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***HY5* regulates Nitrite Reductase 1 (*NIR1*) and Ammonium Transporter1;2 (*AMT1;2*) in *Arabidopsis* seedlings**

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Abstract

HY5 (*Long Hypocotyles 5*) is a key transcription factor in *Arabidopsis thaliana* that has a pivotal role in seedling development. Soil nitrogen is an essential macronutrient, and its uptake, assimilation and metabolism are influenced by nutrient availability and by lights. To understand the role of *HY5* in nitrogen assimilation pathways, we examined the phenotype as well as the expression of selected nitrogen assimilation-related genes in *hy5* mutant grown under various nitrogen limiting and nitrogen sufficient conditions, or different light conditions. We report that *HY5* positively regulates nitrite reductase gene *NIR1* and negatively regulates the ammonium transporter gene *AMT1;2* under all nitrogen and light conditions tested, while it affects several other genes in a nitrogen supply-dependent manner. *HY5* is not required for light induction of *NIR1*, *AMT1;2* and *NIA* genes, but it is necessary for high level expression of *NIR1* and *NIA* under optimal nutrient and light conditions. In addition, nitrogen deficiency exacerbates the abnormal root system of *hy5*. Together, our results suggest that *HY5* exhibits the growth-promoting activity only when sufficient nutrients, including lights, are provided, and that *HY5* has a complex involvement in nitrogen acquisition and metabolism in *Arabidopsis* seedlings.

Keywords

HY5; nitrite reductase *NIR1*; *AMT1;2*; light regulation; nitrogen metabolism; root development

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1. Introduction

HY5 (*Long Hypocotyles 5*) is a nuclear bZIP type of transcription factor in *Arabidopsis thaliana* that has been extensively studied for its role in photomorphogenesis [1]. *HY5* promotes photomorphogenesis in response to light signals of various wavelengths, and lack of *HY5* weakens photomorphogenic responses [2-5]. In addition to light signaling, *HY5* is critically involved in auxin mediated root growth [4, 6,7]. Analyses of genomic binding sites of *HY5* have identified as many as 11797 target genes, which equates to approximately 44% of all genes in the *Arabidopsis* genome [8], highlighting the pivotal role that *HY5* plays in *Arabidopsis* development.

Nitrogen is an essential macronutrient and a key factor limiting agricultural productivity [9,10]. Plants absorb inorganic nitrogen mainly in two forms, nitrate (NO_3^-) and ammonium (NH_4^+), and their nitrogen metabolism is dynamically regulated in response to ambient nitrogen sources and levels as well as other environmental factors [10,11]. Higher plants have both high- and low-affinity nitrate uptake systems (HATS and LATS, respectively), which operate under different nitrate concentrations and are thought to be genetically distinct [12]. In addition, nitrate and nitrite act both as nutrients as well as signals for the global regulation of gene expression in *Arabidopsis* roots [9,13]. Different nitrogen sources and varying nitrogen levels can also affect transcriptional profiles and various physiological processes of plants [11,14]. Light signals play a crucial role in regulating nitrogen uptake, translocation and assimilation into organic compounds [15]. The rates of photosynthesis and respiration are known to vary as a function of tissue nitrogen concentration in various species and growth conditions [16,17].

By analyzing null mutants of *Arabidopsis* for *HY5* and *HYH* (an *HY5* homologue) genes, Jonassen *et al.* showed that these genes are important for high nitrate reductase activity [18]. They further showed that *HY5* and *HYH* are activators of *NIA2*, and are involved in light inhibition of *NRT1.1* [19]. Based on these studies, a scheme of signal transduction pathway from light to nitrate translocation and assimilation has been proposed [15]. Notably, these studies used a *hy5 hyh* double mutant growing in a growth medium (half-strength Murashige and Skoog salts containing 3% sucrose) that contained nitrogen [18-20]. Moreover, *hy5 hyh* double mutant exhibits a root growth phenotype opposite to the *hy5* single mutant [7]. It is therefore necessary to examine *hy5* single mutant for nitrogen related morphological phenotype and gene expression alterations.

Improved understanding of the complex network of light, hormones, and nitrogen requires answers to further questions, including whether *HY5* regulates nitrogen-related genes in a nitrogen concentration-dependent manner; how the nitrogen related genes respond to light; and what the role of *HY5* is in their light regulation. To this end, here we characterized the phenotype of a *hy5* mutant under different nitrogen conditions and examined the expression of representative genes involved in nitrogen assimilation using quantitative real-time polymerase chain reactions (qPCR) and biochemical methods. We found that *HY5* regulates many nitrogen related genes in a nitrogen concentration dependent manner, and that it constitutively activates *NIR1* (Nitrite reductase 1) while suppresses *AMT1;2* (Ammonium transporter 1;2), two important genes in nitrogen metabolism.

2. Materials and methods

2.1. Plant materials and growth conditions

Wild-type (WT) *A. thaliana* used herein was the Col-0 ecotype. The *hy5-ks50* mutant (Columbia background) has been described previously [4, 21-23]. All chemical reagents were purchased from Sigma. For root phenotype analysis, surface sterilized seeds were sown on 12×12 cm plates with 40 ml of solid medium (0.8% agar) containing 10 mM KH₂PO₄, 2 mM MgSO₄, 1 mM CaCl₂, 0.1 mM Fe-EDTA, 50 μM H₃BO₄, 12 μM MnSO₄, 1 μM ZnCl₂, 1 μM CuSO₄, 0.2 μM Na₂MoO₄, and 0.5% sucrose (pH 5.5) [24]. This basal medium was supplemented with (ammonium)₂succinate, NH₄NO₃, or KNO₃ as the nitrogen source as indicated in the text [25]. The final concentrations of nitrogen in the media were 0, 0.5, 2, 5 or 25 mM. After cold stratification for 3 days at 4°C in the dark, the plates were incubated at 22°C in continuous white light (WLC) at 180 μmol · m⁻²·s⁻¹ or under a 16 h light/8 h dark regime. The experiments on nitrogen concentration series or when unspecified, were carried out using the 16 h light/8 h dark light period. The lengths of individual primary roots of seedlings were measured with Image J software (National Institutes of Health; <http://rsb.info.nih.gov/ij>) from images captured with a Canon G7 camera. For treatments requiring different light sources, seeds were surface-sterilized and sown on solid 1% Murashige and Skoog medium supplemented with 0.5% sucrose (GM). After 3 days of cold treatment, seeds were exposed to WLC (180 μmol · m⁻²·s⁻¹), FRc (140 μmol · m⁻²·s⁻¹), Rc (80 μmol · m⁻²·s⁻¹), or dark (Dc) conditions at 22°C for 4 days as described previously [22]. All experiments were performed at least three times and the data represent one independent experiment.

