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# HIGH NITROGEN INSENSITIVE 9 (HNI9)-mediated systemic repression of root $\text{NO}_3^-$ uptake is associated with changes in histone methylation

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**In plants, root nitrate uptake systems are under systemic feedback repression by the N satiety of the whole organism, thus adjusting the N acquisition capacity to the N demand for growth; however, the underlying molecular mechanisms are largely unknown. We previously isolated the *Arabidopsis high nitrogen-insensitive 9-1 (hni9-1)* mutant, impaired in the systemic feedback repression of the root nitrate transporter *NRT2.1* by high N supply. Here, we show that *HNI9* encodes *Arabidopsis* INTERACT WITH SPT6 (*AtIWS1*), an evolutionary conserved component of the RNA polymerase II complex. *HNI9/AtIWS1* acts in roots to repress *NRT2.1* transcription in response to high N supply. At a genomic level, *HNI9/AtIWS1* is shown to play a broader role in N signaling by regulating several hundred N-responsive genes in roots. Repression of *NRT2.1* transcription by high N supply is associated with an *HNI9/AtIWS1*-dependent increase in histone H3 lysine 27 trimethylation at the *NRT2.1* locus. Our findings highlight the hypothesis that posttranslational chromatin modifications control nutrient acquisition in plants.**

*Arabidopsis* genetics | nitrogen signaling | plant chromatin | nutrient uptake

Nutrient homeostasis in plants requires that the root transport systems responsible for the uptake of mineral ions from the soil are finely tuned by the nutrient demand of the whole organism (1). It has been hypothesized that an important part of this adjustment relies on systemic signaling pathways that integrate the nutrient status of the various organs and convey this information to the roots where transduction mechanisms modulate expression and/or activity of ion transporters (2–4). However, for most nutrients, the mechanisms of internal nutrient sensing, long-distance signaling, and downstream regulation of uptake systems in roots remain poorly characterized (5). Components of these whole-plant signaling cascades have been recently discovered for phosphate and sulfate in the model plant *Arabidopsis thaliana*, where miR395 and miR399 have been shown to act as long-distance signals triggering adaptive responses to S and P deficiency, respectively (4). When considering nitrate ( $\text{NO}_3^-$ ), the main N source for many higher plant species, an additional complexity is that root  $\text{NO}_3^-$  uptake is not only adjusted to the N demand of the whole plant, but also strongly modulated locally by the external availability of the ion (6).  $\text{NO}_3^-$  itself is a signal molecule that triggers induction of genes required for its use as an N source, including some of its own membrane transporters and assimilatory enzymes (7–9). Several genes involved in the local response to  $\text{NO}_3^-$  affecting root  $\text{NO}_3^-$  transporters have recently been identified in *Arabidopsis*, e.g., *NRT1.1* (10–12), *NLP7* (12, 13), *CIPK8* (14), and *LBD37-38-39* (15). By contrast, the systemic mechanisms allowing root  $\text{NO}_3^-$  uptake to respond to changes of the N status of the whole plant remain poorly characterized. It has frequently been hypothesized that this mechanism involves a feedback repression of

root  $\text{NO}_3^-$  uptake systems by products of N assimilation, such as glutamine, that may act as long-distance signals translocated from shoots to roots by the phloem (1, 2). Despite the physiological evidence supporting this model, its unequivocal validation is still lacking because responsible genes have not been identified so far. However, *NRT2.1*, encoding the main component of the root high-affinity transport system (HATS) for  $\text{NO}_3^-$ , is now well known as a major target of this mechanism (16, 17). Both HATS activity and *NRT2.1* expression are repressed in plants under high N supply (7, 18). Split-root experiments have demonstrated that this repression is due to systemic N signaling, which is expected for a mechanism triggered by the whole-plant N status (19). At least part of this mechanism is due to transcriptional control, because down-regulation of *NRT2.1* mRNA accumulation is tightly correlated with the inhibition of *NRT2.1* promoter activity (20). To isolate mutants defective in systemic N signaling, we have initiated a forward genetic screen using a transgenic line expressing a *pNRT2.1::LUC* construct as a reporter gene (21). Three high nitrogen-insensitive (*hni*) mutants were isolated (*hni9-1*, *hni48-1*, and *hni140-1*) that display an increased expression of *NRT2.1* compared with the parental line when subjected to high N (HN) repressive conditions (10 mM  $\text{NH}_4\text{NO}_3$ ), but not under low N (LN) permissive conditions (0.3 mM  $\text{NO}_3^-$ ). Split-root experiments confirmed that all three mutants are predominantly impaired in the systemic repression of the HATS activity by high N supply (21).

