fig. 2B). Interestingly, little differences

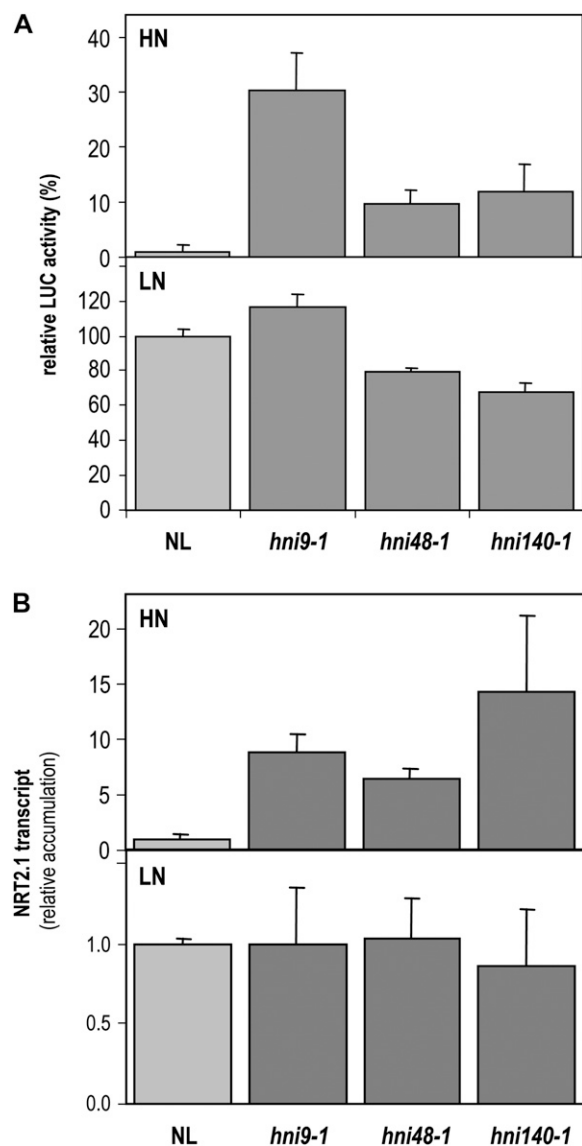


Figure 2. Characterization of *NRT2.1* expression in *hni* mutants. Plants were grown on vertical agarose plates supplemented with 1% Suc for 7 d. A, LUC activity in *hni* mutants and NL in plants cultivated on HN or LN medium. Values are means of six replicates \pm SD. B, Relative accumulation of the *NRT2.1* transcript on HN or LN medium. Values are means of three replicates \pm SD.

were found for LUC activity or *NRT2.1* transcript accumulations between the mutants and the NL line in plants grown on LN medium (Fig. 2). This showed that the mutants were not constitutively overexpressing *NRT2.1*, but rather displayed an altered response of this gene to high N supply.

To determine whether these phenotypes resulted from a specific impairment of the feedback repression exerted by high N status of the plant, the response of the mutants (1) to changes of their sulfur (S) status, and (2) to other factors known to modulate *NRT2.1* transcription, i.e. stimulation by NO_3^- itself (Filleur and Daniel-Vedele, 1999) and stimulation by sugars

(Lejay et al., 1999), were investigated (Fig. 3). We used the S-repressible SO_4^{2-} transporter gene *SULTR1.2* (Maruyama-Nakashita et al., 2006) as a marker gene for S signaling. As shown in Figure 3A (ANOVA analysis in Supplemental Table S4), the three mutants displayed no or only little differences in the repression of *SULTR1.2* expression in response to high S supply. Similar results were obtained with the *APSR* gene, encoding 5'-adenosine phosphosulfate reductase (At1g62180; data not shown). This suggested that

