The Chromatin Factor HNI9 and ELONGATED HYPOCOTYL5 Maintain ROS Homeostasis under High Nitrogen Provision

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Short title:

Transcriptional control of ROS levels under high N

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One sentence summary:

Excessive N supply leads to ROS accumulation and requires the function of major transcriptional regulators to maintain physiological balance.

Author contributions:

An.M. and A.G. conceived research plans and supervised the experiments; F.B, Am.M., J.B., L.L., L.B. and An.M performed most of the experiments; F.B, Am.M., J.B., G.K., L.L., L.B. and An.M analyzed the data; An.M. wrote the article with contributions of all the authors.

ABSTRACT

Reactive oxygen species (ROS) can accumulate in cells at excessive levels, leading to unbalanced redox states and to potential oxidative stress, which can have damaging effects on the molecular components of plant cells. Several environmental conditions have been described as causing an elevation of ROS production in plants. Consequently, activation of detoxification responses is necessary to maintain ROS homeostasis at physiological levels. Misregulation of detoxification systems during oxidative stress can ultimately cause growth retardation and developmental defects. Here, we demonstrate that Arabidopsis (*Arabidopsis thaliana*) plants grown in a high nitrogen environment express a set of genes involved in detoxification of ROS that maintain ROS at physiological levels. We show that the chromatin factor HIGH NITROGEN INENSITIVE 9 (HNI9) is an important mediator of this response and is required for the expression of detoxification genes. Mutation in HNI9 leads to elevated ROS levels and ROS-dependent phenotypic defects under high but not low N provision. In addition, we identify ELONGATED HYPOCOTYL 5 (HY5) as a major transcription factor required for activation of the detoxification program under high N. Our results demonstrate the requirement of a balance between N metabolism and ROS production, and establish major regulators required to control ROS homeostasis under conditions of excess N.

INTRODUCTION

Reactive oxygen species (ROS), generated as byproducts of a large range of enzymatic reactions, are integral to plant metabolism. The dynamics of ROS in plant cells correspond to two distinct scenarios. First, ROS are involved in numerous signaling pathways, and thus their levels affect many developmental and physiological processes such as cell differentiation and response to biotic and abiotic stresses (Noctor et al., 2018). In such cases, variations in ROS are generally dynamic and transient, and occur at moderate concentrations. On the other hand, ROS can also accumulate in cells at excessive and sustained levels, leading to unbalanced redox states and to potential oxidative stress (Schieber and Chandel, 2014). At the cellular level, excessive accumulation of ROS can trigger oxidation and damage to many essential molecules (Moller et al., 2007). For instance, oxidation of enzymatic proteins often leads to loss of activity, oxidation of DNA can lead to degradation or mutations, and oxidation of lipids leads to disorganization of cellular membranes. Maintenance of ROS homeostasis is therefore essential to preserve cell integrity. ROS levels are also associated with a range of physiological and developmental phenotypes. For instance, functioning of the shoot apical meristem is largely influenced by redox status (Schippers et al., 2016), and many developmental processes involve interactions between ROS and phytohormones (Considine and Foyer, 2014). Several reports have also demonstrated a role for ROS in root growth and development (Tsukagoshi, 2016). As a consequence, mutations leading to excessive ROS production or exogenous application of ROS at high concentrations lead to retardation of root growth in Arabidopsis (Dunand et al., 2007; Tsukagoshi et al., 2010; Zhao et al., 2016). Altogether, these observations demonstrate that controlling ROS levels is crucial for plant growth, development, and physiology.

Given the importance of ROS homeostasis, plants possess a large range of mechanisms to remove or detoxify ROS. A main component of this antioxidant response corresponds to

enzymes, such as peroxidases, that use reducing power in oxidation/reduction reactions to decrease the cell redox status (Moller et al., 2007). These enzymes work in complex systems that also involve essential antioxidant molecules, like ascorbic acid, glutathione, or thiamin, that are known to protect against oxidative stress (Tunc-Ozdemir et al., 2009; Ramírez et al., 2013). At the molecular level, transcriptional induction of ROS-responsive genes in plants constitutes a major part of the response to ROS overproduction (Willems et al., 2016). However, in agreement with the high number of genes encoding components of the antioxidant response in Arabidopsis, a specificity of response occur according to the signals at the origin of ROS production. Indeed, plant signaling pathways and genes involved in ROS homeostasis and the response to oxidative stress largely vary depending on the type of stress encountered (Apel and Hirt, 2004). Furthermore, the mechanisms that underlie transcriptional regulation and specificity remain to be identified.

ROS production has been observed in plants in response to many environmental factors, with both biotic interactions and abiotic stress contributing to ROS signaling (Baxter et al., 2014). Among abiotic factors, drought, salinity, and heat stress are known to induce the production of ROS (Choudhury et al., 2017). However, these responses correspond to cases where ROS contribute to signaling pathways, but do not accumulate and generate oxidative stress. In contrast, several examples of abiotic stress have been directly linked to elevation of ROS and eventual perturbation of plant redox balance. Such is the case for nutrient deprivation, where potassium, nitrogen (N), or phosphorus starvation rapidly induce the accumulation of H_2O_2 in roots (Shin and Schachtman, 2004; Shin et al., 2005). This suggests that the redox status of plants is highly sensitive to changes in the nutritional environment.

Previously, the chromatin factor HNI9 was shown to be involved in the response to high N provision in Arabidopsis. Mutations in *HNI9* led to an increase in the transcript level of the nitrate transporter *NRT2.1* under high N, a condition under which this gene is normally

strongly downregulated (Widiez et al., 2011). However, a direct relationship between HNI9 and *NRT2.1* has not been demonstrated. Several reports in plants, animals, and yeast have shown that HNI9 is a positive regulator of gene expression (Yoh et al., 2008; Li et al., 2010; Chen et al., 2012; Wang et al., 2013; Wang et al., 2014), which is inconsistent with the hypothesis of a direct role for HNI9 in *NRT2.1* downregulation. Here, we examined the function of HNI9 in the response of plants to high N provision and demonstrate an unexpected role for HNI9 in the regulation of ROS homeostasis through the induction of a subset of genes involved in detoxification. We also identify the transcription factor HY5 as an important component of this response, and highlight the interaction between plant nutrition and ROS homeostasis.

RESULTS

HNI9 is Required to Upregulate the Expression of Genes Involved in Redox Processes in Response to High N Provision

In order to examine the role of HNI9 in the response to high N provision, we compared the transcriptomic data of WT and *hni9-1* mutant plants under low nitrate (0.3 mM KNO_3) and high N (10 mM NH_4NO_3) provision (Widiez et al., 2011). These distinct conditions were selected for their large differences in N availability, leading to the identification of the *hni9* phenotype (Widiez et al., 2011). As HNI9 is a positive regulator of gene expression, we selected genes that were induced by high N in WT, and looked for those that were not induced in the *hni9-1* mutant line. Using these criteria, we obtained a list of 108 genes induced under high N in an HNI9-dependent manner (Supplemental Table S1). In order to determine which biological functions are affected by the HNI9 mutation under high N provision, we performed a gene ontology analysis using this list of 108 genes. Among the biological functions overrepresented, we found that several categories related to redox processes, including

oxidoreductase activity, peroxidase activity, or antioxidant activity, were the most significantly represented (Figure 1, Table 1). Indeed, this list includes genes whose functions in general detoxification or antioxidant processes are well described, such as peroxidases, catalases, and NAD(P)-linked oxidoreductases. In addition, upon further manual curation, we observed that many genes present in other GO categories might also encode enzymes or proteins associated with ROS detoxification or antioxidant biosynthetic pathways (Supplemental Table S1). Therefore, we hypothesized that high N provision could disrupt ROS homeostasis in plants, and that HNI9 may be required to induce a set of genes involved in cellular detoxification.