In this paper, we report that *HNI9* is allelic to *Arabidopsis* INTERACT WITH SPT6 *AtIWS1* (22), encoding for an evolutionary conserved protein belonging to the RNA polymerase II complex (RNAPII). *HNI9/AtIWS1* plays an important role in the systemic regulation of root  $\text{NO}_3^-$  uptake in *Arabidopsis*,

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The authors declare no conflict of interest.

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Data deposition: The Affymetrix GeneChip data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo) (accession no. GSE24738).

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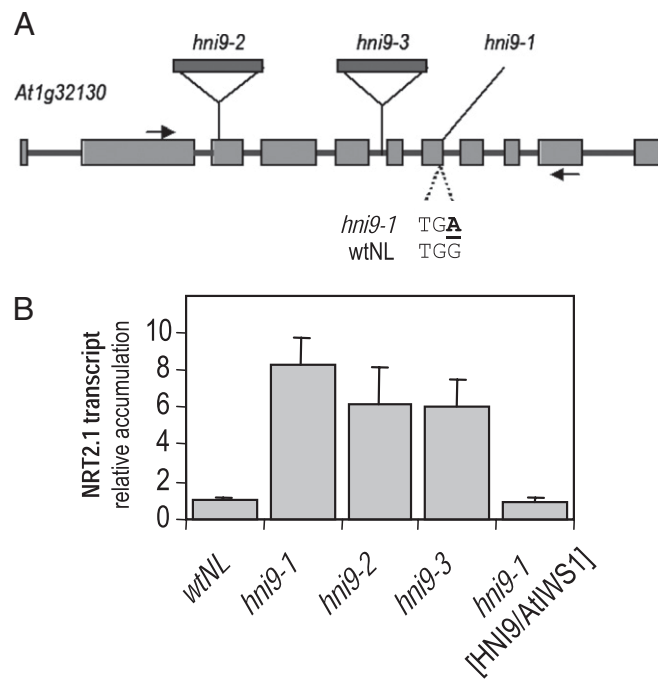
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which is associated with histone lysine 27 trimethylation at the *NRT2.1* locus.

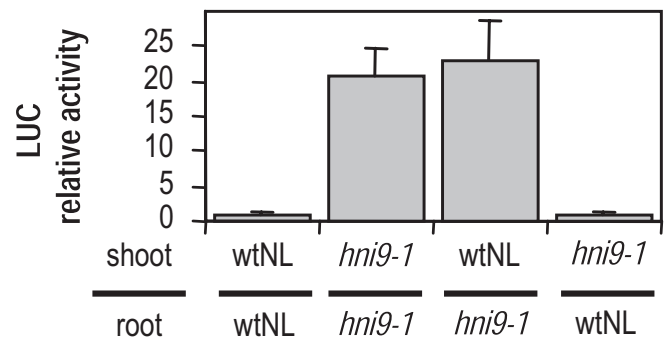
## Results

**HNI9 Encodes AtIWS1, a Component of the RNAPII Complexes.** The *hni9-1* mutant (21) was outcrossed with the wild-type ecotype *Ler* for chromosome walking and subsequent map-based cloning (Fig. S1). Sequencing of genomic DNA from the mutant revealed a G-to-A substitution in the last nucleotide of the seventh exon of the *At1g32130/HNI9* gene (Fig. 1A), which prevents intron splicing and generates a premature stop codon (Fig. S1). Gene cloning was confirmed by identification of two additional allelic transferred DNA (T-DNA) insertion mutants, *hni9-2* and *hni9-3*, displaying a phenotype similar to that of *hni9-1*, and by complementation of the *hni9-1* mutant with a genomic fragment containing the wild-type *At1g32130* gene (Fig. 1B and Fig. S1A). *At1g32130* has recently been characterized and named *AtIWS1* (22). It encodes a protein sharing sequence homologies with the conserved C-terminal domain of IWS1 proteins found in a wide range of eukaryotic organisms, including human, mouse, plants, yeast, and fungi (Fig. S2A and B). These proteins are components of the large RNAPII (23–31). A paralog of *HNI9/AtIWS1*—*At4g19000/AtIWS2*—is present in the *Arabidopsis* genome, but its mutation does not cause *NRT2.1* derepression at high N supply (Fig. S2C).