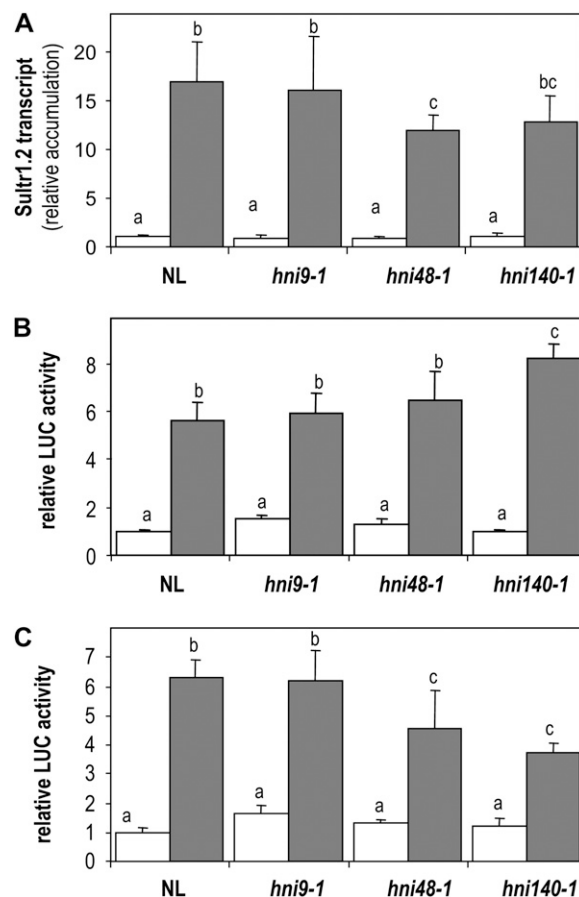


Figure 3. Response of the *hni* mutants to S limitation, NO_3^- induction, and Suc supply. Plants were grown on vertical agarose plates. A, Effect of S limitation on *SULTR1.2* transcript accumulation in the roots. Plants were cultivated on basal medium supplemented with 1% Suc and 2 mM NH_4NO_3 for 7 d. S limitation was applied by transferring them for 48 h to $-\text{S}$ medium (SO_4^{2-} substituted by Cl^-) after a 1-min wash in water. Values are means of three replicates \pm SD. B, Effect of external NO_3^- on the root LUC activity. Plants were cultivated on basal nutrient medium supplemented with 1% Suc containing either 0.3 mM NH_4^+ or 0.3 mM NO_3^- as sole N source according to Girin et al. (2007). Values are means of six replicates \pm SD. C, Effect of an exogenous supply of Suc on the root LUC activity. Plants were cultivated on LN medium in presence or absence of 3% Suc and transferred for 24 h in the dark before analysis according to Girin et al. (2007). Values are means of six replicates \pm SD. To determine if genotype significantly affects the plant response to S, NO_3^- , or Suc, data presented in A to C were analyzed by ANOVA (Supplemental Table S4). Letters denote significant differences by paired *t* test ($P < 0.05$).

hmi mutants were indeed specifically deficient in N signaling. Comparison of plants grown on NH_4^+ or NO_3^- as sole N source showed that the *hmi* mutations had no or little impact on the stimulation of the *pNRT2.1* activity by NO_3^- (Fig. 3B; Supplemental Table S4). The stimulation of the *pNRT2.1* activity by sugars was also kept in all three mutants, as evidenced by the increased LUC activity associated with exogenous Suc supply to the plants (Fig. 3C; Supplemental Table S4). However, this response was slightly attenuated in two mutants (*hmi48-1* and *hmi140-1*) compared to the NL line, possibly indicating that these *hmi* mutations also modified the regulation of *NRT2.1* expression by sugars. Nevertheless, this could not explain the up-regulation of *pNRT2.1* activity recorded in the mutants on HN medium (containing Suc) since a lowered stimulation by sugars in *hmi* plants would result in a decrease rather than in an increase in LUC activity. Altogether, the above data indicated that the prominent effects of *hmi* mutations on *NRT2.1* expression were related to altered feedback repression by high N status of the plant.

To further document the molecular effects of the mutations on the general control exerted by N status signaling, we investigated the expression of several other genes of N transport and metabolism in the roots of *hmi* and NL plants (Supplemental Table S5). These included *NAR2.1(NRT3.1)*, encoding a protein required for *NRT2.1*-mediated transport activity (Orsel et al., 2006), *NRT2.2* and *NRT1.1*, which encode NO_3^- transporters involved in root NO_3^- uptake (Tsay et al., 1993, Li et al., 2007), *NIA1* and *NIA2*, encoding the two isoforms of the nitrate reductase apoprotein (Cheng et al., 1988, Wilkinson and Crawford, 1991, 1993), and *AMT1.1*, *AMT1.2*, and *AMT1.3*, encoding NH_4^+ transporters contributing to root NH_4^+ uptake (Loque et al., 2006, Yuan et al., 2007). *NAR2.1* has been reported to be regulated similarly to *NRT2.1* (Krouk et al., 2006; Okamoto et al., 2006). *NRT2.2* encodes a protein very similar to *NRT2.1* and present in tandem with *NRT2.1* on chromosome 1. This gene is induced by NO_3^- , but its level of expression is very low (Okamoto et al., 2006). *NRT1.1*, *NIA1*, and *NIA2* are stimulated by NO_3^- and sugars (Cheng et al., 1991; Lejay et al., 1999). *NRT1.1*, in contrast to *NRT2.1*, is not repressed by reduced N metabolites (Lejay et al., 1999). *AMT1.1* and *AMT1.3* (but not *AMT1.2*) are down-regulated by high N status of the plant (Gazzarrini et al., 1999; Rawat et al., 1999) but, at least for *AMT1.1*, through a distinct mechanism than *NRT2.1* (Gansel et al., 2001). In plants grown on HN medium, only expression of the *NAR2.1* gene displayed a significant increase in the three mutants compared to the NL line (1.9- to 2.7-fold; Supplemental Table S5). *NRT1.1*, *NRT2.2*, *NIA1*, and *NIA2* were not significantly affected in the mutants compared to the NL line (Supplemental Table S5). The N-repressed *AMT1.1* and *AMT1.3* genes also showed an unchanged transcript accumulation in the mutants compared to the NL line, suggesting that *hmi* mutants were affected in an N signaling pathway specifically

targeting root NO_3^- uptake systems, such as the one involving *NRT2.1/NAR2.1*. Although *AMT1.2* has been characterized as constitutively expressed upon variations of the N supply (Gazzarrini et al., 1999; Sheldon et al., 2001; Yuan et al., 2007), transcript of the gene was up-regulated (+52%) in the *hmi140-1* mutant, suggesting that the control of ammonium uptake may be also modified in this mutant.

