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Short Title: Systems biology approach to study NRT2s regulation

Article title:
Genome-wide Analysis In Response to N and C Identifies New Regulators for root AtNRT2 Transporters

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One sentence summary:
Identification of three transcription factors involved in the regulation of NRT2s transporters using a systems biology approach and NRT2.1 as target gene in response to combinations of N/C treatments

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SR performed the transcriptomic experiments the analysis and the generation of the gene regulatory network. SR and VC obtained and performed the experiments to characterise the mutants along with JPT, IF, PT and LL. JAO’B with CI performed Y1H experiments. TM, CF and RG performed bioinformatics and statistical analysis for the gene regulatory network and the interaction of the transcription factors with the NRT2 promoters. LL, SR, AG and RG designed the experiments. LL, SR and AG wrote the manuscript.
Abstract

In *Arabidopsis thaliana*, the High-Affinity Transport System (HATS) for root NO$_3^-$ uptake depends mainly on four NRT2 transporters, namely NRT2.1, NRT2.2, NRT2.4 and NRT2.5. The HATS is the target of many regulations to coordinate nitrogen (N) acquisition with the N status of the plant and with carbon (C) assimilation through photosynthesis. At the molecular level, C and N signaling pathways have been shown to control gene expression of the *NRT2* transporters. Although several regulators of these transporters have been identified in response to either N or C signals, the response of *NRT2* genes expression to the interaction of these signals has never been specifically investigated and the underlying molecular mechanisms remain largely unknown. To address this question we used an original systems biology approach to model a regulatory gene network targeting *NRT2.1*, *NRT2.2*, *NRT2.4* and *NRT2.5* in response to N/C signals. Our systems analysis of the data highlighted the potential role of three putative transcription factors, TGA3, MYC1 and bHLH093. Functional analysis of mutants combined with yeast one hybrid experiments confirmed that all 3 transcription factors are regulators of *NRT2.4* or *NRT2.5* in response to N or C signals.
Introduction

As all living organisms, plants must integrate internal and external signals to adapt to fluctuating environmental conditions. This is particularly the case concerning mineral nutrition, because most nutrients display dramatic changes in external availability, whereas their internal concentrations must be kept within a limited range to be compatible with physiological processes. Accordingly, root nutrient uptake systems are finely tuned by regulatory mechanisms activated by local signaling of external nutrient availability and systemic signaling of the nutrient status of the whole plant (Schachtman and Shin, 2007). Furthermore, acquisition of the various nutrients has to be coordinated to remain consistent with the global chemical composition of plant tissues and with the fact that most nutrients contribute to the synthesis of biomolecules with a relatively strict elemental stoichiometry (e.g., C, N and S for amino acids). Therefore, the signaling pathways that are specific for the different nutrients must interact to ensure this coordination. Although coordinated regulation of uptake systems for different nutrients have been clearly demonstrated at the physiological level, the underlying molecular mechanisms remain largely obscure (Schachtman and Shin, 2007). The cross-talks between N and C signaling mechanisms are certainly those that have been most often investigated (Coruzzi and Zhou, 2001; Nunes-Nesi et al., 2010; Ruffel et al., 2014), first because N and C are the two mineral nutrients plants require in largest quantities, and also because they connect two key functions of plants as autotrophic organisms, i.e., photosynthesis and assimilation of inorganic nitrogen. Moreover, the importance of N/C signaling interaction is dramatically illustrated by the fact that most N-responsive genes in Arabidopsis are actually regulated by C/N interaction (Gutierrez et al., 2007).