2.2. RNA analyses

Total RNA was extracted from *Arabidopsis* seedlings using the RNeasy Plant Mini kit (Qiagen) [26]. Reverse transcription was performed using the SuperScript II First-strand cDNA Synthesis System (Invitrogen) according to manufacturer's instructions. qPCR analysis was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) with a Bio-Rad CFX96 real-time PCR detection system. Each experiment was repeated with three independent samples, and qPCR reactions were performed with three technical replicates for each sample. The primers used for qPCR are listed in Supplementary Table 1. Relative RNA expression was calculated with *UBQ1* (AT3G52590) as the endogenous reference control. For Figure 3 showing N concentration series, the value of WT at zero N concentration was set at 1 for each gene. In Figure 4A, relative transcript levels of the genes were compared to *NIA1* of WT Dc (set to 1). Representative gene profiles were repeated with *ACTIN 2* (At3g18780) as endogenous reference. Data presented are means of three biological parallels, and error bars represent standard deviation of the sampling distribution of the mean. Analysis of variance were tested by least significant difference at P = 0.01(LSD0.01), based on SAS statistical analysis package (version 9.1.3, SAS Institute, Cary, NC, USA).

2.3. Nitrite reduction activity assay

Tissue samples were extracted for nitrite reduction activity (NiRA) assays as described [27]. In brief, the assay mixture (pH 8.0) consisted of 75 mM Tris-HCl, 5 mM methyl-viologen

and 50 mM sodium dithionite was prepared fresh in a solution of 30 mM sodium bicarbonate and 1 mM sodium nitrite. The reaction was initiated by adding freshly prepared sodium dithionite solution and followed by incubating at 25°C for 20 min. Then, the mixture was vortexed vigorously to oxidize the remaining dithionite and 0.1 ml of the mixture was diluted to 1 ml with water. To this mixture, 1 ml of 1% (w/v) sulfanilamide prepared in 1 N HCl and 1 ml of 0.01% (w/v) N-1-naphthylethylene-diamine-dihydrochloride prepared in water were added. After 30 min, the resultant color was measured with a spectrophotometer at 540 nm. NiRA is expressed as μM of nitrite consumed per hour per gram fresh weight ($\text{g}^{-1}\cdot\text{FW}$).

2.4. Determination of ammonium contents

Fresh seedlings of 500 mg of were added to 1 ml deionized water and shaken for 1 h at 45°C. Samples were centrifuged at 15000 g for 20 min. Ammonium content was determined for 50 μl of supernatant using 1 ml Nessler reagent (Merck). Optical density was measured at 404 nm and NH_4^+ content was determined using a standard curve and expressed as $\mu\text{mol NH}_4^+ \text{g}^{-1}\cdot\text{FW}$ (Fresh Weight) [28].

2.5. Chlorophyll Measurement

Seedlings were collected and weighed for their fresh weight. Chlorophyll was extracted with ethanol and its content was assayed based on the absorbance of the extract at 645 and 663 nm [29].

3. Results

3.1. Phenotype of *hy5* in response to different forms and concentrations of nitrogen nutrients

We examined the seedling phenotype of *hy5* mutant growing under nitrogen deficient conditions. As a normal condition control, we used standard growth media (GM) that provided 60 mM of nitrogen, which is sufficient for plant growth. When grown on plates that had no nitrogen supplement (nitrogen starvation), wild type seedlings were purple, small, and about to die, confirming that nitrogen was necessary for plant survival. In comparison, the aerial part of *hy5* mutants appeared greener than wild type under the same nitrogen starvation condition (Fig. 1A and 1B). While growing in the nitrogen sufficiency condition (GM), *hy5* mutant was paler due to reduced chlorophyll content (92.7%) compared to that of wild type. Under nitrogen starvation however, the chlorophyll level of the *hy5* mutant was 1.89-fold greater than wild type, though still substantially below normal chlorophyll levels (Fig. 1C). Thus, the reduction of chlorophyll content resulted from nitrogen deprivation was less severe in *hy5* mutant, suggesting that *hy5* was slightly more tolerant to nitrogen deficiency at least in the aerial part of the seedlings.

We examined the root growth of *hy5* in a gradient concentration of nitrogen (0, 0.5, 2, 5 and 25 mM) that were provided in three different forms (Fig. 2): Potassium nitrate (KNO_3) was used as a nitrate salt; ammonium succinate [$(\text{NH}_4)_2\text{Suc}$] was used as ammonium salt; and ammonium nitrate (NH_4NO_3) was used as combination of nitrate and ammonium salt. The *hy5* mutant displayed a drastically different root system architecture when grown at

nitrogen-limiting conditions, such as in 0.5 mM of nitrogen supplied as potassium nitrate or as ammonium nitrate (equivalent to 0.25 mM NH_4NO_3) (Fig. 2A). Under those low nitrogen conditions, *hy5* mutants had shorter primary roots but exaggerated lateral roots compared to wild type. We measured primary root length and found that *hy5* had shorter primary roots than wild type at the nitrogen concentration range of 0-2 mM in both potassium nitrate (PN) or ammonium nitrate (AN) (Fig. 2B, 2C). Consistent with a previous report [4], wild type and *hy5* mutant showed no significant differences in primary root length at nitrate concentrations higher than 5.0 mM (Fig. 2B, 2C).

Supplying ammonium salt alone did not promote root growth, and high concentration of $(\text{NH}_4)_2\text{SO}_4$ (higher than 0.25 mM) repressed root growth to less than 0.9 cm length in both wild type and *hy5* mutant (Fig. 2D). This result is consistent with the reports that high concentrations of ammonium are toxic to plants [11, 28, 30, 31]. Under ammonium salt condition, the *hy5* mutant showed 1.85–2.08-fold shorter primary root length than wild type. Considering its toxicity, $(\text{NH}_4)_2\text{SO}_4$ salt was not used as nitrogen nutrient in further experiments.

The different phenotypes of *hy5* mutant in different nitrogen nutrition environments suggest that HY5 may have different roles under high-affinity nitrate uptake system (HATS) and low-affinity nitrate uptake system (LATS), which involve different nitrogen-related genes. It seems that HY5 participates in nitrogen management in a complex manner that depends on ambient nitrogen levels.

3.2. HY5 regulates genes involved in nitrogen metabolism in different ways

We next determined the transcript levels of nitrogen-related genes in seedlings growing in different concentrations of nitrogen nutrients, NH_4NO_3 (AN) or KNO_3 (PN). Representative genes were examined, including those encoding enzymes for primary nitrogen metabolism such as *NRT* (Nitrate transporter), *NIA* (Nitrate reductase), *NIR* (Nitrite reductase), and *AMT* (Ammonium transporter). The expression of each gene in *hy5* mutant was compared relative to that of the wild type under nitrogen starvation (0 mM) (set to 1 as reference value) (Fig. 3).

Amongst the genes examined, *NIR1* and *AMT1;2* were most strongly affected by *hy5* mutation. *NIR1* expression was strongly induced by nitrate, particularly potassium nitrate in wild type seedlings, but not in *hy5* mutation (Fig.3 panel *NIR1*), indicating that HY5 is required for nitrate induced high level expression of *NIR1*. The ammonium transporter gene *AMT1;2*, which unlike *AMT1;1* or *AMT2;1*, did not show significant nitrate induction in wild type seedlings. This gene was expressed in higher levels in *hy5* than WT at all nitrogen conditions tested, indicating that HY5 has a negative role in *AMT1;2* expression (Fig.3 panel *AMT1;2*). We also found that *NRT2.2* expression was progressively induced by nitrate, but it was not affected by *hy5* mutation under all nitrogen concentrations tested (Fig.3 panel *NRT2.2*).