Finally, an important feature of *NRT2.1* repression by high N status of the plant is that it relies on a systemic regulation, involving a shoot-to-root signaling pathway (Gansel et al., 2001; Girin et al., 2007). Therefore, we performed split-root experiments to determine whether *hmi* mutations altered the response of the *pNRT2.1* activity to either local or whole-plant signals. In NL plants, high N supply (HN) to one side of the split-root system resulted in the down-regulation of *pNRT2.1::LUC* expression in the side exposed to a low NO_3^- medium (LN), confirming the action of a whole-plant signaling mechanism (Fig. 4). Remarkably, this systemic repression of the *pNRT2.1* activity is almost completely suppressed in all three *hmi* mutants. Thus, these mutants could not modulate *NRT2.1* expression in roots in response to changes in N provision to the other organs. This clearly demonstrated that the *hmi* mutations impaired crucial steps of the systemic signaling pathway governing root NO_3^- uptake as a function of the whole-plant N status.

Phenotypic Characterization of *hmi* Mutants

To characterize the functional consequences of the altered regulation of *NRT2.1* transcription in *hmi* mutants, the NO_3^- uptake capacities of the mutants were studied in hydroponically grown plants cultivated on HN or LN conditions without Suc. Overaccumulation of the *NRT2.1* transcript in roots of the mutant plants grown hydroponically on HN conditions was confirmed (data not shown). The NO_3^- HATS activity was measured using ^{15}N labeling (Fig. 5). In good agreement with the effects of the mutations on *NRT2.1* expression, all three mutants displayed higher HATS activity (2- to 3-fold) compared to the NL line when the plants were cultivated on HN conditions, while no significant difference was found between the four genotypes when cultivated under LN conditions. This showed that the misregulation of *NRT2.1* expression by high N supply in *hmi* mutants had indeed functional consequences and resulted in a reduced down-regulation of the NO_3^- HATS under repressive conditions. Effect of the *hmi* mutations on NH_4^+ uptake was investigated using ^{15}N labeling (Supplemental Fig. S2). The *hmi9-1* and *hmi48-1* mutants displayed NH_4^+ uptake levels similar to NL, supporting the idea that these mutations have a specific impact on NO_3^- acquisition. However, consistent with the stimulation of *AMT1.2* expression, a significant increase of NH_4^+ uptake was measured in *hmi140-1*, indicating that in this mutant both NH_4^+ and NO_3^- intake were up-regulated.

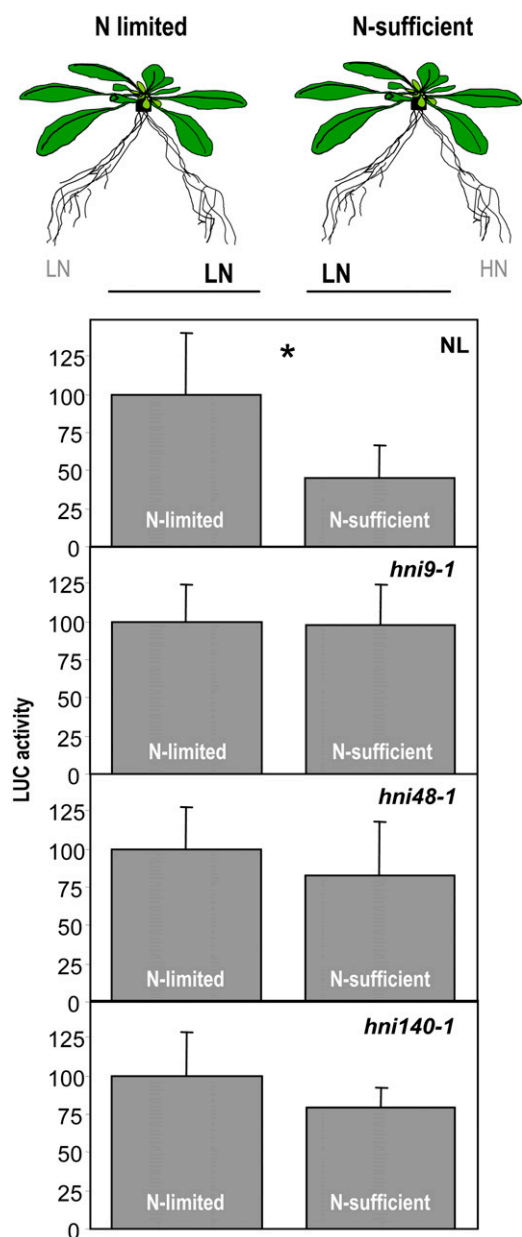


Figure 4. Response of the *NRT2.1* promoter activity to systemic repression exerted by downstream N metabolites in the *hni* mutants. Plants were grown in vitro for 13 d on LN medium supplemented with 1% Suc and then transferred on an heterogeneous medium for 7 d. Half of the root system was maintained on LN medium, while the other half was supplied either with LN medium (N-limited plants) or HN medium (N-sufficient plants). For each genotype, the LUC activities of the LN roots of N-limited and N-sufficient plants were compared. Values are means of six replicates \pm SD. *, Significant differences according *t* test ($P < 0.05$). [See online article for color version of this figure.]