**N Status-Dependent Regulation of *NRT2.1* Expression Requires *HNI9/AtIWS1* Function in Roots but Not in Shoots.** The *HNI9/AtIWS1* transcript accumulates at similar levels in both shoots and roots and is not regulated by high N supply in roots (Fig. S3). Previous split-root experiments have shown that the systemic feedback repression of *pNRT2.1* activity by high N supply is impaired in *hni9-1* (21). To investigate if this phenotype is related to a *HNI9/*



**Fig. 1.** *HNI9* is *AtIWS1/At1g32130*. (A) Gene structure and mapping of the various *hni9* alleles. The *hni9-2* (SalK\_056238) and *hni9-3* (GK\_556E04) mutants are T-DNA insertion lines. Arrows are PCR primers used for RT-PCR (Fig. S1). (B) Relative accumulation of *NRT2.1* transcript in the three *hni9* mutants and a *hni9-1* transgenic line complemented with a 4.6-kb genomic fragment carrying the *At1g32130* gene. Plants were grown on HN medium. Values are means  $\pm$  SD ( $n = 4$ ).



**Fig. 2.** Repression of *NRT2.1* by high N supply requires *HNI9/AtIWS1* function only in the roots. Micrografting experiments were performed as described by Turnbull et al. (49) on 5-d-old plants (wtNL and *hni9-1*) grown on vertical agar plates on HN medium. LUC activity was measured in roots 12 d after grafting. Verification of graft integrity was done by dissecting silicon tubing collars. Only fully joined scion/rootstock were used for analysis. Luciferase assays were performed as described in Girin et al. (20). Values are means  $\pm$  SD ( $n = 12$ ).

*AtIWS1* function in roots and/or in shoots, reciprocal grafting experiments were performed using *hni9-1* and the wild-type parental transgenic line wtNL (both contain the *pNRT2.1::LUC* transgene; Fig. 2). Derepression of *pNRT2.1* at high N supply was observed when wild-type shoots were grafted on the *hni9-1* roots, demonstrating that the suppression of *HNI9/AtIWS1* function in roots is necessary and sufficient to yield the mutant phenotype.

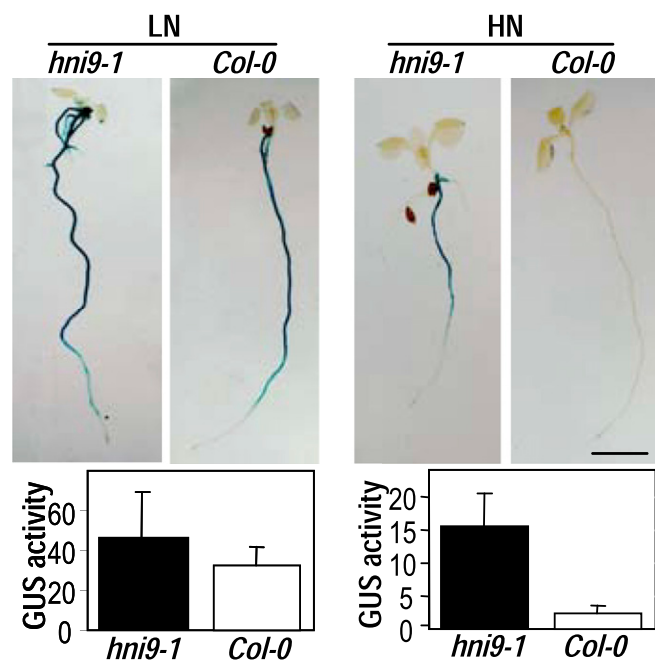
***HNI9/AtIWS1* Is Involved in the Regulation of Several Hundreds of N-Responsive Genes in Roots.** A transcriptome study (ATH1; Affymetrix) was performed using roots of 9-d-old *hni9* and WT plants grown either on HN or LN media (three biological repeats). A two-way analysis of variance identified subsets of differentially expressed genes (Dataset S1): 2,253 genes were regulated by the N treatment (HN vs. LN), 2,348 were affected by *hni9* mutation (*hni9* vs. WT), and 781 were regulated by the interaction of both factors ( $N^*hni9$ ). *NRT2.1* was found in the last two subsets (N and  $N^*hni9$ ). Despite the fact that *HNI9/AtIWS1* inactivation has a limited impact on genome expression (<12% of the transcriptome), a particular role in the expression of N-responsive genes is observed. This is supported by the strong interaction between the effects of N treatment and *hni9* mutation ( $N^*hni9$ ), as well as by the large overlap between the