The effect of *hy5* mutation on the expression of *NRT2.1*, *NIA1*, *NIA2* and *AMT1;1* varied depending on the nitrogen growth conditions. *AMT1;1* was only moderately decreased in *hy5*, and it occurred only at high nitrogen concentrations (greater than 5 mM) (Fig. 3). This

observation also applied to *NRT1.1*, *NIA1*, *NIA2*; all of which showed moderately decreased expression only when grown in higher nitrogen concentrations, suggesting that HY5 positively influenced these genes expression under nitrogen sufficient conditions. However, under nitrogen starvation and low nitrogen condition (0.5 mM), *NRT2.1* and *NIA1* expression appeared higher in *hy5* than in WT (Fig. 3). This result might suggest that HY5 could be involved in suppressing these genes during low nitrogen stress. Together we found that HY5 affects many nitrogen assimilation genes in a nitrogen-dependent manner, but HY5 constitutively activates *NIR1* and inhibits *AMT1;2* regardless of the nitrogen nutrient status.

3.3. Light induction of nitrogen related genes was largely preserved in *hy5* seedlings

To determine the effect of HY5 on light regulation of nitrogen related genes, we examined RNA expression of these genes in wild type and *hy5* seedlings grown in nitrogen sufficient conditions (GM) under continuous white light (WLC), far red light (FRC), red light (Rc), or darkness (Dc) (Fig. 4). Several genes examined were induced by light, including *NIR1*, *AMT1;2*, *NRT2.2*, *NIA1*, *NIA2*, and weakly, *AMT1;1* and *NRT2.1* (Fig. 4A, 4C). However the effects of *hy5* mutation on those genes were different. In all light environments, *NIR1* transcript levels were markedly reduced in *hy5*, whereas *AMT1;2* transcript levels were markedly elevated in *hy5* compared to wild type. Gel analysis of RT-PCR products further confirmed marked reduction of *NIR1* in *hy5* across all light conditions, while *ACT2* (At3g18780) expression stayed largely constant (Fig. 4B). Together with data from nitrogen concentration series (Fig.3), our results indicate that HY5 constitutively promotes *NIR1* and inhibits *AMT1;2* regardless of the nitrogen nutrient concentration or light environments.

Nitrate transport gene *NRT2.2* was induced by all wavelengths of lights in both wild type and *hy5* mutant equally (Fig.4), indicating that lack of HY5 had little influence on *NRT2.2* expression. This is also consistent with the *NRT2.2* result in Figure 3, confirming that *hy5* mutation has no detectable effect on *NRT2.2* expression over different nitrogen concentrations or lights. *NRT1.1*, a dual transporter in both LATS and HATS, and *NRT2.1*, the major transporter in HATS, were moderately decreased in *hy5* mutants compared to WT in the dark as well as in white light and red light (Fig.4). Again, these results are in agreement with the nitrogen concentration experiments, in which *NRT1.1* and *NRT2.1* expression moderately declined in *hy5* in high concentration of nitrogen (Figure 3).

There are two functional genes in the nitrate reductase gene family, namely *NIA1* (At1g77760) and *NIA2* (At1g37130), with the latter reported to account for 90% of nitrate reductase activities in higher plants [32]. Expression of these two genes was very low under darkness, and was significantly induced by light. The expression of *NIA1* and *NIA2* in white light was notably decreased in the *hy5* mutant compared with WT (by 62% and 68%, respectively). No significant reductions were observed in red light or far-red light. Similarly, *AMT1;1* was under-expressed in *hy5* mutant only under constant white light, suggesting that HY5 promoted these genes in white light.

To evaluate the effect of *hy5* on light responsiveness of these genes, we calculated the light induction of each gene, or fold of change in gene expression increase (or decrease) in white light (WLC) over darkness (Dc) (Fig.4C). With exception of *AMT1;1*, light induction of

most genes did not weaken due to lack of HY5. This surprising results suggest that, in contrast to most photosynthetic genes, HY5 is not essential for light-stimulated expression of many nitrogen related genes, such as *NIR1*, *AMT1;2*, *NRT2.2*, *NIA1*, and *NIA2*. Nonetheless, HY5 is important for overall high level expression of genes such as *NIR1*.

3.4. Changes in the nitrite reductase activity and the ammonium content in the *hy5* mutant

Prompted by nitrogen-dependent involvement of HY5 on genes involved in nitrogen uptake and assimilation, we examined the nitrite reductase activity (NiRA) and ammonium contents in *hy5* seedlings growing in different nitrogen concentrations (Fig. 5). Under nitrogen depletion (0mM), NiRA and ammonium contents were almost undetectable in the *hy5* mutant or wild type. With increasing nitrogen supply, the NiRA of seedlings rose steadily until the concentration of 5.0 mM, and then plateaued thereafter. *hy5* showed lower NiRA compared to wild type, and the reduction was most significant under limited nitrogen supply of less than 2.0 mM ammonium nitrate or potassium nitrate (Fig.6A). This result suggests that HY5 has a greater role for seedling's NiRA when growing at limited nitrogen environment.

At all concentrations of external nitrogen, the NH_4^+ contents of wild type and *hy5* mutant seedlings were all significantly greater on ammonium nitrate medium compared with potassium nitrate medium, reflecting greater uptake and assimilation of NH_4^+ in the former medium by both genotypes. At 0.5 mM and 2 mM of ammonium nitrate, the NH_4^+ contents were 19% and 23% greater in the *hy5* mutant than in WT, and when the nitrogen source was potassium nitrate, the NH_4^+ contents were 16% and 22% greater in the mutant than wild type respectively, thus showing significant differences between the two genotypes. At higher nitrogen level (5 mM and 25 mM), NH_4^+ contents did not differ significantly between wild type and *hy5* on either the ammonium nitrate or potassium nitrate medium. It appeared that *HY5* affects steps of NiRA and transportation of ammonium, resulting in abnormal nitrogen metabolism. The altered nitrite reductase activity and ammonium contents, particularly in the nitrogen-limiting environments, may contribute to the altered root system architecture of *hy5* under those conditions.

4. Discussion

HY5 transcription factor is widely involved in growth and developmental processes of Arabidopsis. Here we studied the role of HY5 in nitrogen assimilation pathways by characterizing the *hy5* mutant seedlings with respect to its nitrogen-dependent phenotype and the expression of selected genes related to nitrogen assimilation. Among the nine representative nitrogen-related genes selected for this study, five of which are known as sentinels for Primary Nitrate Response [33]: *NRT1.1*, *NRT2.1*, *NIA1*, *NIA2*, and *NIR1*. We presented evidence that HY5 positively regulates *NIR1* expression, negatively regulates *AMT1;2*, and affects expression of several other genes in a manner dependent on the nitrogen nutrient supply. Surprisingly, HY5 does not appear to affect light responses of most of these genes, but it is necessary to support high level gene expression under optimal nutrient and light conditions. Under sustained low nitrogen conditions, lack of HY5 resulted in a more dramatic root phenotype, reduced NiRA activity, and increased ammonium

content. The *hy5* single mutant still expressed the HY5 homolog HYH, which might partially compensate the loss of HY5 functions in the mutant [34]. Despite of the partial redundancy, our data nonetheless showed that HY5 is an important player in the nitrogen assimilation pathway in Arabidopsis.