HNI9 Mutation Leads to Higher ROS Levels and to ROS-dependent Phenotypes Under High N Provision

In order to assess the role of HNI9 in ROS homeostasis under high mixed N provision, we measured H_2O_2 and total ROS levels in WT and *hni9-1* mutant lines under low nitrate and high N provision. In the WT, H_2O_2 levels remained constant independent of the N level, whereas total ROS levels slightly increased under high N provision, although not significantly, demonstrating that ROS homeostasis is maintained from low to high N provision (Figure 2A, B). In contrast, ROS accumulation was significantly altered in the *hni9-1* mutant line. The levels of H_2O_2 and total ROS in the *hni9-1* mutant were similar to WT under low N, but they increased significantly under high N provision (Figure 2A, B). To confirm these results, we used an Arabidopsis line expressing the *GRX1-roGFP2* construct (hereafter referred to as *roGFP2*), in order to visualize ROS levels *in planta*. RoGFP2 is a redox-sensitive probe, which allowed us to monitor plant redox status and, in particular, to assess H_2O_2 levels *in vivo* (Meyer et al., 2007; Marty et al., 2009). In WT plants grown under high N provision, the fluorescence signal of roGFP2 was very low, in agreement with our

measurements of H_2O_2 and ROS levels (Figure 2C). In contrast, the fluorescence signal of roGFP2 in *hni9-1* plants was strong, corresponding to the elevation of H_2O_2 and ROS levels measured in this line (Figure 2D). Quantification of roGFP2 signals in each line validated a strongly significant increase in ROS levels in the *hni9-1* mutant, as compared to the WT (Supplemental Figure S1). Altogether, these results led us to conclude that HNI9 contributes to prevent ROS overaccumulation under high N supply, likely through the induction of a set of genes required to detoxify ROS produced under such conditions.

We next sought to determine whether upregulation of *NRT2.1* expression under high N supply, which is a main phenotype described for the *hni9-1* line, was linked with ROS overaccumulation in this mutant. To this end, we investigated the expression of *NRT2.1* under conditions associated with altered ROS levels. First, we measured *NRT2.1* transcript levels in WT plants treated with ROS-generating or ROS-scavenging chemicals. We used ascorbic acid (ASC) as a ROS scavenger, and menadione, a redox-active quinone that causes an elevation of ROS in plant roots (Lehmann et al., 2009), and H_2O_2 as ROS-generating molecules. Our results showed that ASC treatment caused a decrease in *NRT2.1* expression while menadione and H_2O_2 treatment led to an increase in *NRT2.1* transcript levels (Figure 3A). Next, we measured *NRT2.1* transcript levels in an ascorbate-deficient mutant, *vtc2*, which has known defects in ROS detoxification processes and accumulates significantly higher H_2O_2 levels than WT plants (Kotchoni et al., 2009). In comparison to WT, *NRT2.1* transcript levels were significantly higher in the *vtc2* mutant line, similar to what we observed for the *hni9-1* mutant (Figure 3B). Altogether, these observations suggest that *NRT2.1* expression is responsive to plant redox status, which lends support to the hypothesis that the previously reported *hni9-1* phenotype under high N conditions may be due to elevated ROS levels in this mutant.

Conversely, we investigated whether well-known phenotypes associated with ROS overaccumulation were also observed in *hni9-1* plants under high N supply. In particular,

elevated ROS levels have been shown to alter root growth, such that plants grown under conditions of elevated ROS display reduced primary root length (Dunand et al., 2007; Tsukagoshi et al., 2010). Therefore, we asked whether elevated ROS levels in the *hni9-1* mutant line under high N provision were correlated with alterations in root growth. We used the *vtc2* mutant line, affected in ROS detoxification processes, as a positive control to confirm that elevated ROS levels inhibit root growth under our experimental conditions. Indeed, the *vtc2* mutant line showed a decrease in primary root length, as compared to the WT, regardless of the level of N. However, this decrease in root growth was significantly higher under high N than under low N (Figure 4). The *hni9-1* mutant line also exhibited reduced root growth in comparison to the WT (Figure 4), which is consistent with the general role of HNI9 in plant growth and development that had been previously described (Li et al., 2010). Moreover, the decrease in root growth was also significantly higher under high N than under low N (Figure 4). This also suggests that phenotypic changes due to HNI9 mutation under high N supply may be explained by ROS overaccumulation. Finally, we tested whether external application of an antioxidant molecule could rescue the *hni9-1* root growth phenotype. We therefore measured root growth of WT and *hni9-1* lines under high N conditions, in the presence or absence of thiamin, an antioxidant known to alleviate growth defects caused by oxidative stress (Tunc-Ozdemir et al., 2009). Indeed, we found that the primary root growth defect of *hni9-1* was fully abolished in the presence of thiamin (Figure 5). This demonstrates that the presence of antioxidants can rescue the *hni9-1* root growth phenotype, further indicating that HNI9 affects root development under high N supply specifically through altered ROS homeostasis.

HNI9 Might Be Required to Achieve Full Levels of H3K4me3 Modification at the Loci of Detoxification Genes

Along with elongation and chromatin remodeling factors, HNI9 is part of a large protein complex that influences the histone modification state of genes (Yoh et al., 2008; Li et al., 2010). Indeed, several reports in plants and animals demonstrated that HNI9 operates at the switch between transcriptional repression and activation, in cooperation with H3K27me3 demethylases and H3K36 methyl-transferases (Li et al., 2013; Wang et al., 2014). Therefore, we tested the hypothesis that HNI9 controls the expression of ROS detoxification genes by modulating their chromatin state in response to N provision. To do so, we assayed the level of 3 active chromatin marks (H3K4me3, H3K9ac, and H3K36me3) and 1 repressive chromatin mark (H3K27me3) at the loci of several HNI9-dependent genes implicated in ROS detoxification in response to high N. Representative genes for this analysis were selected from the subset of genes identified by the GO analysis as belonging to the category "oxidoreductase activity" (Supplemental File S1) and displaying a clear induction of their transcript levels by high N provision in an HNI9-dependent manner (Supplemental Figure S2). Here, the results showed that the level of H3K27me3 and H3K36me3 were not changed in the *hni9-1* mutant at the loci of selected HNI9-dependent detoxification genes (Figure 6). In addition, the levels of H3K9ac, associated with active transcription, were also generally similar between WT and *hni9-1*. However, we observed that H3K4me3 levels were globally lower in the *hni9-1* mutant at the loci of several HNI9-dependent ROS detoxification genes (Figure 6). We therefore conclude that HNI9 may induce the expression of ROS detoxification genes by regulating the profile of H3K4me3.

Analysis of HNI9-dependent Genes Involved in ROS Detoxification Under High N Conditions Reveals the Role of HY5 in the Control of ROS Homeostasis

HNI9 is a general regulator of gene expression, as it does not provide by itself any sequence specificity to its target loci. Specificity of response thus requires the action of transcription factors to drive chromatin remodeling complexes to target loci (Li et al., 2010). Therefore, we

tested whether a promoter sequence analysis of genes involved in ROS detoxification under high N could help identify such transcription factors. We used the promoter sequences from the 108 genes induced by high N and dependent on HNI9 in order to find putative conserved cis regulatory elements (CREs) using MEME software. This analysis revealed substantial enrichment of two CREs showing strong sequence similarity with the binding site consensus of the transcription factor HY5 defined by DAP-seq (O'Malley et al., 2016) (Figure 7A, B). In total, 45 of the 108 aforementioned genes contained a putative HY5 binding site (Supplemental Tables S2 and S3). Interestingly, it was shown previously that HY5 is involved in the control of ROS production in response to light or temperature treatments (Catala et al., 2011; Chen et al., 2013; Chai et al., 2015). We first analyzed *HY5* transcript levels in response to N provision, and observed that *HY5* expression was slightly higher under high N condition, but independent of HNI9 function (Supplemental Figure S3). To explore the possible role of HY5 in the regulation of genes involved in ROS detoxification under high N condition, we investigated the binding of HY5 to the putative consensus sequences identified in some of the ROS detoxification genes. To this end, we performed chromatin immunoprecipitation followed by quantitative PCR on a subset of genes with or without putative HY5 binding sites. The results showed that HY5 indeed binds to the promoter of detoxification genes containing a putative HY5 binding site, whereas no significant binding was observed for genes lacking a putative HY5 binding site, although some are also involved in detoxification processes under high N provision (Figure 7C). In order to test the effect of HY5 mutation, we next measured the expression of the identified genes (with or without HY5 binding sites) in WT and the *hy5-215* mutant line. All genes, with one exception, were significantly down-regulated in the *hy5-215* mutant line under high N provision (Figure 7D), strongly supporting a major role of HY5 in the activation of the detoxification program under high N provision. Finally, to test the effect of misregulation of the detoxification program under high N

following HY5 mutation, we measured the levels of ROS and H_2O_2 in WT and the *hy5-215* mutant. The results clearly showed that ROS and H_2O_2 levels were higher in *hy5-215* than in WT, demonstrating the functional importance of HY5 in the detoxification process under high N provision (Figure 8). Altogether, our results demonstrate the activation of a transcriptional program required for ROS detoxification under high N, which is controlled by the chromatin remodeler HNI9 and the transcription factor HY5.