The total N and NO_3^- contents of roots and shoots of NL and *hni* plants cultivated on HN were analyzed (Fig. 6). Except a slight decrease in the *hni9* mutant compared to NL plants, the *hni* mutations were not associated with a significant change in either total N or NO_3^- contents in any organ (according to a *t* test, $P <$

0.05), suggesting that despite up-regulation of the NO_3^- HATS, the overall N acquisition and assimilation was quantitatively not significantly modified in the mutants under these conditions. Growth of the plants in vertical agarose plates on HN medium also appeared little affected in the *hni* mutants compared to the NL line (Supplemental Fig. S3).

To further explore modifications induced by the mutations on primary metabolism, the levels of 28 metabolites (amino acids, organic acids, or sugars) were assayed by a multitargeted gas chromatography-mass spectrometry (GC-MS) profiling approach in the mutant and NL plants grown on HN medium on vertical agarose plates (Supplemental Table S6). Effects on primary metabolism were found to be marginal, as was expected from the minor effects of the genetic lesions on total N and NO_3^- content of the mutants. However, global examination of the data revealed that some quantitative variations between the mutants and the NL line were found in the three classes of compounds. These changes indicated that several pools of metabolites that are directly or indirectly connected to N metabolism were affected by the *hni* mutations. However, each mutant displayed a particular pattern that was in general different between roots and shoots. Among the three mutants, the widest variations of metabolite levels compared to the NL line were found in *hni48-1* plants, in both roots and shoots (Supplemental Table S6). The levels of major metabolites, such as Gln (-59% in the roots), Glu (-44% in the shoots),

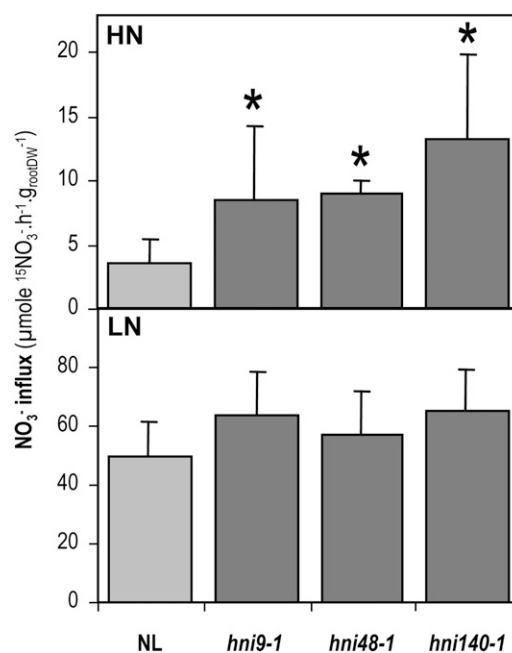


Figure 5. High-affinity NO_3^- influx in the *hni* mutants. Plants were grown hydroponically on LN medium for 6 weeks and transferred on HN or LN medium for 1 week before the experiment. NO_3^- influx was measured at the external concentration of $0.2 \text{ mM } ^{15}\text{NO}_3^-$. Values are means of 10 replicates \pm SD. *, Significant differences according *t* test ($P < 0.05$). DW, Dry weight.

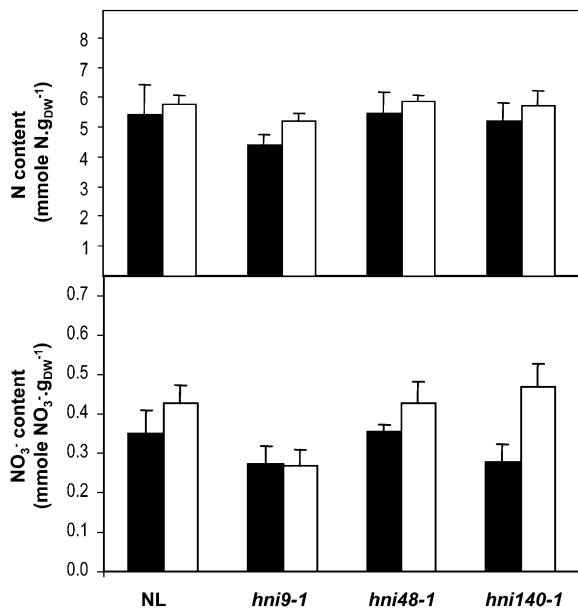


Figure 6. Total N and NO₃⁻ content of *hni* mutants. Plants were grown on vertical agarose plates on HN medium supplemented with 1% Suc for 7 d. Roots (black bars) and shoots (white bars) were harvested separately. Values are means of 10 replicates ± sd. DW, Dry weight.

malic acid (+139% in the roots and -51% in the shoots), Fru (-29% in the roots), and Suc (-49% in the shoots), as well as minor amino acids, organic acids, and sugars indicated a substantial metabolites redistribution in the *hni48* mutant. Even though Gln was found to be reduced in roots of *hni9-1* plants (in parallel with a reduction of Asn in roots and an increase accumulation of Glu in the shoots), no global remodeling of the metabolome was found in this mutant in contrast to the *hni48-1* mutant. The same lack of global metabolic remodeling was apparent in the *hni140-1* mutant. This mutant exhibited only a small decrease in minor sugars of the root organ.