4.1. The *hy5* mutant displays a complex effect on expression of nitrogen related genes

Among the nine representative nitrogen assimilation genes, we found that the expression of *NIR1* and *AMT1;2* changed most drastically in *hy5*, and the changes were consistent regardless of nitrogen supplement and light conditions tested (Fig. 3 and 4). Other genes examined were either not affected (*NRT2.2*) or weakly and conditionally affected by the lack of *HY5*, as summarized in Figure 6. We should mention that the experiment of nitrogen concentration series was not conducted by transient reduction or increase of the nutrient, but was conducted with seedlings that grew on specified conditions. Therefore our data cannot distinguish whether *HY5* has a direct or indirect role in their gene expression. However, given the robust effect on *NIR1* (stimulation) and *AMT1;2* (inhibition), we think these two genes are likely directly regulated by *HY5*. We have searched the *HY5* chromatin-IP genomic binding data [8]. Based on the binding criteria described in the report, all of the selected genes including *NIR1* and *AMT1;2* have been categorized as *in vivo* *HY5* binding loci in light-grown plants (NIH Gene Expression Omnibus database accession number GSE24974).

Even on this small group of selected nitrogen-related genes, *HY5* exhibited a complex pattern of regulation. According to how the genes are affected in *hy5* mutant, they can be classified into 4 different types (Fig. 6A). Type A, as represented by *NIR1*, is activated by *HY5* constitutively; Type B, as represented by *AMT1;2*, is inhibited by *HY5* constitutively; Type C, as represented by *NRT2.2*, is not regulated by *HY5*; and Type D, which are affected by *HY5* in a nitrogen concentration dependent manner. The functions of the tested genes in the nitrogen assimilation pathway are depicted in a simplified diagram in figure 6B. In general, it appears that *HY5* tends to act negatively at low nitrate conditions (except for *NIR1*), while predominantly promotes nitrogen assimilation genes when nitrate are abundantly supplied (Fig. 6B). This is consistent with a general growth-stimulating role of *HY5*, either directly or indirectly [35,36].

We also found that different genes of the multigene family are distinctively regulated by *HY5*. For example, *HY5* constitutively represses *AMT1;2*, but not *AMT1;1* or *AMT2;1*. As a member of six isoforms in the AMT family, *AtAMT1;2* is a low-affinity NH_4^+ transporter uniquely located in the plasma membrane of the root endodermis rather than in the rhizodermis and cortex as in high-affinity NH_4^+ transporter *AMT1;1* and *AMT1;3* [37,38]. *AMT1;2* has been shown to be up-regulated in roots when internal ammonium concentration increases [39]. However, increased internal ammonium concentration in *hy5* (Fig. 5) is unlikely the direct cause of the abnormal elevation of *AMT1;2* in *hy5*. This is because *hy5* seedlings exhibited increased internal ammonium level only at low nitrogen conditions, but exhibited increased *AMT1;2* expression at all nitrogen conditions tested (Fig. 3 and 4). Further study is required to understand the mechanism of cell-specific regulation of different gene isoforms.

By investigating transient light responsiveness of nitrogen related genes in *hy5 hyh* double mutant, Lillo's team showed that HY5 and HYH positively regulate nitrate reductase activity as well as the main nitrate reductase gene *NIA2* in Arabidopsis leaves [18-20], which is consistent with our observations. Interestingly, Jonassen *et al.*, (2009b) did not find strong effect of *hy5 hyh* double mutant in transient (6 hours) induction of *NIR1* by nitrate [19]. The discrepancy of the two studies on *NIR1* maybe explained by one of the following reasons. 1) It is possible that the strong positive regulation of *HY5* on *NIR1* expression (Figs. 3 and 4) represent a persistent function of *HY5*, as oppose to a transient response. 2) Given that opposite root growth phenotypes have been observed in *hy5* versus *hy5 hyh* double mutant [7], similar discrepancy in the single and double mutant might also occur in this case. 3) The internal reference genes in the RT-PCR analyses of RNA expression were different. Jonassen *et al.*, (2009b) [19] used At3g02540, whereas we used *UBQ1* (At3g52590) as the reference gene (Fig. 3 and Fig. 4A), and we further validated *NIR1* result with *ACT2* (At3g18780) (Fig. 4B). According to a microarray study of nitrate response submitted by Wolf Scheible lab to the database (https://www.arabidopsis.org/servlets/TairObject?type=hyb_descr_collection&id=1005823564) and a study of light response in *hy5* (<http://www.ncbi.nlm.nih.gov/geoprofiles>) [8], *UBQ1* and *ACT2* show no significant responses to nitrate or light, and they are not regulated by *HY5*. However At3g02540 used in [19] showed weak nitrate response, with KNO_3/KCl fold change as high as 2.378, while it is not subject to regulation by light or *HY5* [8]. Detailed examination of the behaviors of internal control genes has lent further support to our result that *HY5* is unequivocally required for high-level expression of *NIR1*.

The gene expression defects for certain genes in *hy5* were found unremarkable and inconclusive (indicated by dots in Fig. 6A). This might be related to our protocol of using whole seedlings for RNA analysis, which would not allow us to clearly distinguish genes that are tissue-specifically regulated in roots or shoots. In the future, organ-specific sampling might provide more precise information on the function of *HY5*.

4.2. *HY5* versus lights regulation of nitrogen related genes

HY5 and light signals both regulate the expression of nitrogen assimilation genes, but our results indicate that *HY5* actions are not always in accord with light actions (Fig. 6A). For examples, *AMT1;2* gene was induced by lights (WLC and Rc), but it was repressed by *HY5*; *NRT2.2* gene was strongly induced by all wavelength of lights, but was not regulated by *HY5*. (Figs. 3, 4, and 6). In the case of *NIR1*, which was activated by both light and *HY5*, even though the *hy5* mutation dramatically lowed the overall expression level of *NIR1* (Fig. 4A), it did not weaken the light inducibility, the ratio of WLC over Dc, of the gene (Figs 4C). Similarly, the light induction of *AMT1;2* was not weakened by *hy5* mutation. In fact the folds of light induction for most genes examined, except for *AMT1;2*, were greater in *hy5* than in wild type (Fig.4C). These results indicate that *HY5* is not required for light activation of *NIR1* and *AMT1;2*. This finding is in agreement with Jonassen *et al.* (2009b) [19] that *HY5* and *HYH* are not required for transient light induction of several nitrogen assimilation genes, and that *HY5* and *HYH* were involved in light repression of *NRT1.1*. Similarly, we found that light repression of *NRT1.1* (Fig. 4A) did not occur in *hy5*, mainly due to its higher expression in the dark. Those results raise questions whether *HY5* may

have a distinct function in mediating gene expression of nitrogen assimilation pathway, besides its well-established role in light signaling.

It should be emphasized that our data support a pro-growth role of HY5 with regard to nitrogen nutrient and light nutrient. Under sufficient nutrient conditions, HY5 predominantly acts to promote expression of nitrogen-related genes (except for *AMT1;2*), as summarized in figure 6.