	<i>hni9</i> regulated	<i>seb1</i> regulated	N regulated
<i>hni9</i> regulated	2348 genes		
<i>seb1</i> regulated	341 genes	1617 genes	
		$Z_{score} = 21.7$	
N regulated	686 genes	274 genes	2253 genes
		$Z_{score} = 16.6$	$Z_{score} = 44.6$
BL induced	60 genes	49 genes	51 genes
	$Z_{score} = 7.8$	$Z_{score} = 8.5$	$Z_{score} = 6.2$

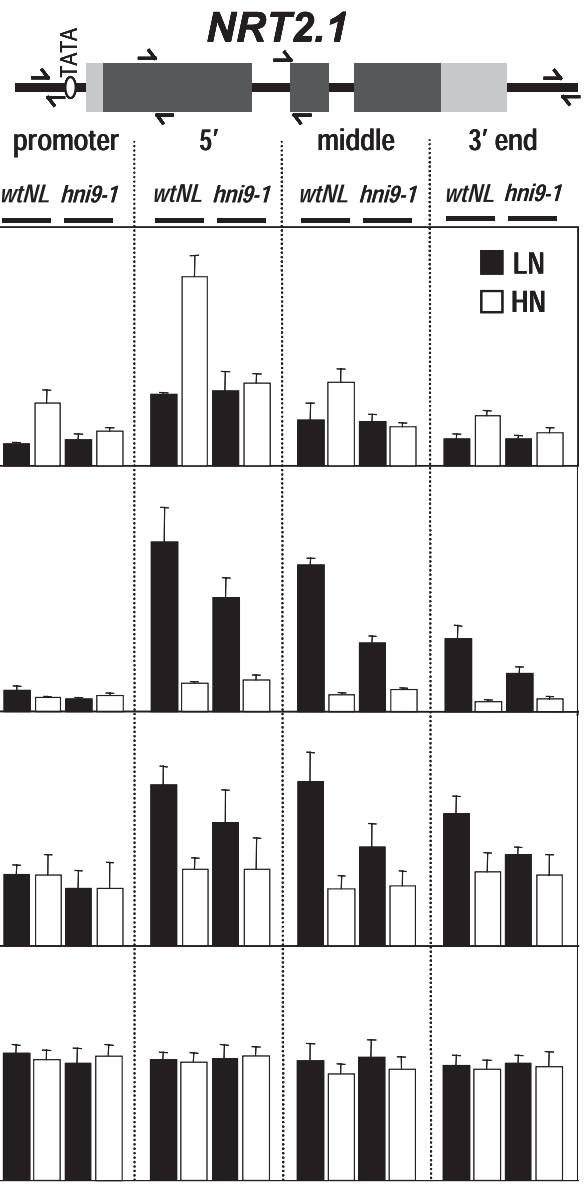
**Fig. 3.** N-regulated genes are overrepresented in *HNI9/AtIWS1*-regulated genes. Intersections between *hni9*-regulated (this study), N-regulated (this study), BL-induced (22), and *seb1*-regulated genes (22) are presented. A post-hoc procedure has been used to investigate whether overlap between gene lists was higher than expected by random distribution. The Z-score estimates the oversize of the intersection (significance threshold is eight SDs).

genes responsive to N and to *hni9* (686 genes overlap), far exceeding what could be expected by random distribution (Fig. 3). Genes regulated by HNI9/AtIWS1 in roots belong to 15 expression clusters (Fig. S4A). Functional categories related to N and C primary metabolism are clearly overrepresented in these clusters (Fig. S4B) defining HNI9/AtIWS1 controlled biomodules (32). The comparison of our data with the transcriptome of the other *hni9/atw1* mutant *seb1* reported by Li et al. (22) highlights a robust implication of HNI9/AtIWS1 in N signaling despite experimental differences between the two studies [plant material is roots in our case and whole plants in Li et al. (22)]. First, *seb1*-responsive genes also largely overlap with N-responsive genes (Fig. 3). Second, the common set of genes affected by both mutations (Fig. 3) includes a large proportion of N-regulated genes (134 genes, 39%). Only a small overlap was found between *hni9*-affected genes and brassinolide (BL)-induced genes identified by Li et al. (Fig. 3), indicating that the role of HNI9/AtIWS1 in BL response previously described by these authors in whole plants (22) is marginal in roots in our conditions (Discussion).