DISCUSSION

Plants, like every living organism, have to cope with environmental constraints. ROS accumulation has been shown to be involved in numerous signaling pathways or to cause oxidative stress in response to different environmental challenges (Baxter et al., 2014; Choudhury et al., 2017; Noctor et al., 2018). The main finding of our work is that high N provision leads to the generation of ROS in plant roots, which is managed by the transcriptional induction of a specific detoxification program. Interestingly, increasing N availability is often viewed positively and as a favorable condition for plant growth, but we demonstrate here that excessive N provision can, in fact, be detrimental to plants. Indeed, even if efficient detoxification systems exist to maintain ROS homeostasis, their induction and functioning undoubtedly incur costs for plants, and their efficiencies can be limited under certain circumstances. In spite of the demonstration that high N availability leads to ROS accumulation, the physiological cause of ROS production under high N conditions remains to be determined. Nitrate assimilation and, to a lower extent, ammonium assimilation pathways can be candidate, as they consume a lot of reducing power (Hachiya and Sakakibara, 2017) and affect the cellular redox balance of plants. However, ROS accumulation under high N may also reflect a more general link between nutrition and redox balance. Indeed, several reports have shown that excessive nutrition in animals leads to ROS production and

perturbation of cellular redox states (Sies et al., 2005; Samoylenko et al., 2013; Gorlach et al., 2015). In the case of plants, nutrient starvation has also been identified as a condition that generates oxidative stress through the production of ROS (Shin and Schachtman, 2004; Shin et al., 2005). In addition, recent reports demonstrated that ROS accumulate early during the N starvation response, and most likely contribute to the regulation of nitrate-responsive genes like *NRT2.1* (Jung et al., 2018; Safi et al., 2018). Therefore, these studies and our present work suggest the existence of thresholds of N provision inside which the cellular redox balance is optimal. This situation is reminiscent of what has been described for iron (Fe) homeostasis. Indeed, excessive Fe provision can also lead to an accumulation of ROS, and specific mechanisms have been described to limit this negative effect (Briat et al., 2010). Nutrient deficiency or excess (at least for N and Fe) would correspond to conditions in which ROS production is above a physiological limit and alters plant redox status. Again, it is interesting to note that the same findings hold true for animals (Gorlach et al., 2015).

In our work, we highlight the role of HNI9 in the transcriptional induction of this detoxification program. In Arabidopsis, HNI9 has been mainly associated with brassinosteroid (BR) signaling (Li et al., 2010; Wang et al., 2014). In response to BRs, HNI9 associates with the chromatin remodeling factors ELF6, REF6, and SDG8, and also with the transcription factor BES1 (Li et al., 2010; Wang et al., 2014). ELF6 and REF6 are responsible for removing of the repressive chromatin mark H3K27me3, while SDG8 is thought to catalyze the trimethylation of H3K36, which promotes elongation of transcription (Lu et al., 2011; Crevillen et al., 2014; Li et al., 2015). This suggests a role for HNI9 in complexes associated with transcriptional switches (e.g., from repression to activation), as demonstrated in animals (Chen et al., 2012; Wang et al., 2013). In our work, HNI9 is mainly linked with differences in H3K4me3 levels. This suggests that HNI9 could be associated with other chromatin complexes or influence other steps of transcriptional regulation. Nevertheless, it

identifies another transcriptional pathway in which HNI9 is essential for the induction of expression of a large set of genes.

In addition, our work identifies HY5 as a major component involved in the induction of the gene network underlying ROS detoxification in response to high N. HY5 is a master regulator of gene expression in Arabidopsis, at the center of several transcriptional networks. It is notably involved in the response to photosynthesis, light, temperature, and hormones (Gangappa and Botto, 2016). Interestingly, HY5 has been also implicated in the control of ROS homeostasis in response to light or cold treatments (Catala et al., 2011; Chen et al., 2013; Chai et al., 2015). In each case, HY5 is required to suppress ROS accumulation during stressful conditions. However, the set of genes induced by HY5 to balance ROS levels seems to differ from one stimulus to another. This suggests that other transcription factors, in addition to HY5, could specify the response according to the environmental signal. Interestingly, we found that the expression of every gene of the ROS detoxification network in response to high N that we tested was dependent on HY5, independent of the presence of a HY5 binding site in their promoter. This suggests that HY5 could be a direct (and general) regulator of many ROS detoxification genes, but that it may also function as an indirect regulator for a subset of them, for instance by regulating another transcription factor that, in turn, regulates these genes. This would imply the existence of several layers in this gene network, and that HY5 is a master regulator at the top of the network.

In conclusion, we demonstrated how a detoxification program is induced in order to maintain plant redox status under physiological conditions even under high nutritional provision. However, these detoxification processes are certainly not without cost for plants. Overall, this work demonstrates that excess N availability is not necessarily advantageous for plant physiology and development, and lends support to the existence of an optimum balance between nutrition and ROS homeostasis.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) accession used in this study was Col-0. Mutant alleles and transgenic plants used in this study were *hni9-1* (Widiez et al., 2011), *vct2* (Collin et al., 2008), *hy5-215* (Oyama et al., 1997), and *GRX1-roGFP2* (Meyer et al., 2007). Experiments were performed using roots from 7 day-old seedlings grown under a long-day photoperiod (16 h light and 8 h dark) on vertical half-strength MS (MS/2) plates without N (PlantMedia) supplemented with 0.8% agar, 0.1% sucrose, 0.5 g/L MES, and the specific concentration of N (0.3 mM KNO_3 or 10 mM NH_4NO_3), as described in the main text and figure legends.

Analysis of Gene Expression by Quantitative PCR

Root samples were frozen and ground in liquid nitrogen, and total RNA was extracted using TRI REAGENT (MRC) and DNase treated (RQ1 Promega). Reverse transcription was performed with M-MLV reverse transcriptase (RNase H minus, Point Mutant, Promega) using an anchored oligo(dT)20 primer. Transcript levels were measured by RT-qPCR (LightCycler 480, Roche Diagnostics) using the TB Green™ Premix Ex Taq™ (Tli Rnase H plus) (TaKaRa). Gene expression was normalized using the *ACT2* gene as an internal standard. Sequences of primers used for gene expression analysis are listed in Supplemental File 2.

Chromatin Immunoprecipitation (ChIP) Quantitative PCR

ChIP experiments were performed as previously described (Bellegarde et al., 2018). Chromatin was precipitated with 2.5 μg of antibodies against H3 (Abcam 1791), H3K27me3 (Millipore 07-449), H3K4me3 (Diagenode C15410030), H3K36me3 (Abcam 9050), H3K9ac (Agrisera AS163198), or HY5 (Agrisera AS12 1867). Immunoprecipitated DNA was purified

by phenol-chloroform extraction and ethanol precipitation, and the resulting DNA was analyzed by qPCR. For chromatin marker analyses, ChIP experiments were quantified using H3 level as an internal standard, and normalized using *ACT7 (*H3K4me3, H3K9ac), *ACT2* (H3K36me3), or *LEC2* (H3K27me3) enrichment. For HY5 binding site analyses, ChIP experiments were normalized to the input levels. Sequences of primers used in qPCR for ChIP experiments are listed in Supplemental File S2.