DISCUSSION

hni9-1, *hni48-1*, and *hni140-1* Belong a New Class of N Signaling Mutants

NRT2.1 encodes a main component of the NO₃⁻ HATS in Arabidopsis (Cerezo et al., 2001; Filleur et al., 2001; Li et al., 2007) and is known to be a major target of the feedback control of NO₃⁻ uptake by downstream N metabolites (Lejay et al., 1999, 2003; Zhuo et al., 1999; Cerezo et al., 2001; Gansel et al., 2001; Girin et al., 2007). However, little is known either at the genetic or at the molecular levels concerning the systemic signaling pathway responsible for this regulatory mechanism. On one hand, known genes involved in the regulation of *NRT2.1* expression, namely *NRT1.1* (Muños et al., 2004; Krouk et al., 2006; Wang et al., 2009), *NLP7* (Castaings et al., 2009; Wang et al., 2009), and *CIPK8* (Hu et al., 2009), were to date all shown to

participate only in the local regulation of *NRT2.1* by NO₃⁻. On the other hand, none of the other genes or regulators contributing directly or indirectly to N signaling in Arabidopsis, such as *GLR1.1* (Kang and Turano, 2003), *NLA* (Peng et al., 2007), *CCA1* (Gutierrez et al., 2008), *OSU1* (Gao et al., 2008), *PLD_ε* (Hong et al., 2009), or the miR167/ARF8 regulatory circuit (Gifford et al., 2008), were shown to modulate the response of *NRT2.1* to changes in N provision to the plant.

The genetic screen described here was especially designed to identify mutants impaired in the regulation of *NRT2.1* expression by the N status of the plant, and, indeed, several lines of evidence indicate that the three nonallelic *hni* mutants are signaling mutants affected in this specific regulation. First, mutants that are not constitutively misexpressing *NRT2.1* appear to be predominantly deficient in the repression of the gene in response to high N supply, whereas the stimulation of the gene by NO₃⁻ is unchanged and its stimulation by sugars is only marginally modified (see discussion below). Second, this altered response to high nutrient status is not general since molecular responses to high S supply are conserved in *hni* plants, suggesting that these mutants are specifically impaired in N signaling. Third, all three *hni* mutations almost totally prevent systemic regulation of *pNRT2.1* activity, which is known to be due to long-distance signaling of the N status of the whole plant. Fourth, total N accumulation is not significantly modified in *hni* plants compared to the NL line, ruling out the hypothesis that *NRT2.1* is up-regulated because the mutants suffer from N deficiency under HN conditions (for instance, because they may be affected in the overall N acquisition or assimilation system). Thus, up-regulation of *NRT2.1* expression in *hni* plants is very likely due to an altered regulation by the N status, rather than to the normal activation of this signaling in response to N limitation. The mapping of the three *hni* mutations also further supports the originality of the mutants, since none of the *hni* mutations colocalize with N signaling genes previously identified, with in theory the possible exception of *hni48-1* and *PLD_ε* (Supplemental Fig. S4). Therefore, we concluded that *hni9-1*, *hni48-1*, and *hni140-1* belong to an original class of N signaling mutants because, to our knowledge, they are the first ones identified to be affected in the systemic feedback repression of root NO₃⁻ uptake systems and because they are deficient in previously unidentified N signaling regulatory genes. However, down-regulation of *pNRT2.1* activity by high N supply is not fully suppressed but only attenuated in *hni* plants. Double and triple mutants will be necessary to determine whether putative additive effects between the three independent mutations allow total inactivation of feedback repression of *NRT2.1*.

Specificity of the Effects of *hni* Mutations

One striking observation is that all three *hni* mutants seem to be specifically altered in the regulation of

NO_3^- transport system associated with *NRT2.1*. Indeed, among the investigated genes, only *NRT2.1* and *NAR2.1(NRT3.1)* are misregulated in *hmi* plants. *NAR2.1(NRT3.1)* encodes a protein of unknown function, which interacts with *NRT2.1* and is required for correct expression and transport function of *NRT2.1* (Okamoto et al., 2006; Orsel et al., 2006; Wirth et al., 2007). Thus, *hmi* mutations apparently affect mechanisms ensuring a coordinated repression of both components of the *NRT2.1/NAR2.1(NRT3.1)* transport system by high N status of the plant. On the contrary, neither *NRT1.1* nor the two *NIA* genes display a modified expression level in *hmi* plants compared to NL plants, ruling out a general alteration of the regulation of the whole NO_3^- uptake and reduction system in the mutants. Furthermore, unlike *NRT2.1*, both *AMT1.1* and *AMT1.3* genes do not show increased expression as a result of *hmi* mutations under repressive HN conditions. However, these two genes are, as *NRT2.1*, strongly down-regulated by high N supply (Gazzarrini et al., 1999; Rawat et al., 1999; Loque et al., 2006). Hence, *hmi* plants are regulatory mutants for *NRT2.1*, but not for *AMT1.1* and *AMT1.3*, at least under the conditions investigated. This strongly suggests that the mechanisms responsible for feedback repression of the high-affinity uptake systems for either NO_3^- or NH_4^+ are at least partly independent, as postulated previously (Gansel et al., 2001; Ruffel et al., 2008). This later result also gave further support to the conclusion that none of the *hmi* mutants suffers from N deficiency when grown on HN medium, since this would have triggered a derepression of *AMT1.1* and *AMT1.3* genes. Nevertheless, this did not rule out that some cross talk may exist between mechanisms involved in the systemic repression of *NRT2.1* and the regulation of NH_4^+ uptake, since *AMT1.2* expression, a gene not regulated by the N status of the plant (Gazzarrini et al., 1999; Shelden et al., 2001; Yuan et al., 2007), was stimulated in the *hmi140-1* mutant.