**HNI9/AtIWS1 Is a Nuclear Protein That Represses the Activity of *cis*-Element(s) of the *NRT2.1* Promoter.** The HNI9/AtIWS1 protein sequence contains two putative nuclear localization signals (NLS) suggesting the targeting of the protein to the nucleus (Fig. S5). Transgenic *hni9-1* plants complemented with a functional p35S::GFP::HNI9/AtIWS1 fusion protein display an exclusive GFP localization in nuclei (Fig. S6A–C). The signal was homogeneously distributed in young cells close to the meristem but was progressively restricted to nuclear foci in differentiated cells (Fig. S6D and E). Comparison of GFP::HNI9/AtIWS1 and GFP::HNI9-1 fusion proteins showed that the *hni9-1* mutation prevents the correct targeting of the protein to the nucleus (Fig. S6F and G), suggesting that the NLS-2 sequence is required for proper nuclear



**Fig. 4.** HNI9/AtIWS1 is involved in the repression of high N supply of *cis*-acting elements located upstream the TATA box of the *NRT2.1* promoter. The *pNRT2.1(456/95)::pmini35S::GUS* transgene initially in *Col0* genetic background (20) was introgressed into *hni9-1*. GUS staining and GUS activity quantification (pmolMU<sup>-1</sup>·mg<sup>-1</sup>·root<sub>DW</sub><sup>-1</sup>) were done on 6-d-old plants grown on HN and LN medium as described by Girin et al. (20). Values are means ± SD ( $n = 8$ ). (Scale bar: 0.5 cm.)



**Fig. 5.** Effect of *hni9-1* on accumulation of histone posttranslational modifications on *NRT2.1* chromatin. CHIP was performed on root extracts from 12-d-old plants grown on HN or LN medium. The relative levels of H3K27me3, H3K4me3, and H3K36me3H3 posttranslational modifications and histone H3 were quantified by real-time quantitative PCR using primers sets amplifying different regions of *NRT2.1*. Values are means ± SD ( $n = 3$ ).

localization of HNI9/AtIWS1. Together, these findings confirm the nuclear function of the HNI9/AtIWS1 protein in *Arabidopsis* (22).

The *pNRT2.1::LUC* transgene used to isolate the *hni9-1* mutant included the 67-bp 5'UTR of *NRT2.1*. Therefore, it was unclear whether HNI9/AtIWS1 inactivation affects the transcription of *NRT2.1* and/or the stability of the transcript via the 5'UTR of *NRT2.1*. To clarify this point, a *GUS* transgene under the control of a chimeric promoter (20) was introgressed into *hni9-1*. This promoter contained the 366-bp sequence located upstream of the *NRT2.1* TATA box fused to a minimal 35S promoter (restricted to the TATA box and the transcription start site). Analysis of *GUS* expression (Fig. 4) demonstrated that HNI9/AtIWS1 represses the *cis*-acting elements present upstream of the TATA box, and therefore that the 5'UTR of

*NRT2.1* was not required for HNI9/AtIWS1-dependent repression of *NRT2.1* expression.

**High N Supply Results in a HNI9/AtIWS1-Dependent Increase of the H3K27me3 Level on *NRT2.1* Chromatin.** The IWS1 proteins were initially classified as transcription elongation factors (23), but were subsequently shown to be involved in several other aspects of transcription, e.g., recruitment of chromatin-remodeling factors (28), mRNA processing and export (26), and posttranslational histone modification (27). Genome-wide analysis on seedlings grown with high N supply has identified the chromatin of *NRT2.1* as being enriched in histone H3 lysine 27 trimethylation (H3K27me) (33). We therefore investigated histone methylation at the *NRT2.1* locus in *hni9-1* roots (Fig. 5). In wtNL plants, the N status of the plant was correlated with important changes in the abundance of the various H3 methylation marks present on the chromatin of the *NRT2.1* locus. The accumulation of H3K27me3 residues on all segments of *NRT2.1* (from promoter to 3' end) was strongly stimulated on HN compared with LN, whereas the levels H3K4me3 and H3K36me3 residues showed the opposite response, except at the promoter level, where they remained only slightly affected (Fig. 5). These data are consistent with the knowledge that H3K27me3 is generally associated with transcription repression, whereas H3K4me3 and H3K36me3 are accumulated on actively transcribed genes (34). The N effects on H3 methylation were not associated to changes in nucleosome abundance on the *NRT2.1* chromatin, because H3 abundance was equivalent in all examined conditions (Fig. 5). The increased accumulation of H3K27me3 at *NRT2.1* in plants supplied with HN medium was almost completely abolished in *hni9-1*, whereas mutant and wild-type plants harbored similar levels of these histone methylation marks when grown on LN medium (Fig. 5). This result demonstrates that HNI9/AtIWS1 is an important factor in the deposition of H3K27me3 at *NRT2.1* locus in response to high N supply. The variations of H3K27me3 levels observed in the region located upstream of the TATA box of *NRT2.1* are consistent with the effect of the *hni9-1* mutation on *cis*-acting element(s) of *pNRT2.1* (see previous paragraph). Interestingly, inactivation of HNI9/AtIWS1 did not prevent the increase in levels of H3K4me3 and H3K36me3 at *NRT2.1* in response to low N supply. Although *hni9-1* mutation tended to lower these levels in plants grown on LN medium, these limited changes did not lead to a reduced accumulation of *NRT2.1* transcript in the roots (20). Additional investigations on chromatin modifications present at the *At1g69660* gene (encoding a TRAF family-like protein) extended the conclusions