ROS and H_2O_2 Assays

Root samples were frozen and ground in liquid nitrogen, and ROS and H_2O_2 were extracted using approximately 30 mg of plant material in 200 µL of phosphate buffer (20 mM K_2HPO_4, pH 6.5). For ROS measurements, 50 µL of supernatant were mixed with 50 µL of 20 µM DFFDA (Molecular Probes). Reactions were incubated for 30 min at ambient temperature, and fluorescence was detected using 492/527 nm as excitation/emission parameters. H_2O_2 was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen), as described previously (Brumbarova et al., 2016).

Microscopy

Confocal imaging of Arabidopsis root cells expressing *GRX1-roGFP2* was performed using a Leica SP8 Confocal Microscope (Leica, Germany). GFP quantifications were done with an Axiovert 200 M microscope (Zeiss, Germany) and images were analyzed using ImageJ software. The data represent the fluorescence quantification values measured in the root tip.

Root Growth Analyses

Vertical agar plates containing plants were scanned after 7 days of growth and root length was analyzed using ImageJ software. For experiments with thiamin, plants were grown on vertical MS/2 plates without N (PlantMedia) supplemented with 0.8% (w/v) agar, 0.1% (w/v) sucrose, 0.5 g/L MES, and 10 mM NH_4NO_3 for 4 days and then transferred to the same medium with or without 100 µM thiamin. The position of the root tip was marked on the back of the new

plates, and root length was analyzed 2 days after transfer by measuring growth from the marked position.

Determination of Conserved cis Regulatory Sequences

Putative cis regulatory sequences were identified using the MEME suite (Bailey et al., 2009). 500 base pairs of promoter sequences were used as the primary input, with the following parameters: 0-order background model, classic discovery mode, 0 or one occurrence per sequence, motif width between 6 and 50 nucleotides.

Analysis of Gene Ontology

Gene ontology analysis was performed using BINGO in the Cytoscape environment, using Biological Process file, and a significance level of 0.05.

Statistical Analysis

Mean ± SE is shown for all numerical values. Statistical significance was computed using a two-tailed Student's *t*-test. Significance cutoff: $*P < 0.05$, $** P < 0.01$, $*** P < 0.001$.

ACCESSION NUMBERS

HNI9	AT1G32130
HY5	AT5G11260
NRT2.1	AT1G08090
2OG/Fe(II)-dpt oxygenase	AT1G52820
Iron sulfur cluster	AT2G29630
THALIANOL HYDROXYLASE	AT5G48000
NAD(P) oxidoreductase	AT1G14345
CYTOCHROME P450	AT2G42250
FLAVONOL SYNTHASE 1	AT5G08640
2OG/Fe(II)-dpt oxygenase	AT2G36690

PEROXIDASE AT2G37130

SUPPLEMENTAL DATA

The following supplemental materials are available.

Supplemental Figure S1. Quantification of GFP signal from *GRX1-0roGFP2* probe in WT and *hni9-1* lines under high N (10 mM NH_4NO_3) provision.

Supplemental Figure S2. Expression of genes identified as induced under high N condition in an HNI9-dependent manner.

Supplemental Figure S3. *HY5* expression is slightly higher under high N provision, independently of HNI9 function.

Supplemental Table S1. List of 108 genes induced under high N conditions (10 mM NH_4NO_3) in an HNI9-dependent manner, and the corresponding description of their functions in TAIR.

Supplemental Table S2. Genes from the list of 108 genes induced under high N conditions (10 mM NH_4NO_3) in an HNI9-dependent manner showing the presence of a putative HY5 binding site (motif #1) in their promoter.

Supplemental Table S3. Genes from the list of 108 genes induced under high N conditions (10 mM NH_4NO_3) in an HNI9-dependent manner showing the presence of a putative HY5 binding site (motif #2) in their promoter.

Competing Interests

The authors declare no competing financial interests.

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420

Table 1: Gene ontology enrichment identified from HNI9-dependent genes induced by high N (10 mM NH_4NO_3). The gene ontology term enrichment analysis was performed in the Cytoscape environment by BINGO software using the Molecular Function ontology file with *P* values < 0.01. Details of the analysis are provided in Supplemental File S1.

GO-ID	***P value***	***Number of genes in the GO category***	***Description***
3824	6.5259E-4	50	catalytic activity
16491	6.5259E-4	17	oxidoreductase activity
51739	6.5259E-4	2	ammonia transmembrane transporter activity
15200	6.5259E-4	2	methylammonium transmembrane transporter activity
16684	1.5486E-3	5	oxidoreductase activity, acting on peroxide as acceptor
4601	1.5486E-3	5	peroxidase activity
50284	2.2260E-3	2	sinapate 1-glucosyltransferase activity
16209	3.4940E-3	5	antioxidant activity
15250	5.4994E-3	3	water channel activity
5372	5.4994E-3	3	water transmembrane transporter activity

FIGURE LEGENDS

Figure 1. Genes induced by high N provision in HNI9 dependent manner are associated with redox processes. Functional network realized from the list of 108 genes induced specifically under high N condition (10 mM NH_4NO_3) in an HNI9-dependent manner. Orange to yellow circles correspond to *P* values < 0.01 (see also Table 1 for details). A major hub corresponding to function associated with redox and antioxidant activities emerges at the center of the network

Figure 2. ROS levels are higher in *hni9-1* under high N provision. Measurement of H_2O_2 (A) and ROS (B) in roots of WT and *hni9-1* lines under low N (0.3 mM NO_3^-) and high N (10 mM NH_4NO_3). Data represent mean ± standard deviation of 3 biological replicates from independent experiments. Statistical significance was computed using a two-tailed Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Visualization of H_2O_2 levels *in vivo* using *GRX1-roGFP2* probe in WT (C) or *hni9-1* (D) lines under high N condition. White bars = 50 µm.

Figure 3. *NRT2.1* expression is sensitive to ROS homeostasis. A, Relative expression of *NRT2.1* in the presence of ascorbic acid (400 µM), H_2O_2 (10 mM), and menadione (100 µM) in roots of WT plants. Plants were grown on MS/2 medium containing 1 mM NO_3^- and then transferred on the same medium with or without ascorbic acid, H_2O_2, or menadione for 4 h. B. Relative expression of *NRT2.1* in roots of WT, *hni9-1*, and *vtc2* mutants. Plants were grown on MS/2 medium containing high N (10 mM NH_4NO_3). Data represent mean ± standard deviation of 3 biological replicates from independent experiments. Statistical significance was computed using a two-tailed Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 4. Root growth retardation is more pronounced under high N provision in *hni9-1* and *vtc2* mutants. Primary root length measurements of 7 day-old plants grown under low (0.3 mM NO_3^-) and high (10 mM NH_4NO_3) N provision in WT, *hni9-1*, and *vtc2* lines. The extent

of root growth reduction is enhanced under high N and correlated with the presence of ROS in the mutant lines. Data represent mean ± standard deviation of at least 10 independent plants. Statistical significance was computed using a two-tailed Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 5. External antioxidant application rescues the *hni9-1* root growth phenotype. Plants were grown under high N (10 mM NH_4NO_3) for 4 days and transferred to the same medium with or without 100 µM thiamin. Primary root growth of WT and *hni9-1* lines was measured after 2 days of growth. Data represent mean ± standard deviation of at least 10 independent plants. Statistical significance was computed using a two-tailed Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 6. Mutation in HNI9 is associated with reduction of the H3K4me3 modification at the loci of detoxification genes. ChIP analysis of H3K4me3, H3K27me3, H3K36me3, and H3K9ac in WT and *hni9-1* roots of 7 day-old plants grown under high N provision (10 mM NH_4NO_3). Quantification by qPCR is shown as the percentage of H3K4me3, H3K36me3, H3K27me3, or H3K9ac over H3 at target loci, normalized by the percentage of H3K4me3, H3K36me3, H3K27me3, or H3K9ac over H3 at positive control loci (i.e., *ACT2* for H3K4me3, H3K36me3, and H3K9ac, and *LEC2* for H3K27me3). Data are presented relative to the WT level. Error bars represent standard errors of the mean based on at least 3 biological replicates. Statistical significance was computed using a two-tailed Student's t-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 7. HY5 binds to and regulates the expression of genes involved in detoxification under high N provision. Comparison of first (A) and second (B) conserved motifs discovered by MEME analysis in the promoters of HNI9-dependent genes induced under high N (10 mM NH_4NO_3), with the HY5 consensus binding site identified by DAP-seq. C, ChIP analysis following HY5 enrichment in WT roots at the loci of HNI9-dependent genes induced under