Another unexpected finding is the interaction occurring between *hmi* related pathway(s) and C metabolism or signaling. First, a lowered stimulation of *NRT2.1* expression by Suc was observed in two mutants compared to the NL line, suggesting that the control exerted by photosynthates on *NRT2.1* expression (Lejay et al., 1999, 2003) is also partly under the control of *HNI* genes. Second, the tissue concentration of several organic acids and sugars are modified in the shoots or in the roots of the mutants. The interpretation of these observations is not straightforward since previous studies have indicated that regulation of root NO_3^- uptake systems by reduced N metabolites and by photosynthates involve distinct mechanisms (Delhon et al., 1995, 1996; Lejay et al., 2003). However, several reports have already pointed out that mutation or modified expression of regulators of N metabolism, such as *GLR1.1*, *DOF1*, *OSU1*, and *NLA*, lead to marked changes in C metabolism (Kang and Turano, 2003; Yanagisawa et al., 2004; Peng et al., 2007; Gao et al., 2008). Therefore, the phenotype of the *hmi*

mutants may provide an additional illustration of the fact that N and C signaling pathways are interconnected possibly because they share common regulatory components involved in their tight integration (Palenchar et al., 2004; Gutierrez et al., 2007).

Physiological Consequences of *NRT2.1* Misregulation in *hmi* Mutants

Both increased accumulation of *NRT2.1* mRNA and higher NO_3^- HATS activity resulted from the up-regulation of *pNRT2.1* activity in the mutants under HN repressive conditions. Thus, despite the fact that *NRT2.1* is probably subject to regulation at the protein level (Wirth et al., 2007), this result strongly suggests that the control of *NRT2.1* transcription is actually important for governing NO_3^- HATS. The stimulation of NO_3^- HATS activity recorded in *hmi* plants versus NL plants (2- to 3-fold) may appear relatively modest when compared to the corresponding increase in *NRT2.1* mRNA level (6- to 14-fold). This may indicate that posttranslational mechanisms still active in the mutants contribute to the HATS repression in response to high N supply. Such mechanisms have been proposed to explain why high-affinity NO_3^- influx is still down-regulated by NH_4^+ supply in transgenic *Nicotiana plumbaginifolia* plant constitutively expressing the *NpNRT2.1* gene (Fraisier et al., 2000). However, *NRT2.1* is not the only transporter active in the HATS (Tsay et al., 2007), and its contribution to the residual HATS activity in wild-type plants under repressive conditions is limited (Cerezo et al., 2001). Thus, high-affinity NO_3^- influx measured in NL plants grown on HN medium most probably also results from the activity of other NO_3^- transporters than *NRT2.1*, while the increase resulting from *hmi* mutation is likely to be due to a specific stimulation of *NRT2.1* uptake activity. Although this specific stimulation cannot be precisely determined by our influx assays, it is then probably much stronger than that recorded for the whole HATS. As already seen in transgenic *N. plumbaginifolia* plants overexpressing *NpNRT2.1* (Fraisier et al., 2000), the increase in NO_3^- HATS activity resulting from the up-regulation of *NRT2.1* in *hmi* plants did not lead to higher overall N acquisition or accelerated growth of the plants. This is easily explained by the limited extend of this increase ($10 \mu\text{mol h}^{-1} \text{g}^{-1}$ root dry weight at most; see Fig. 6), which represents only a small fraction of the overall total N (NO_3^- plus NH_4^+) uptake by the plants on HN conditions (estimated at approximately $200 \mu\text{mol h}^{-1} \text{g}^{-1}$ root dry weight; see Fig. 1 in Girin et al., 2007).