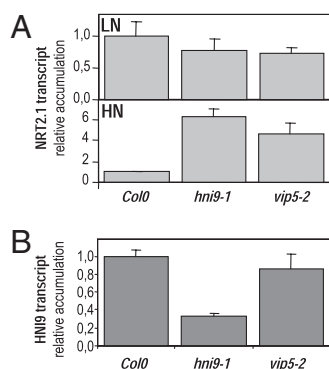
concerning the impact of HNI9 inactivation on H3K27me3 deposition on at least another gene repressed by high N supply (Fig. S7).

**Inactivation of AtVIP5 Impairs the Repression of *NRT2.1* by High N Supply.** In yeast and human, evidence have been uncovered indicating that IWS1 interacts physically or functionally with many other proteins of the RNAPII complexes, including SPT6, the arginine methyl transferase PRMT5/SKB1, the histone methyl transferase HYPB/SET2, and RTF1, a component of PAF1C complex (25–28). We failed to isolate a null mutant for the *AtSPT6* gene (*At1g65440*), most probably because its inactivation is embryo-lethal. Null mutants of *At1g61040/VIP5*, encoding for a protein homologous to RTF1, *At4g31120/SKB1*, and *At1g77300/SDG8*, encoding for a protein homologous to HYPB/Setd2, have previously been described (35–37). We analyzed *NRT2.1* expression by real-time quantitative PCR in *vip5-2*, *skb1-2*, and *sdg8-1* plants grown for 9 d on HN medium (Fig. 6 and Fig. S8A). Derepression of *NRT2.1* was found in both *vip5-2* and *hni9-1* but not in *skb1-2* and *sdg8-1*. Interestingly, like the *hni9-1* mutant, *vip5-2* has no *NRT2.1* expression phenotype when plants were grown on LN medium (Fig. 6A), indicating a specific effect of the mutation on the response to high N supply. The lack of repression of *NRT2.1* by high nitrogen supply in the *vip5-2* mutant was not due to an indirect effect on HNI9/AtIWS1 expression, because HNI9/AtIWS1 transcript levels were similar in the mutant and the WT (Fig. 6B). When coexpressed transiently in onion cells, VIP5 and HNI9/AtIWS1 show substantial areas of colocalization in nuclei (Fig. S8 B–G). We did not succeed in obtaining a double-*vip5-2hni9-1* mutant (most probably because of the embryo lethality of this genotype), preventing genetic tests for function of HNI9/AtIWS1 and VIP5 in a same pathway. Nevertheless, our data indicate that at least one other component of RNAPII complexes is instrumental in the repression of *NRT2.1* by high N supply.

## Discussion

The adjustment of root  $\text{NO}_3^-$  uptake to the N demand of the whole plant requires mechanisms ensuring N sensing, systemic signaling, signal transduction, and regulation of gene expression. Until now, these mechanisms were totally obscure at the molecular level because none of the genes involved was identified. We obtained genetic evidence demonstrating that HNI9/AtIWS1 is one component of the pathway involved in the systemic feedback repression of the  $\text{NO}_3^-$  HATS by high N status of the plant. Our grafting experiments demonstrate that lack of repression of *NRT2.1* in the *hni9-1* mutant is the result of the inactivation of HNI9/AtIWS1 in the root. Thus, HNI9/AtIWS1 is likely to be a relatively downstream step of the *NRT2.1* regulation pathway, most probably associated with the transduction of the N systemic signal. Transcriptome analyses revealed that HNI9/AtIWS1 determines the expression of several hundred N-responsive genes in the roots.