high N. Quantification by qPCR is shown as the percentage of input. Error bars represent standard errors of the mean based on at least three biological replicates. Statistical significance was computed using a two-tailed Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$), in comparison to a negative control (*AT4G03900*, which showed no relation to N or HY5 signaling). D, Relative expression of genes involved in detoxification that were induced by high N in roots of WT and *hy5-215*. Error bars represent standard errors of the mean based on 3 biological replicates from independent experiments. Statistical significance was computed using a two-tailed Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 8. ROS levels are higher in *hy5-215* under high N provision. Measurement of H_2O_2 (A) and ROS (B) in roots of WT and *hy5-215* under high N (10 mM NH_4NO_3). Error bars represent standard errors of the mean based on 3 biological replicates from independent experiments. Statistical significance was computed using a two-tailed Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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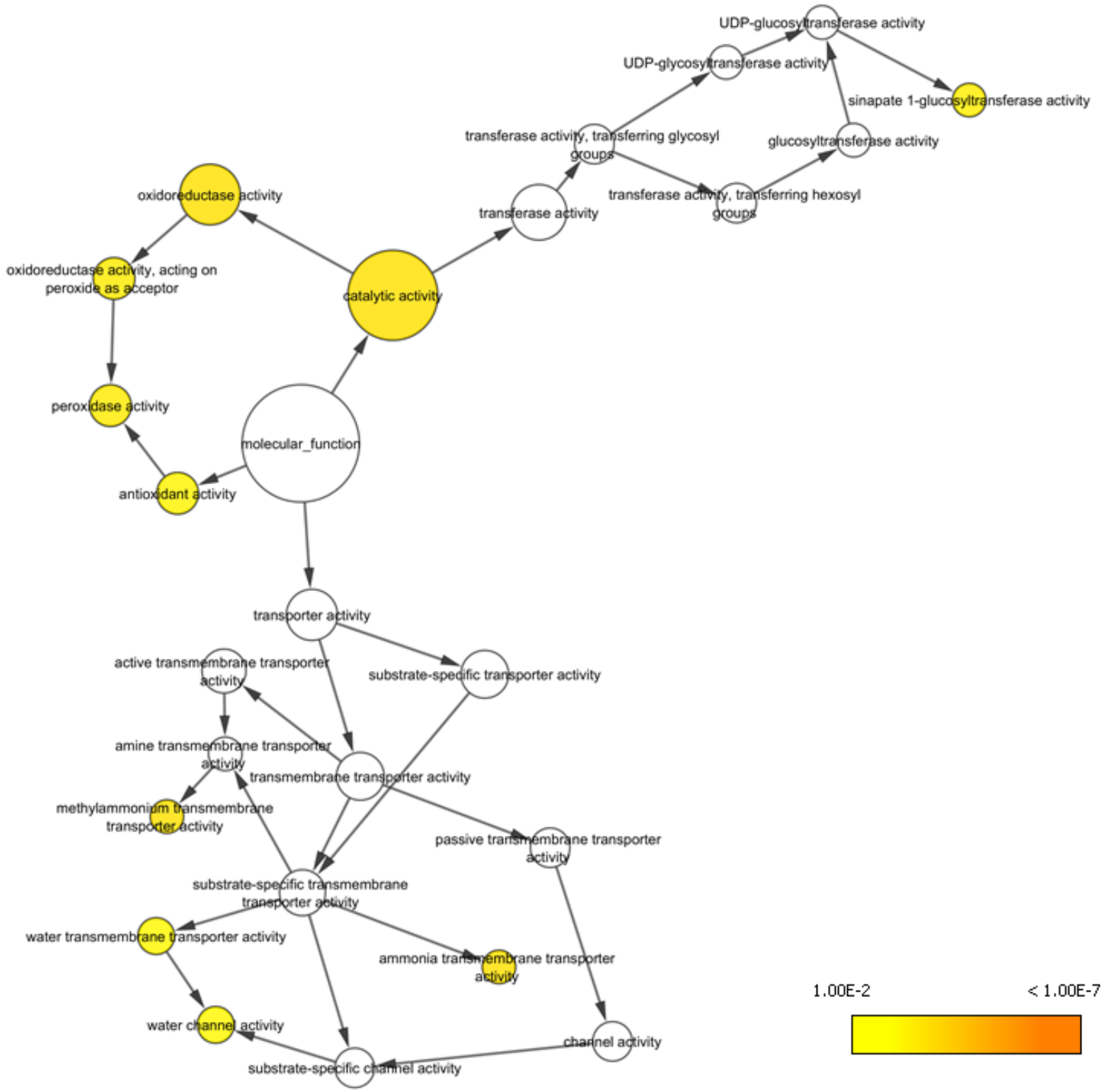