Despite the absence of quantitative change in the overall plant N acquisition, a surprising observation was that *hmi9-1* and *hmi48-1* (but not *hmi140-1*) mutants display a limited but statistically significant decrease in the root concentration of Gln (and Asn for *hmi9*). These observations need to be carefully interpreted because at this stage of the study only one allele per mutant locus has been isolated. Although mutants

have been backcrossed three times, we cannot completely rule out the possibility that some specific phenotypes may be the result of additional mutations unfortunately genetically linked to the *hni* mutations. Nevertheless it is of interest to consider whether such metabolic phenotype may explain the up-regulation of *NRT2.1* in *hni9* and *hni48* plants. It has been hypothesized for a long time that feedback repression of NO_3^- uptake by high N status of the plant may be related to increased export of amino acids through the phloem to the roots and increased accumulation of these compounds in the roots (Cooper and Clarkson, 1989; Imsande and Touraine, 1994). Reports showing that exogenous supply of amino acids triggers a strong and rapid repression of NO_3^- uptake capacity and expression of *NRT2.1* support the model of amino acid acting as plant N status signaling molecules (Breteler and Arnozis, 1985; Müller and Touraine, 1992; Zhuo et al., 1999; Nazoa et al., 2003; Girin et al., 2007). Both Gln and Asn have often been shown to have the most negative effect and are thus the most popular candidates as repressors of *NRT2.1* transcription (Zhuo et al., 1999; Nazoa et al., 2003). However, two observations argue against the hypothesis that attenuated feedback repression of *NRT2.1* in *hni9* and *hni48* plants may be directly due to the lowered amino acid accumulations in the roots. First, effects of the mutation on amino acid levels (*hni48-1* > *hni9-1* > *hni140-1*) are inversely correlated to the effects observed on *pNRT2.1* promoter activity (*hni140-1* > *hni9-1* > *hni48-1*). Second, *AMT1.1*, which is known to be responsive to Gln (Rawat et al., 1999), is not up-regulated in *hni9* or *hni48* plants. Whether the metabolic phenotypes exhibited by the *hni* mutants are part of the misfunctions of the N status systemic signaling pathway operating in these plants or the consequences of the misregulations of the target genes remains to be determined.

To our knowledge, this article is the first report demonstrating that systemic regulation of NO_3^- uptake by the N status of the plant is under a genetic control. Identification of the corresponding genes in the future will allow important progress toward the characterization of the molecular components of this regulatory pathway.

MATERIALS AND METHODS

Plant Material and Culture Conditions

The basal nutrient medium without N contained 1 mM KH_2PO_4 , 1 mM MgSO_4 , 0.25 mM K_2SO_4 , 0.25 mM CaCl_2 , 0.1 mM Na-Fe-EDTA, 50 μM KCl, 30 μM H_3BO_3 , 5 μM MnSO_4 , 1 μM ZnSO_4 , 1 μM CuSO_4 , and 0.7 μM MoNaO_4 , pH adjusted to 5.8 with KOH. The N source was added to the medium generally as 0.3 mM KNO_3 (LN), 0.3 mM NH_4Cl (NI), or 10 mM NH_4NO_3 (HN). For in vitro culture, the medium was solidified with 0.8% agarose and 2.5 mM MES, and 10 g L^{-1} Suc was eventually added (specified in the text). Plants were grown on vertical plates under the following environmental conditions: 16-h/8-h light/dark cycle, 125 $\mu\text{mol s}^{-1} \text{m}^{-2}$ photosynthetically active radiation light intensity, 21°C/18°C day/night temperature, and 70% hygrometry. For hydroponic cultures, the liquid nutrient solution was renewed once a week during the first 5 weeks of the culture and daily the last week before the experiment. Plants were grown in a 10-liter tank as previously described

(Lejay et al., 1999) under the following environmental parameters: 8-h/16-h light/dark cycle, 300 $\mu\text{mol s}^{-1} \text{m}^{-2}$ photosynthetically active radiation light intensity, 22°C/20°C day/night temperature, and 70% hygrometry.

Mutant Screening

The NL transgenic line carrying the *LUC* reporter gene under control of the *pNRT2.1* promoter region was used for mutant screening. The *pNRT2.1::LUC::tNOS* construct was obtained by fusing the 1201 bp located upstream the initiating codon of *NRT2.1* (*HindIII-NcoI* fragment; Nazoa et al., 2003) to the promoterless cassette *LUC::tNOS* (derived from pSP luc+; Promega). The chimeric gene was inserted as a *HindIII-EcoRI* fragment into the pBIB-Hyg binary vector (Becker, 1990). The vector was then introduced into *Agrobacterium tumefaciens* GV3101. Arabidopsis (*Arabidopsis thaliana*) Columbia-0 was transformed according to Clough and Bent (1998). Cloning and sequencing of the genomic sequence flanking the right border of the T-DNA has been achieved by the method described by Devic et al. (1997). NL seeds were mutagenized with 0.3% ethyl methylsulfonate for 10 h at 23°C. M2 seeds were collected from pools of 100 plants. Approximately 70% of M1 plants segregated embryo lethal mutants, indicating the efficiency of the mutagenesis. To maximize the probability to screen independent mutations (Rédei, 1992), approximately two M2 plants per M1 plant were screened by bioluminescence imaging. Mutation mappings were performed according the strategy described by Konieczny and Ausubel (1993) using PCR-derived markers described either on http://www1.montpellier.inra.fr/biochimie/td/Genetic_Map/liste_marqueurs.html or <http://www.inra.fr/internet/Produits/vast/msat.php>.