HNI9/AtIWS1 is an evolutionary conserved protein belonging to the family of IWS1 nuclear proteins involved in the function of the RNAPII. In yeast, IWS1 was shown to be involved in the chromatin remodeling at a specific gene locus in response to changes in C supply (38, 30, 31). In *Arabidopsis*, HNI9/AtIWS1 interaction with the conserved homolog of STP6 has been demonstrated (22), suggesting that the network of interaction of IWS1 identified in yeast and human may be conserved in higher plants. We obtained genetic evidence indicating that VIP5, another component of the RNAPII complex, is involved in the response of *NRT2.1* to the N status of the plant. VIP5 encodes the *Arabidopsis* homolog of RTF1, a component of the PAF1C complex, implicated in yeast, human, and *Arabidopsis*, in several aspects of transcription, including histone methylation (39, 35). RTF1 has been shown to display



**Fig. 6.** Inactivation of *VIP5* phenocopies *hni9-1*. Total RNA was isolated from roots of 9-d-old Col-0, *hni9-1*, and *vip5-2* (Salk\_062223) plants. Transcript levels were quantified by real time RT-PCR in roots. (A) *NRT2.1* transcript levels in plants cultivated on HN or LN medium. (B) HNI9/AtIWS1 transcript levels in plants cultivated on HN medium. Values are means  $\pm$  SD ( $n = 4$ ).

a functional interaction with IWS1 in yeast, but the basis of this interaction is not elucidated (28). Neither the inactivation of *SKB1* (encoding the homolog of PRMT5/SKB1) nor the mutation of *SDG8* (encoding the homolog of HYPB/Setd2) impacted *NRT2.1* expression. This result indicates that only specific activities of the RNAPII complex are implicated in the response of *NRT2.1* to high N supply. We showed that HNI9/AtIWS1 represses the activity of the *cis*-acting sequences already identified as involved in the response of the promoter to N status of the plant (20). Therefore, despite that additional contribution of the protein to other functions of the RNAPII complex cannot be excluded, a role of HNI9/AtIWS1 on transcription of *NRT2.1* or on its initiation has to be considered. Our work indicates in an unpredicted way that mechanisms involved in plant responses to nutrient availability include posttranscriptional histone modifications and chromatin remodeling. Modulations of the chromatin status of particular genes in developmental responses to environmental cues (such as temperature and light) have been already described in plants. The best-characterized gene is *FLC*, whose repression has been associated with the loss of H3K4me2/3 and to the progressive enrichment of H3K27me3 marks at the chromatin during prolonged cold treatment (40). Recently, an increasing number of studies have shown that dynamic variations of posttranscriptional histone modifications are associated with a broader range of plant responses (not strictly developmental) to environmental or intrinsic cues (41–45). Our study provides evidences that the repression of *NRT2.1* expression by high N supply is associated with the stimulation of H3K27me3 accumulation and with the decrease of H3K4me3 and H3K36me3 levels on chromatin of *NRT2.1* (and possibly on other N-responsive genes). Remarkably, HNI9/AtIWS1 is specifically involved in H3K27me3 deposition, but not in H3K4me3 and H3K36me3 deposition, in response to high N supply. Whether these histone methylation marks are found together in the same cells or in different cell types deserves further investigation (46). Because repression of both *NRT2.1* and *At1g69660* by high N supply and increased deposition of H3K27me3 marks at both gene loci were impaired by *hni9* mutation, our data strongly suggests that HNI9/AtIWS1 represses gene expression by promoting H3K27me3 methylation on specific N-responsive loci. However, whether increased H3K27me3 methylation is the cause of *NRT2.1* repression by high N supply or is the consequence of prior changes of *NRT2.1* expression mediated by other HNI9/AtIWS1-dependent mechanisms remained to be investigated.

HNI9/AtIWS1 was recently shown to interact with the BES1 transcription factor and to be involved in BL response in whole plantlets (22); this raises the question of whether BL could interact with, or contribute to, N signaling mechanisms. Our data do not bring evidence of such an assumption, at least in the roots. First, BL-induced genes found by Li et al. (22) were not significantly overrepresented among the N-responsive genes we identified (Fig. 3). Second, expression of BL-induced genes was not markedly dependent on HNI9/AtIWS1 in our conditions because the overlap between BL-induced genes and *hni9*-affected genes is in the range of what could be expected by random distribution (Fig. 3). Collectively, these data do not support a specific role of BL in the HNI9/AtIWS1-dependent regulation of gene expression by N signaling in roots. However, the experiments of Li et al. (22) were performed with whole seedlings, which does not preclude a more direct function of HNI9/AtIWS1 on BL-responsive genes in the shoot. HNI9/AtIWS1 might function as a platform used by distinct signaling pathways involved in distinct functions in shoot and roots.