The nitrogen nutrition of most herbaceous plants relies on the uptake of nitrate (NO$_3^-$), which is ensured in root cells by two classes of transport systems. The High-Affinity Transport System (HATS) is predominant in the low range of NO$_3^-$ concentrations (up to ~ca 1 mM), whereas the Low-Affinity Transport System (LATS) makes an increasing contribution to total NO$_3^-$ uptake with increasing external NO$_3^-$ concentration (Crawford and Glass, 1998). In all species investigated to date, genes encoding the various transporter proteins involved in either HATS or LATS have mostly been identified in the NRT2 and NPF (formerly NRT1/PTR) families, respectively (Nacry et al., 2013; O'Brien et al., 2016). The respective roles of HATS and LATS in the total NO$_3^-$ acquisition by the plant are still a matter of debate. However, field studies suggest that even in agricultural conditions, the HATS has a major contribution over the whole developmental cycle (Malagoli et al., 2004; Garnett et al., 2013). Both the structure
and regulation of the HATS have been extensively studied in *Arabidopsis thaliana*. In this species, almost all the HATS activity depends on four NRT2 transporters, namely NRT2.1, NRT2.2, NRT2.4 and NRT2.5 (Filleur et al., 2001; Kiba et al., 2012; Lezhneva et al., 2014), which all require an interaction with the NAR2.1 protein to be active in NO$_3^-$ transport (Kotur et al., 2012). Under most conditions, NRT2.1 is the main contributor to the HATS (Cerezo et al., 2001; Filleur et al., 2001). However, NRT2.4 and NRT2.5 display a very high-affinity for NO$_3^-$ and are important for taking up this nutrient when present at very low concentration (<50 µM) in the soil solution (Kiba et al., 2012; Lezhneva et al., 2014). Furthermore, unlike NRT2.1 and NRT2.4, NRT2.5 does not require the presence of NO$_3^-$ to be expressed, and is therefore considered crucial for ensuring the initial uptake of NO$_3^-$ as soon as it appears in the external medium (Kotur and Glass, 2015).

Most interestingly, the HATS has been shown to be the target of almost all regulations governing root NO$_3^-$ acquisition in *Arabidopsis* (Nacry et al., 2013), and this is associated with control of NRT2.1, NRT2.2, NRT2.4 and NRT2.5 expression at the mRNA level. In particular, previous reports have shown that NRT2.1 is induced both by N starvation (Lejay et al., 1999; Cerezo et al., 2001; Gansel et al., 2001), and by light and sugars, indicating coordination with photosynthesis (Lejay et al., 1999; Lejay et al., 2003). This makes NRT2.1 a very relevant model gene for investigating the interaction between N and C signalling networks in roots. This also holds true for NRT2.4 (Lejay et al., 2008; Kiba et al., 2012), but not for NRT2.5, which until now has only been reported to be up-regulated by N starvation (Lezhneva et al., 2014). For these reasons, and also due to its high functional importance as the main component of the HATS, NRT2.1 has been extensively investigated to unravel its regulatory mechanisms. Accordingly, a quite significant number of genes have been found to encode regulators of NRT2.1 expression, such as LBD37-39 (Rubin et al., 2009), TGA1 and TGA4 (Alvarez et al., 2014), NLP6 and NLP7 (Marchive et al., 2013; Guan et al., 2017), NRG2 (Xu et al., 2016), BTI-2 (Araus et al., 2016), NRT1.1 (Munos et al., 2004), CIPK8 (Hu et al., 2009), HNI9/IWS1 (Widiez et al., 2011) and HY5 (Chen et al., 2016). Most of these genes contribute to the regulation of NRT2.1 expression in response to changes in N provision. The only exception is HY5, which encodes a transcription factor reported to ensure long-distance signalling of the stimulation of NRT2.1 expression in roots by illumination of the shoot. Strikingly, none of the above regulators were shown to be involved in the cross-talk between N and C signalling pathways. Even more surprising, the response of NRT2.1 expression itself (as well as those of the other NRT2s) to the interaction of N and C signals...
was not specifically investigated. As a consequence, the molecular mechanisms responsible for the coordinated regulation of the NO₃⁻ HATS by N and C status of the plant are unknown. Our study aimed at filling this gap. Therefore, using NRT2.1 as a marker gene to identify relevant combinations of N/C treatments, we developed a systems biology approach based on genome-wide transcriptome analysis in roots of Arabidopsis plants to model a regulatory gene network targeting NRT2.1, NRT2.2, NRT2.4 and NRT2.5 in response to N/C signals. This highlighted the potential role of three putative transcription factors, TGA3, MYC1 and bHLH093 in controlling the expression of these transporter genes. Functional analysis of loss-of-function mutants confirmed that all 3 transcription factors are regulators of NRT2.4 or NRT2.5 in response to N or C signals. Furthermore, yeast one hybrid experiments confirmed that at least TGA3 and MYC1 are able to bind NRT2.4 and NRT2.5 promoters.
Results

Regulation of root nitrate transporters by interaction between nitrogen and light provision