4.3. Enhanced root phenotype in *hy5* when grown under chronic nitrogen stress

The *hy5* mutant exhibited a drastically different root system that was exacerbated at low nitrogen conditions, as characterized by a significantly shorter primary root length and greater development of lateral roots (Fig. 2). The primary root phenotype of *hy5* diminished when nitrogen was provided in sufficient amount (above 5 mM nitrate concentrations). Roots are known to sense and respond to local and global nutrient supply [30, 40-42] as well as other factors [43]. Nitrate itself is an important signal that modulates root growth [44]. Normally, low nitrogen limits, while high nitrate stimulates, the generation of lateral roots [41,45]. Extensive crosstalk between nitrate and auxin signaling has been reported to explain nitrate-dependent root morphogenesis [9,46], as auxin is a key modulator of root architecture: high auxin concentration inhibits primary root elongation and stimulates lateral root development. The *hy5* and *hy5 hyh* double mutants, are known to have a root phenotype that is caused by increased auxin signaling in the mutant [4,6,7]. HY5 regulates a remarkable numbers of auxin responsive genes, and is critical in auxin-dependent regulation of root architecture and growth responses [6,7]. It is plausible that low nitrogen environment somehow enhanced the altered auxin signaling of *hy5* roots, resulting in the drastic root system phenotype under sustained nitrogen deficiency. In addition, since excess ammonium in plants can cause ammonium poisoning and inhibit the growth of primary roots [11], the greater amounts of internal ammonium in *hy5* at low nitrogen medium (less than 2 mM, Fig. 5B) likely contribute to the root phenotype of the mutant in the same medium.

Interestingly, the aerial part of *hy5* appeared more tolerant to nitrogen starvation than wild type seedlings, as evidence by the alleviated reduction of chlorophyll under nitrogen starvation (Fig. 1). We suggest that the overall phenotypes of *hy5* may imply that HY5 has important functions in the interplay of nitrogen signaling and auxin pathways in modulation of plant root growth.

Supplementary Material

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Abbreviations

NRT	Nitrate transporter
NIA	Nitrate reductase
NIR	Nitrite reductase
AMT	Ammonium transporter
HY5	Long Hypocotyles 5
HYH	<i>HY5</i> homologue
HATS	high-affinity nitrate uptake systems
LATS	low-affinity nitrate uptake systems
WT	Wild-type
Dc	constant darkness
WLC	continuous white light
Rc	continuous red light
FRc	continuous far-red light
PN	potassium nitrate
AN	ammonium nitrate
AS	ammonium succinate
NiRA	nitrite reduction activity
qPCR	quantitative PCR

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Highlights

1. The *hy5* mutant displayed shorter primary root only at limiting nitrogen conditions.
2. The *hy5* seedlings showed drastically reduced expression of *NIR1* at all conditions.
3. The *hy5* seedlings had higher expressions of *AMT1;2* at all tested conditions.
4. Most genes tested displayed higher or unchanged light induction in *hy5* mutant.
5. Higher NH_4^+ content and lower NiR activity occur in *hy5* only at low N conditions

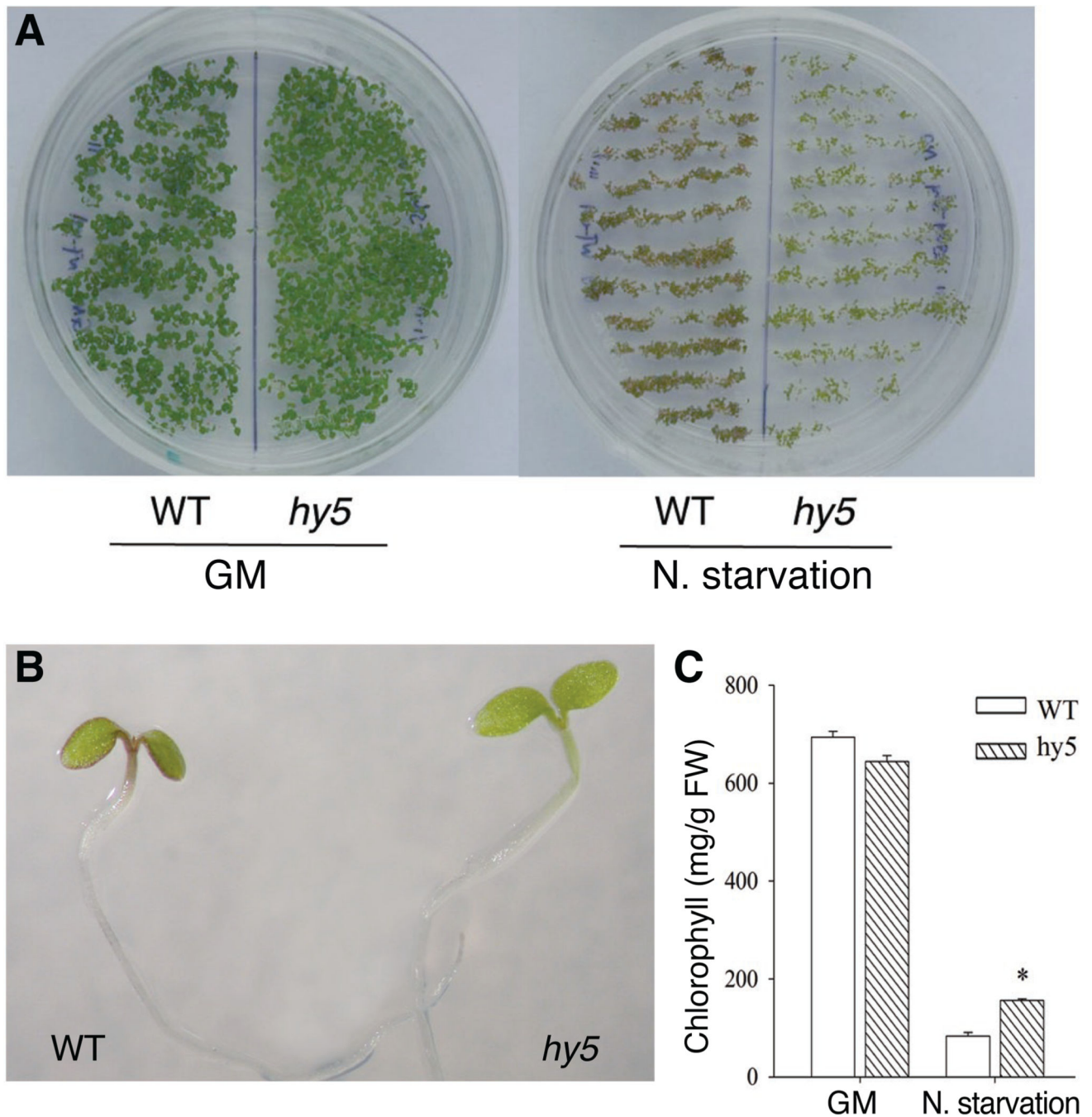


Figure 1.

Phenotype of the *hy5* mutant growing under the nitrogen starvation condition. **A.** Wild type (WT) and *hy5* seedlings of 4-day old growing on GM plate or on medium that lacked nitrogen nutrient (N. starvation). **B.** Enlarged image of WT and *hy5* seedlings growing on nitrogen starvation plate for 4 days. **C.** Chlorophyll contents of 4-day-old WT and the *hy5* mutant seedlings grown on GM plate and nitrogen starvation conditions.