The discovery of HNI9/AtIWS1 will allow the advancement of further insights into the systemic regulation of root  $\text{NO}_3^-$  uptake

by the N status of the whole plant. Whether other *hni* mutants, not allelic to *hni9* (21), are related to the HNI9/AtIWS1 pathway is an exciting open question. Our results show that the mechanism controlling the accumulation of H3K4me3 and H3K36me3 marks on *NRT2.1* chromatin in response to N supply does not depend on HNI9/AtIWS1, suggesting that at least one additional pathway targeting chromatin modifications is involved in the plant response to N status.

## Materials and Methods

**Plant Material.** Unless otherwise stated in the text, the *Arabidopsis thaliana* ecotype used in this study is Columbia (Col-0). The wtNL line has been described (21). Plants were grown on vertical agar plates on basal medium supplemented with 10 mM  $\text{NH}_4\text{NO}_3$  (HN) or 0.3 mM  $\text{KNO}_3$  (LN) as the sole nitrogen source, as previously described (21). Transformation and selection of transgenic line were carried out according to Girin et al. (20). The T-DNA insertion mutants of the Salk or the Gabi-kat tagged populations were obtained from the Nottingham Arabidopsis Stock Center. Genomic sequence containing the *At1g32130* gene was introduced into the pKGW0 binary vector (47) using gateway technology (Invitrogen).

**Gene Expression and Microarray Analysis.** RNA extraction, purification, and real-time quantitative PCR analyses were performed as described by Girin et al. (21). Primers used are listed in Table S1. Transcriptome analyses were performed on roots of WT (three biological replicates), *hni9-1* (two biological replicates), and *hni9-2* (one biological replicate) mutant plants cultivated on HN or LN medium. Standard Affymetrix protocols were used for amplifying, labeling, and hybridizing RNA samples (2  $\mu\text{g}$ ) to the ATH1 GeneChip (Affymetrix). Image analysis and normalization to a target median intensity of 150 was performed with the MAS v5.0 program set at default values. Data (12 ATH1 chips  $\times$  22,810 probes) modeling and clustering were performed using the R package (<http://www.r-project.org/>) as described (48). The signal of a probe set ( $\pi$ ) has been modeled by ANOVA as  $\alpha\text{N} + \beta\text{hni9} + \gamma\text{N}*\text{hni9} + \text{ms} + \text{E}$ , where N is the effect of the nitrogen treatment (HN or LN), *hni9* is the effect of the genotype (WT or *hni9*),  $\text{N}*\text{hni9}$  is the effect of the first order of interactions between genotype and treatment, ms is the mean signal, E is the unexplained variance, and  $\alpha$ ,  $\beta$ , and  $\gamma$  the coefficients of the ANOVA. The Affymetrix GeneChip data discussed herein have been deposited in NCBI's Gene Expression Omnibus database (accession no. GSE24738). To investigate if overlap between two gene lists obtained experimentally is higher than expected by chance, a Monte Carlo test has been designed using the R package. A total of 1,000 gene lists of the size of the experimental list were randomly selected out of the genome. The normal distribution of the 1,000 intersections between these randomized lists and the experimental one was characterized by a mean size and a SD. The Z-score corresponds to the number of SDs between the size of the overlap observed experimentally and the mean of the distribution.

**Chromatin Immunoprecipitation (ChIP)** ChIP assays were performed as previously described (45). The antibodies were anti-H3 (05-499; Millipore) anti-H3K4me3 (07-473; Millipore), anti-H3K36me3 (ab9050; AbCam), and anti-H3K27me3 (07-449; Millipore). No antibody was used in negative controls. ChIP DNA was subjected to real-time quantitative PCR analysis with gene-specific primers (Table S1). Relative enrichment for H3, H3K4me3, H3K36me3, or H3K27me3 on *NRT2.1* chromatin was calculated using reference genes (*ACTIN2/7/At5g09810* for H3, *ACTIN2/7/At5g09810* and *TUB2/At5g62690* for H3K4me3, and H3K36me3, *FUSCA3/At3g26790* for H3K27me3).

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