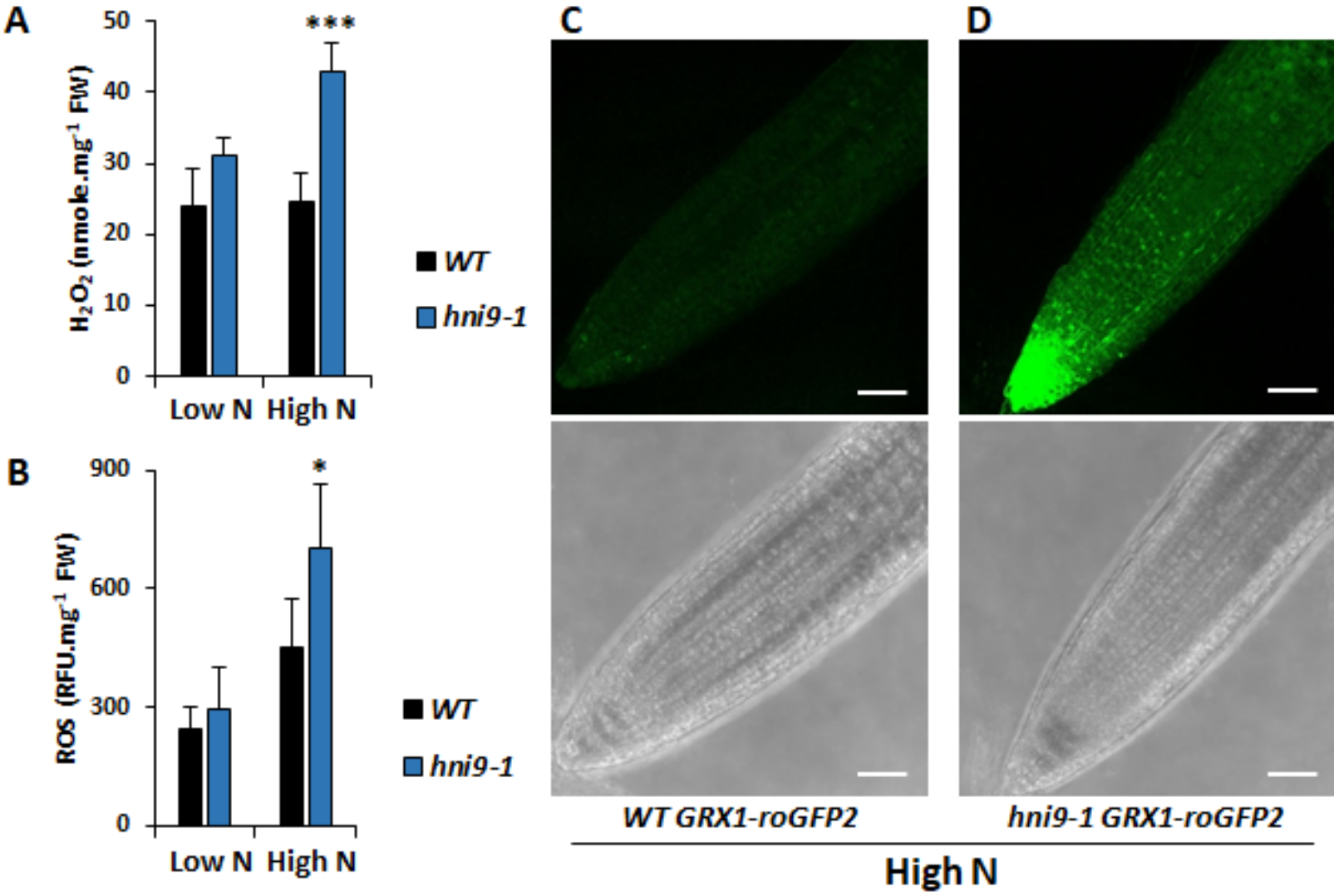

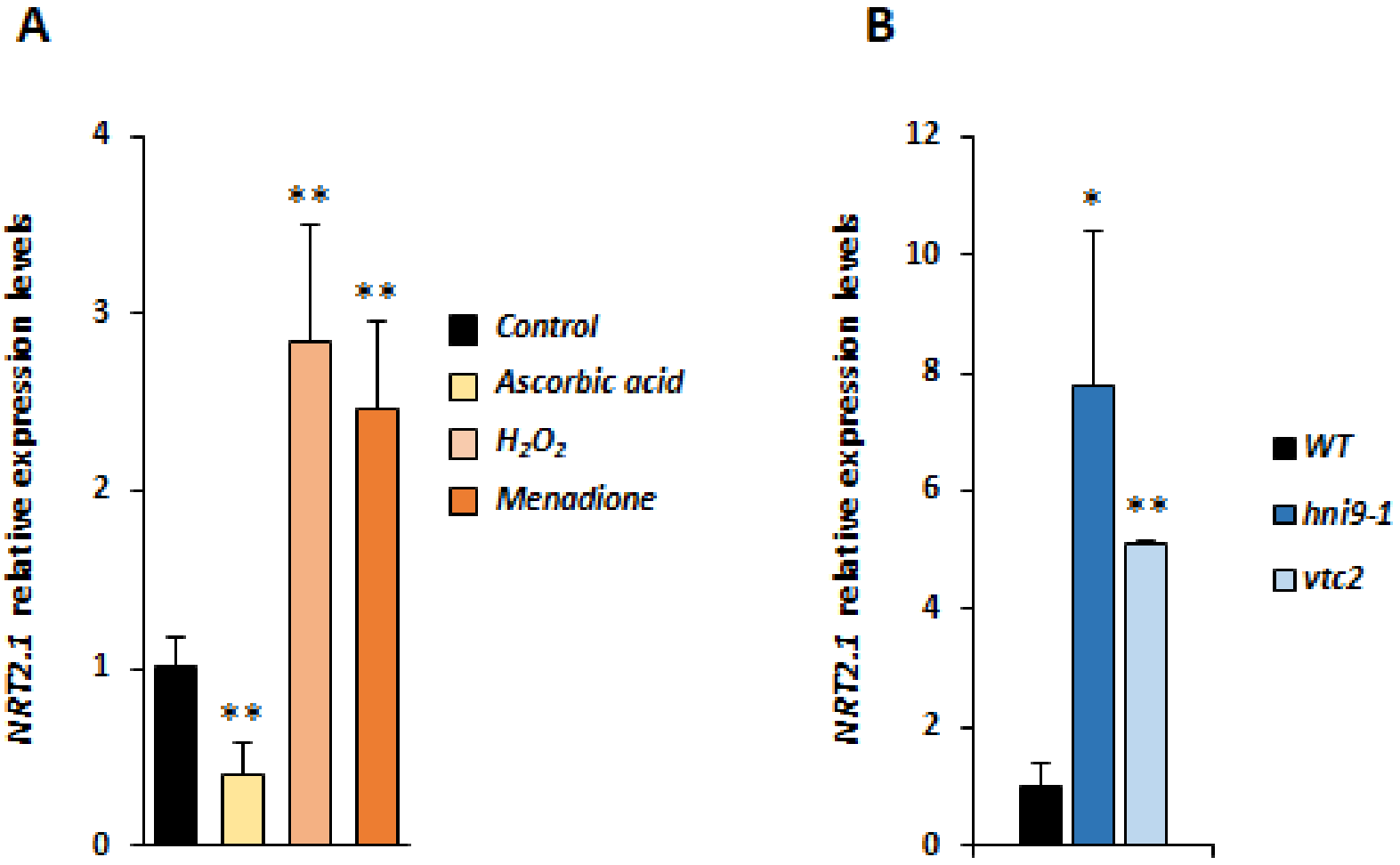

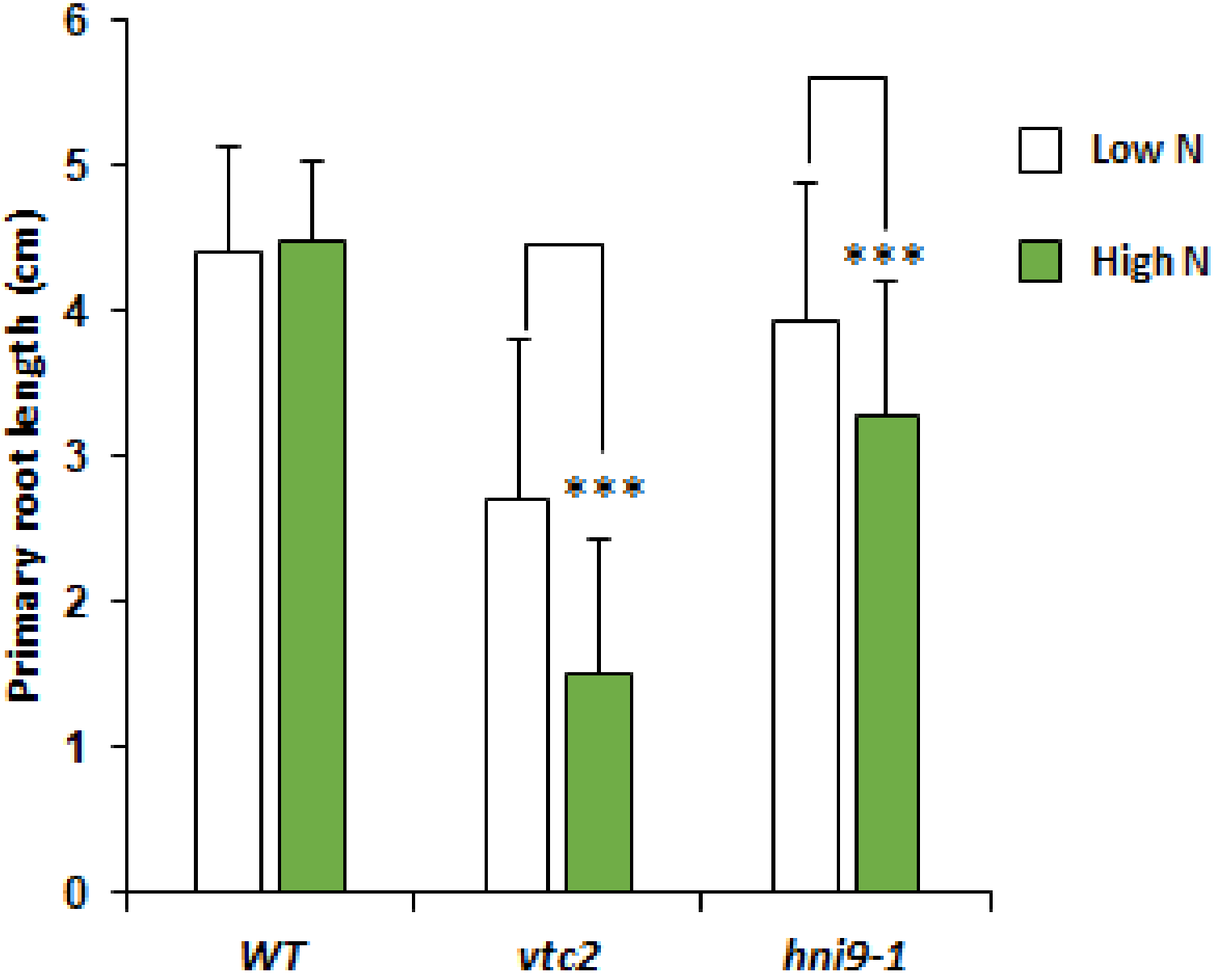

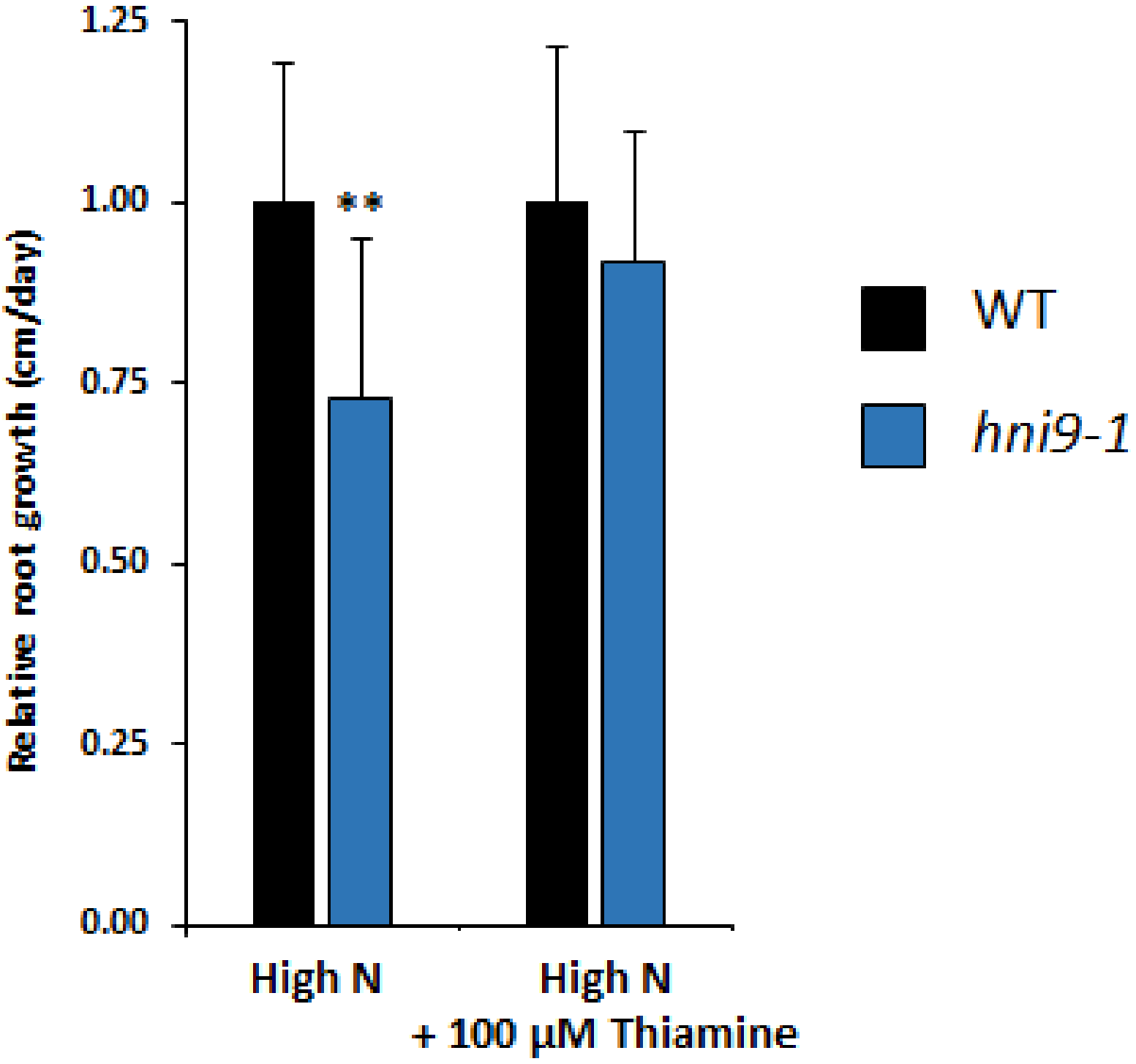

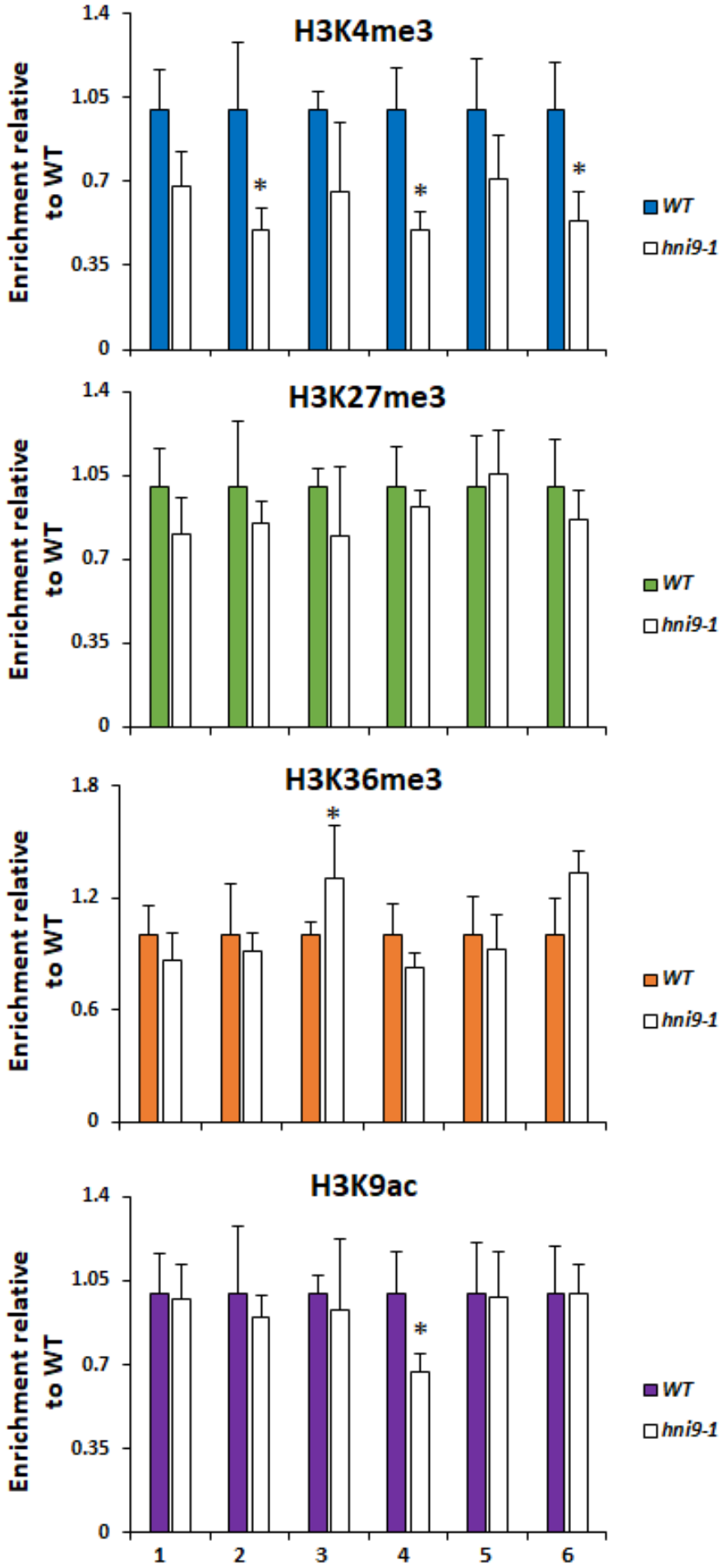