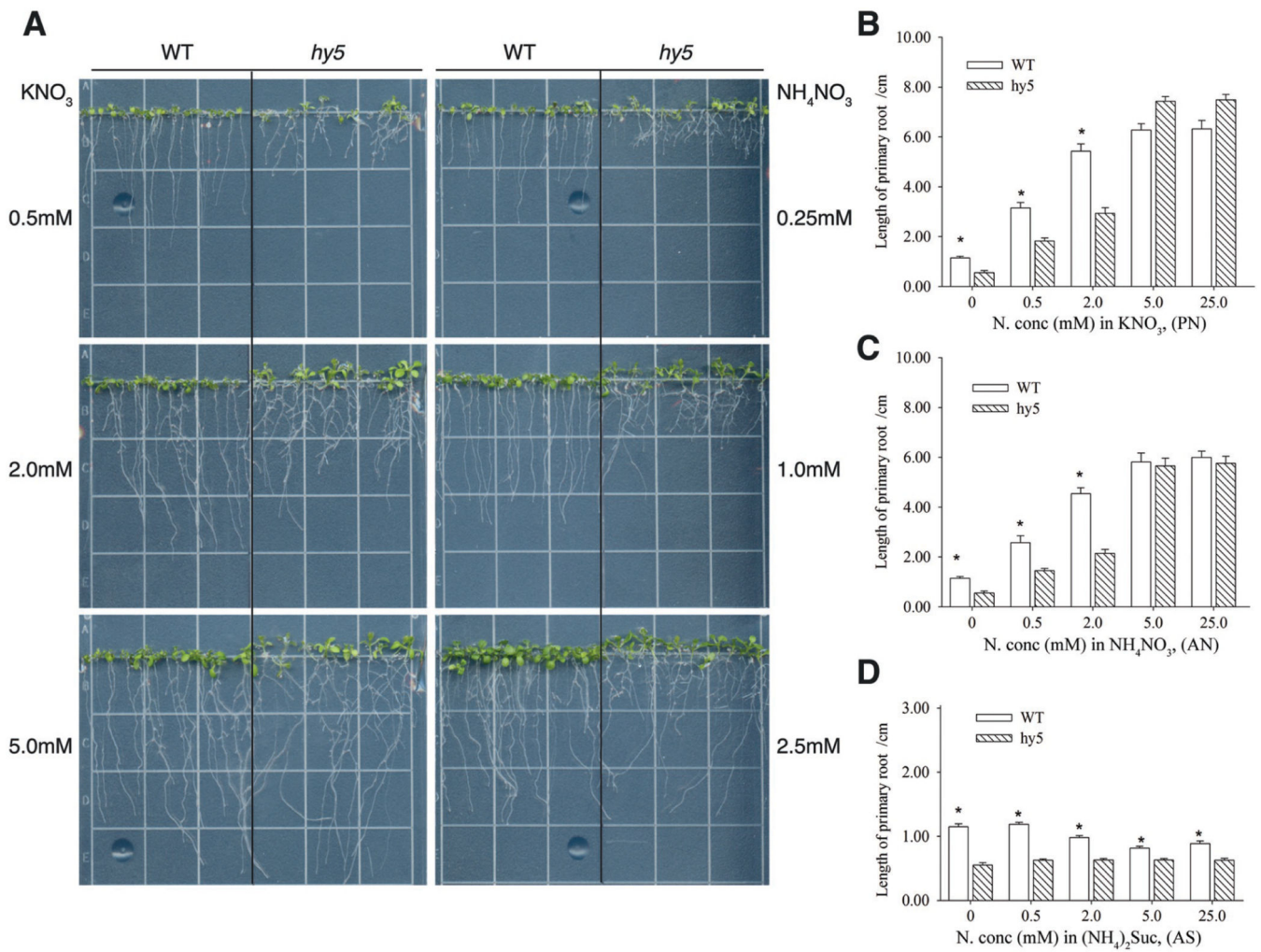


Figure 2. The root phenotype of *hy5* seedlings growing in different nitrogen nutrient environments. **A.** The root system of WT and *hy5* seedlings grown for 10 days on vertical plates that contained indicated concentrations of nitrogen in potassium nitrate (left) or in ammonium nitrate (right). On the same plate, left and right of the black line are WT and *hy5* mutant seedlings, respectively. **B-D.** Primary root lengths of 10-day old WT and *hy5* seedlings grown on **B**, potassium nitrate (PN); **C**, ammonium nitrate (AN); **D**, ammonium succinate (AS). Asterisks denote significant differences (* $P < 0.05$) between WT and *hy5* mutant.

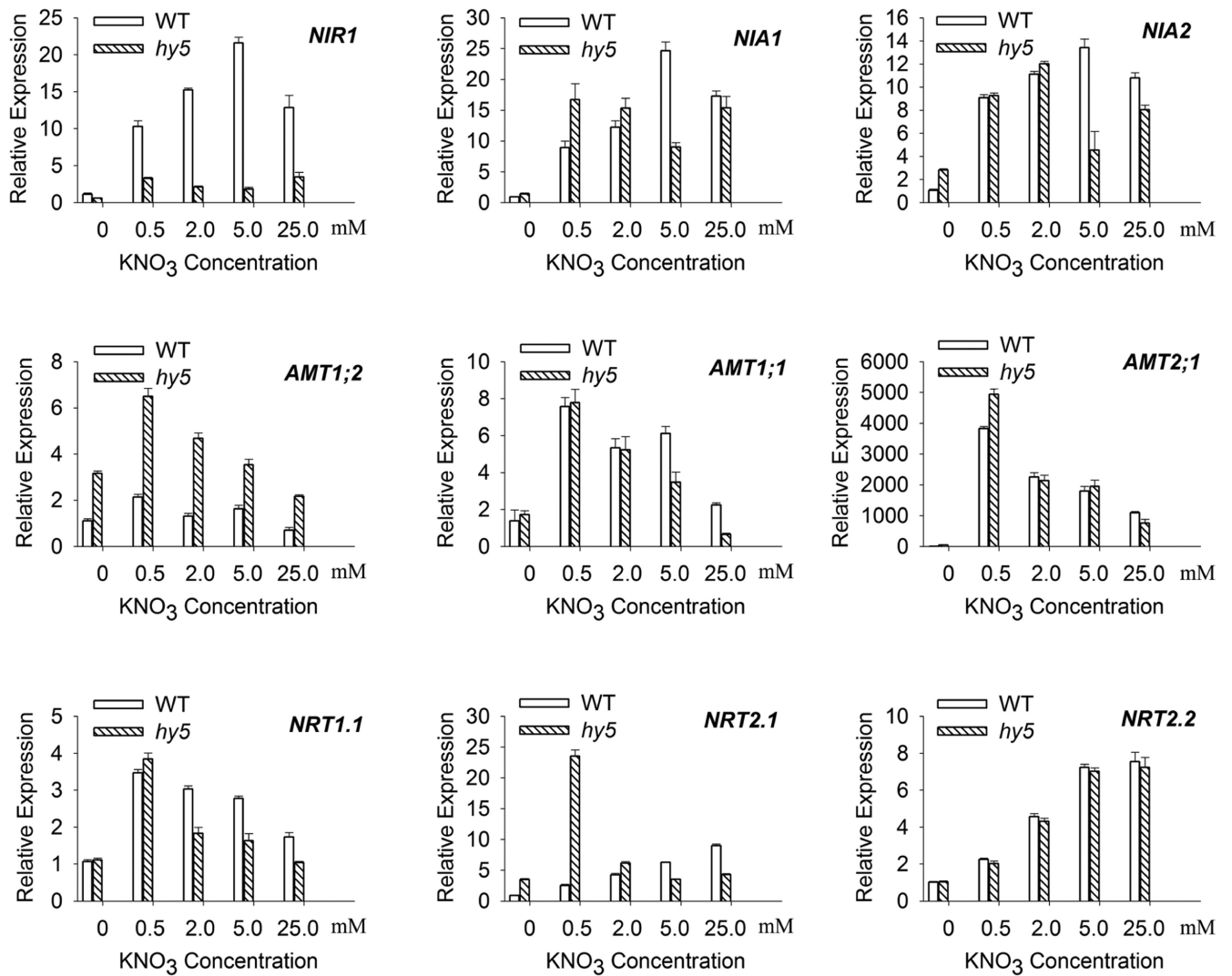


Figure 3.