^{15}N Labeling, NO_3^- , and Metabolite Measurements

The capacity of the NO_3^- HATS was assayed according to Delhon et al. (1995). Plants were sequentially transferred to 0.1 mM CaSO_4 for 1 min and to the basal nutrient solution supplemented with 0.2 mM KNO_3 (99% ^{15}N atom excess) as sole N source for 10 min. At the end of the labeling, the roots were washed for 1 min in 0.1 mM CaSO_4 and were separated from the shoots. The organs were dried at 70°C for 48 h and weighed and analyzed for total ^{15}N content using a continuous-flow isotope ratio mass spectrometer (Isoprime mass spectrometer; GV Instruments) coupled to a CN elemental analyzer (Euro Vector). NH_4^+ influx was measured using a similar protocol except that the basal nutrient solution was supplemented with 10 mM $^{15}\text{NH}_4^+$. For NO_3^- content analysis, ions were extracted from dried tissues in water for 24 h at 4°C. Nitrate concentration was determined colorimetrically in presence of sulfanilamide and *N*-naphthyl-ethylene diamine-dichloride after reduction of NO_3^- to NO_2^- on a cadmium column using an autoanalyzer (Brann-Lubbe). Routine GC-MS-based metabolite profiling was applied to a targeted assessment of differential metabolite accumulation. GC-MS profiling was performed on 100 mg (fresh weight) of root or shoot material as described previously (Sanchez et al., 2008). Multiparallel chromatography data processing and compound identification were performed by TagFinder software (Luedemann et al., 2008) using reference spectra from the Golm Metabolome Database for compound identification (Kopka et al., 2005).

RNA Analysis

Samples were frozen in liquid nitrogen in 2-mL microtubes containing one steel bead (2.5-mm diameter). Tissues were disrupted for 1 min at 30 s^{-1} in a Retch mixer mill MM301 homogenizer. Total RNA was extracted from tissues using TRIzol reagent (Invitrogen) and purified using an RNeasy MinElute Cleanup Kit (Qiagen) after DNase treatment (Qiagen). Reverse transcription was achieved with 4 μg of RNAs in the presence of Moloney Murine Leukemia Virus reverse transcriptase (Promega) after annealing with an anchored poly-dT₍₁₈₎ primer. Accumulation of transcript was measured by quantitative real-time PCR (LightCycler; Roche Diagnostics) using the Light Cycler FastStart DNA Master Syber Green 1 kit (Roche Diagnostics). Specific primers used in this study are listed in Supplemental Table S7. All amplification efficiencies were higher than 0.8. Expression of gene of interest was normalized either by *EF1 α* or *CLATHRIN* as internal standards.

LUC Activity

Bioluminescence images were acquired using a -50°C cooled CCD camera C4880 (Hamamatsu). Images were processed using the HiPic32 v5.1.0 soft-

ware (Hamamatsu). Plants were grown in vitro on vertical agarose plates. Before the luminescence acquisition, plants were sprayed with 1 mM luciferine solution containing 0.01% Triton X-100. After 10-min incubation (at 21°C in the dark), the luminescent image was acquired for 5 min in a dark chamber. Then, a white light image of plants was obtained by a 20-ms acquisition. A final chimeric image was obtained by superposition of luminescence image (false colors) and light image (black and white). Quantification of the extractable LUC enzymatic activity was determined using the Steady-Glo Luciferase Assay System (Promega). Samples (1–10 mg of root tissues) frozen in liquid nitrogen were disrupted for 1 min at 30 s⁻¹ in a Retch mixer mill MM301 homogenizer in 2-mL microtubes containing one steel bead (2.5-mm diameter). Proteins were extracted in 350 µL of 50 mM sodium phosphate buffer, pH 7.8, 2 mM dithiothreitol, 10% v/v glycerol, and 1% v/v Triton X-100. Extracts were clarified by filtration on glass wool. Enzymatic activities were determined in microtiter plates at 37°C using the Steady-Glo Luciferase reagent (Promega). After a 15-min reaction, luminescence was measured during 1 s using a Wallac Victor 2 luminometer (Perkin-Elmer). The LUC activity was normalized by the fresh weight of the samples. Similar results were obtained when normalizing the activity by the DNA content of the extract (measured using picogreen reagent; Molecular Probes).

Supplemental Data

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

Supplemental Figure S1. Identification of the T-DNA insertion site of the NL transgenic line.

Supplemental Figure S2. NH₄⁺ influx in the *hmi* mutants.

Supplemental Figure S3. Picture of *hmi* mutants.

Supplemental Figure S4. Genome mapping of Arabidopsis genes reported to play a role in N signaling compared to *hmi* loci.

Supplemental Table S1. Backcrosses of the *hmi* mutants to the NL parental line.

Supplemental Table S2. Mapping of the *hmi* mutations within the Arabidopsis genome.

Supplemental Table S3. *hmi140-1* × *hmi48-1* complementation test.

Supplemental Table S4. Variance analysis of the data presented in Figure 3.

Supplemental Table S5. Expression of genes related to N acquisition in roots of the *hmi* mutants.

Supplemental Table S6. Metabolite profiling of the *hmi* mutants.

Supplemental Table S7. Gene-specific primers for quantitative real-time PCR.

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