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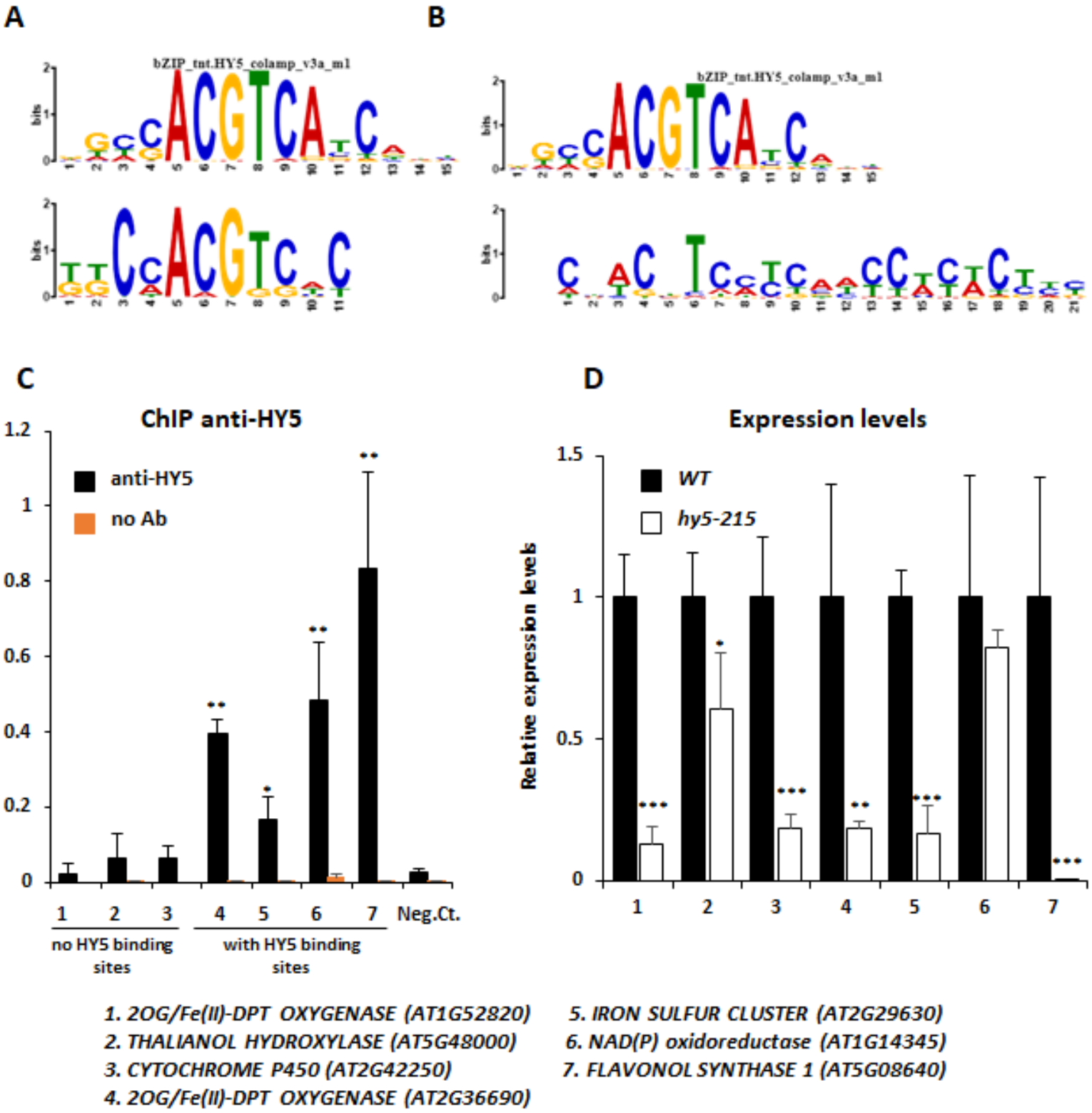
3. CYTOCHROME P450 (AT2G42250)
4. 2OG/Fe(II)-DPT OXYGENASE (AT2G36690)
5. GENE IN SUF1 OR CEU1 CLUSTER (AT2G29630)
6. PEROXIDASE (AT2G37130)