Effect of the nitrogen supply on RNA expression of genes related to nitrogen metabolism.

WT and *hy5* mutant seedlings were grown for 10 days on plates with indicated concentration of potassium nitrate. The RNA samples were analyzed by real time RT-PCR against *UBQ1* (At3g52590) on following genes: *NIR1*; *NIA1*; *NIA2*; *AMT1;2*; *AMT1;1*; *AMT2;1*; *NRT1.1*; *NRT2.1*; *NRT2.2*. The relative transcript level of WT under 0 mM KNO₃ concentration is set to 1. Data presented are means of three repeats, and error bars are standard deviation.

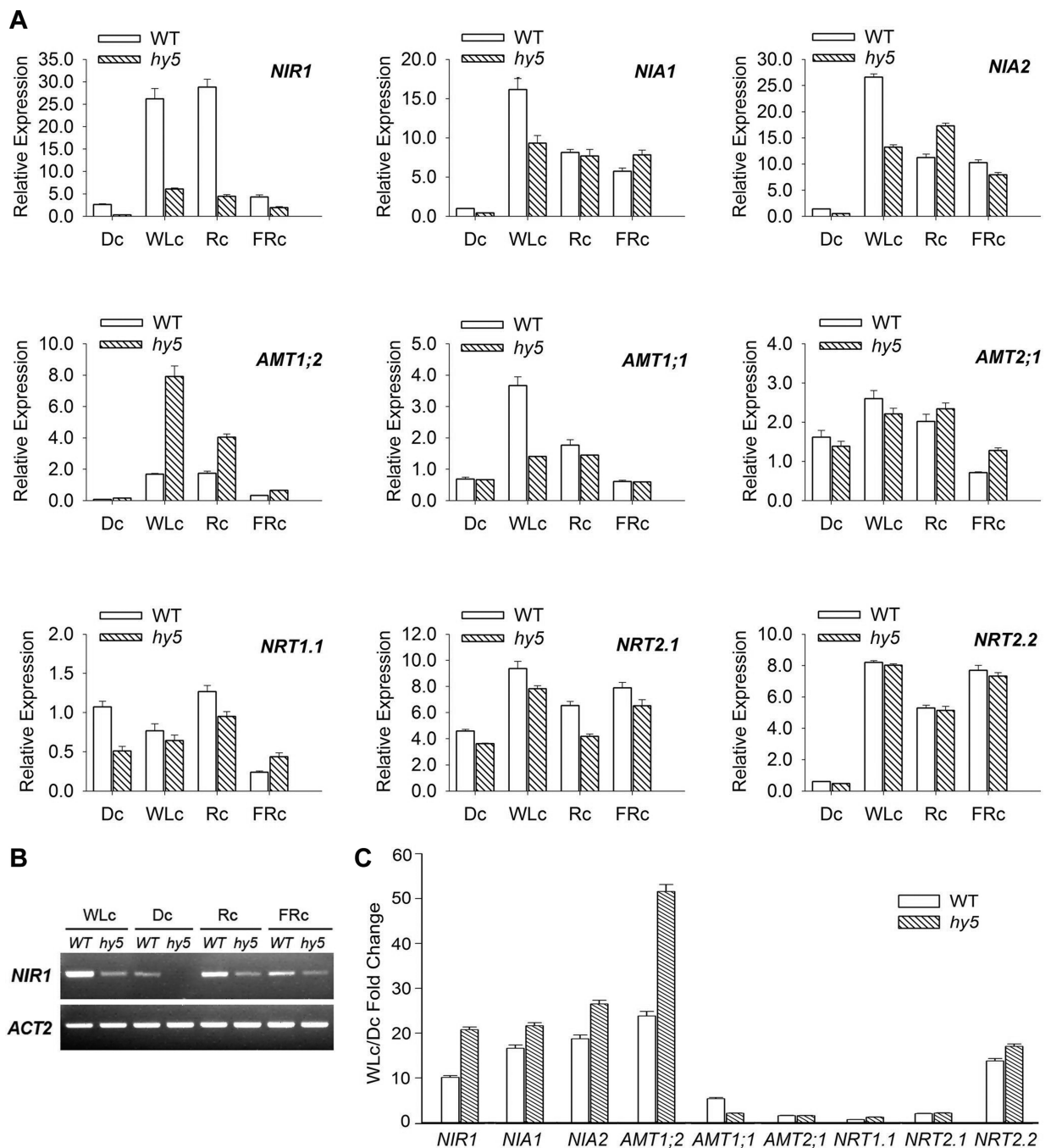


Figure 4. Effect of different wavelengths of light on RNA transcript levels of genes related to nitrogen metabolism. **A**, WT and *hy5* seedlings were grown on nitrogen sufficient media (GM) under indicated light source for 4 days, and RNA samples were analyzed by real time RT-PCR against *UBQ1* (At3g52590) on following genes: *NIR1*; *NIA1*; *NIA2*; *AMT1;2*; *AMT1;1*; *AMT2;1*; *NRT1.1*; *NRT2.1*; *NRT2.2*. Dc, constant darkness; WLc, continuous white light; Rc, continuous red light; FRc, continuous far-red light. Relative transcript level of *NIA1* in WT dark sample is set to 1. **B**, Decreased expression of *NIR1* in *hy5* under all light

conditions was confirmed by RT-PCR DNA gel analysis, with *ACTIN2* (*ACT2* At3g18780) as the internal control. WLC, continuous white light; Dc, constant darkness; Rc, continuous red light; FRc, continuous far-red light. C, Fold of the light induction of tested genes WT or *hy5*. Light induction was calculated as the ratio (fold change) of WLC value over its corresponding Dc value (WLC/Dc) of each gene, according to the data in panel A.