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5. IRON SULFUR CLUSTER (AT2G29630)
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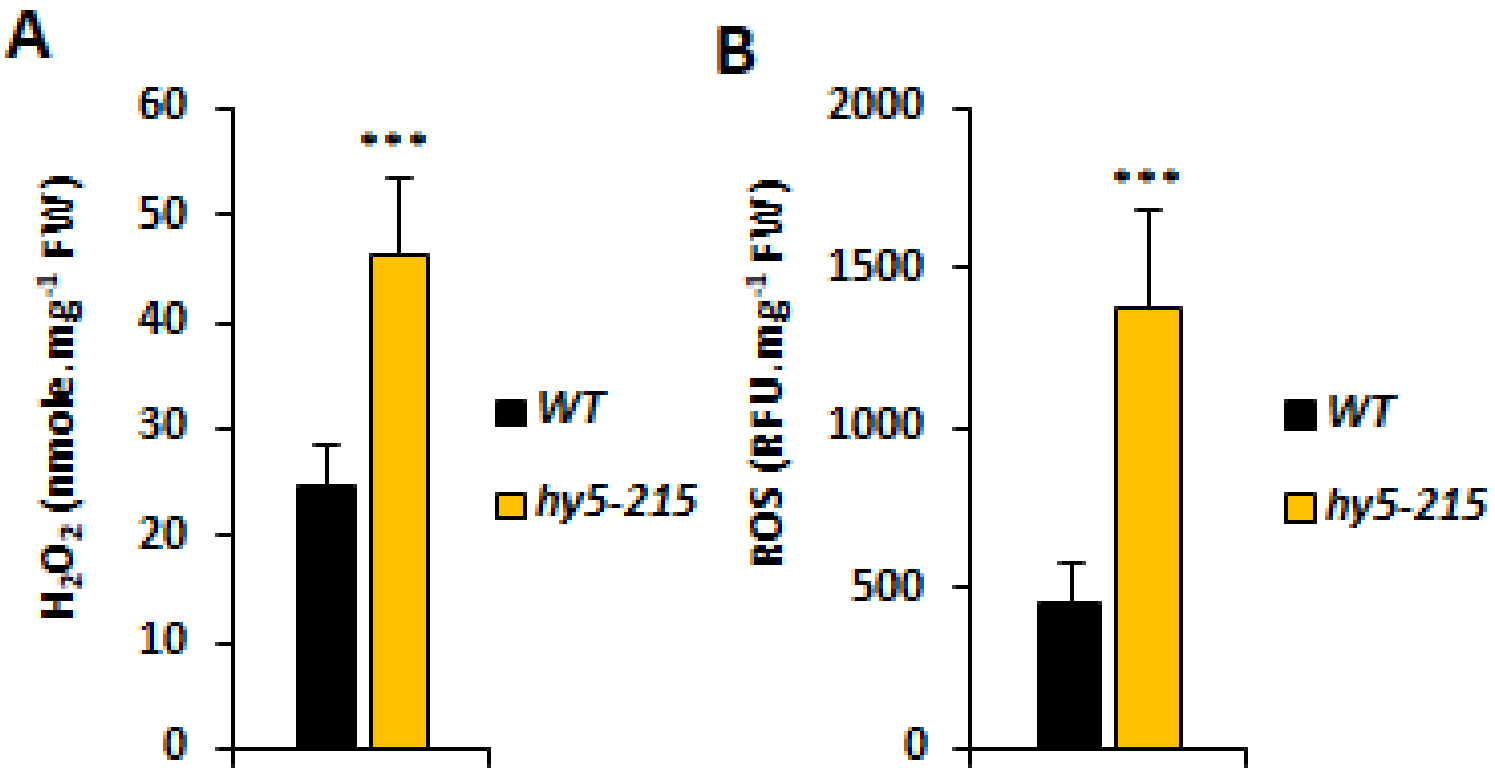

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