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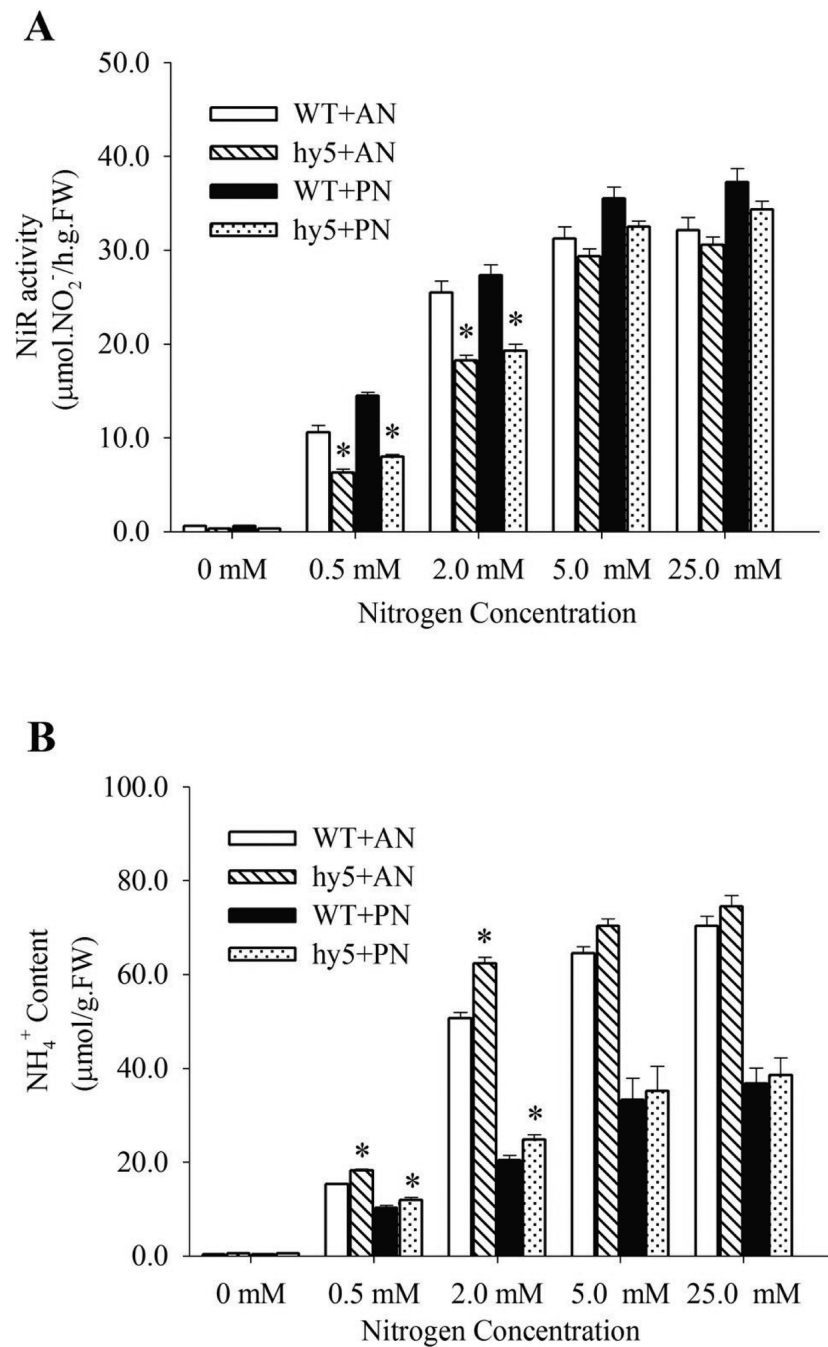


Figure 5. Determination of nitrite reductase activity (NiRA) (**A**) and NH_4^+ content (**B**) in WT and *hy5* mutants. Seedlings of 10-day old were grown in different nitrogen environments as indicated were used for the tests. Values are expressed relative to fresh weight of seedlings (FW). AN, ammonium nitrate; PN, potassium nitrate.

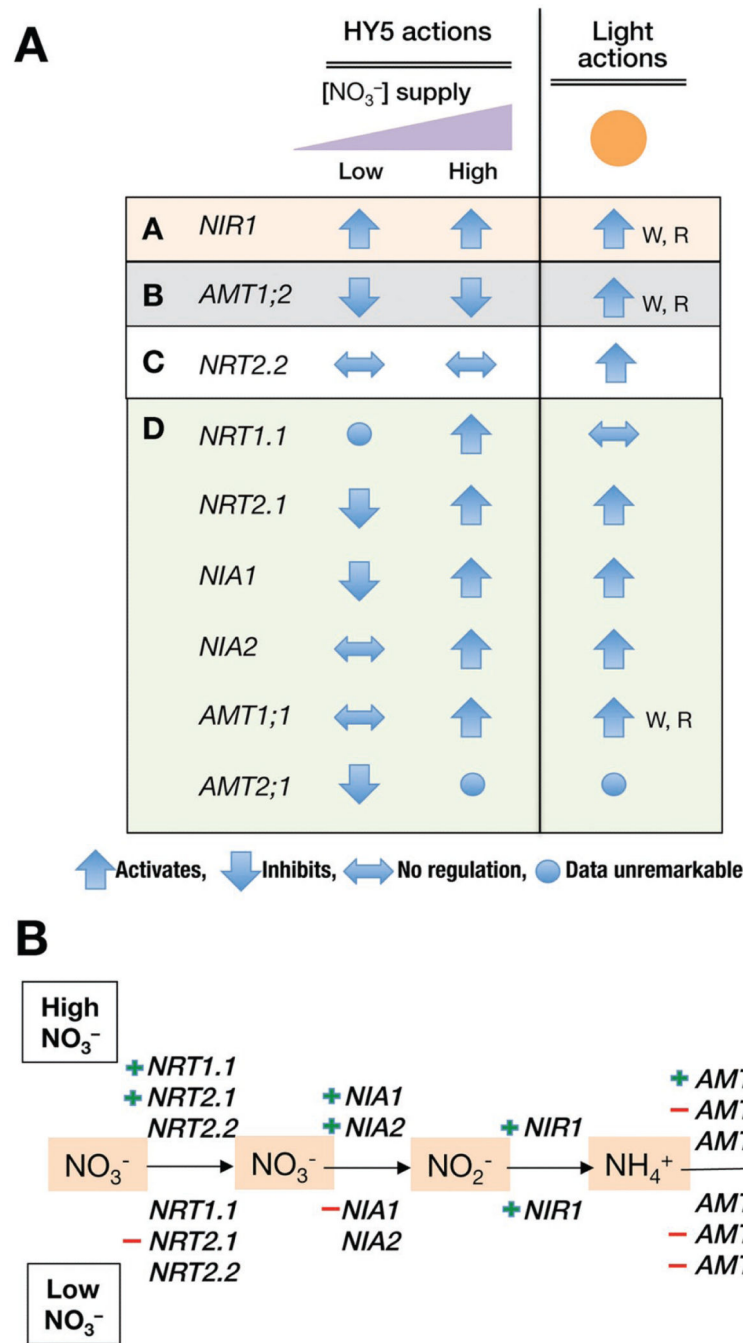


Figure 6. Summaries on regulations of nitrogen transport and assimilation genes by HY5. **A.** Summary of the effect of HY5 on indicated nitrogen-related genes at low nitrogen (0, 0.5 2.0 mM KNO₃) or high nitrogen (5.0 and 25 mM KNO₃) conditions, according to the data shown in figure 3. Light actions of the genes are based on the data in Figure 4, by comparing the values of WLC to Dc in wild type seedlings. The label “W.R.” denotes that the induction only applies to WLC and Rc, but not FRC. **B.** A diagram showing tested nitrogen-related genes along a simplified the pathway of nitrogen uptake and assimilation. “+” signs indicate

positive regulation by HY5, and “-“ signs indicate negative regulation by HY5, either in high NO_3^- conditions (upper side) or low NO_3^- (lower side) conditions.

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