We wished to determine whether induction of \( NRT2.1 \) by \( \text{N} \) starvation is dependent on light, and conversely if \( NRT2.1 \) induction by light is dependent on the availability of \( \text{NO}_3^- \) (Figure 1A and Figure 1B). In order to reveal possible interactions between C and N signalling pathways for the regulation of \( NRT2.1 \), we performed two different sets of experiments. In the first set of experiments, plants were starved for \( \text{N} \) for up to 72h either in the dark or at three different light intensities, 50 μmol m\(^{-2}\)s\(^{-1}\) (LL), 250 μmol m\(^{-2}\)s\(^{-1}\) (IL) and 800 μmol m\(^{-2}\)s\(^{-1}\) (HL) (Figure 1A). In the second set of experiments, plants were treated with 10mM \( \text{NO}_3^- \), 1mM \( \text{NO}_3^- \) or no \( \text{N} \) and transferred during 8h from the dark to HL conditions (Figure 1B). In LL and IL conditions, \( NRT2.1 \) expression was, as expected, induced when plants were starved for \( \text{N} \) even if both the kinetic and the level of induction were different depending on light intensity (Figure 1A). When plants were kept in the dark, \( NRT2.1 \) expression was not induced by \( \text{N} \) starvation but it remained very low both on 10mM \( \text{NO}_3^- \) and on \( \text{N} \) free solution. More surprisingly, the induction of \( NRT2.1 \) expression by \( \text{N} \) starvation was also almost completely abolished when plants were treated in HL conditions. However, under HL \( NRT2.1 \) mRNA levels were always high, even under repressive conditions such as 10mM \( \text{NO}_3^- \). This unexpected result is specific of \( NRT2.1 \) since \( NRT2.2, NRT2.4 \) and \( NRT2.5 \), known to be also induced by \( \text{N} \) starvation in roots (Li et al., 2007; Kiba et al., 2012; Lezhneva et al., 2014), are still regulated by \( \text{N} \) starvation in HL (Supplemental Figure 1A). However, just like \( NRT2.1 \), \( NRT2.2, NRT2.4 \) and \( NRT2.5 \) were not regulated by \( \text{N} \) starvation in the absence of light. These data confirm the need of light for the regulation by \( \text{N} \) starvation of root \( \text{NO}_3^- \) transporters. It also suggests that the mechanisms involved in \( NRT2.1 \) regulation by \( \text{N} \) starvation are somewhat different from the mechanisms involved in the regulation of \( NRT2.2, NRT2.4 \) and \( NRT2.5 \).

The second set of experiments confirmed the strong interaction between C/N signals as it revealed that the level of \( \text{N} \) nutrition affects the regulation of \( NRT2.1 \) expression by light (Figure 1B). Indeed, when plants were starved for \( \text{N} \) during 48h, \( NRT2.1 \) expression was much less induced by light as compared to plants grown on 10 or 1mM \( \text{NO}_3^- \) (Figure 1B). Among other root \( \text{NO}_3^- \) transporters, only \( NRT2.2 \) and \( NRT2.4 \) were induced by light and their level of induction seemed to be also dependent on \( \text{N} \) nutrition (Supplemental Figure 1B). However, in contrast to \( NRT2.1 \), the level of expression of both \( NRT2.2 \) and \( NRT2.4 \) was high...
when plants were starved for N and low when plants were grown on 1 or 10mM NO$_3^-$ (Supplemental Figure 1B). For NRT2.4, it confirms that this transporter is more sensitive to high N repression than NRT2.1 (Kiba et al., 2012). The same result was obtained for NRT2.5, whose expression is barely detectable on either 10mM or 1mM NO$_3^-$ (Supplemental Figure
However, concerning regulation by light, even when NRT2.5 expression was high in N starved plants, light did not induce but rather seemed to repress NRT2.5 mRNA accumulation after 8h in the light (Supplemental Figure 1B).

In a previous study, we showed that expression of NRT2.1 and NRT2.4 is induced by light only in the presence of CO₂ in the atmosphere, suggesting that light regulation of these genes corresponds to a control exerted by photosynthesis (Lejay et al., 2008). As in the rest of our study we used micro-array experiments to look for genes involved in the regulation of root NO₃⁻ transporters by photosynthesis, it was important for us to be able to discriminate between genes regulated by light itself or by photosynthesis. To do so, we performed a third set of experiments where plants were transferred from dark to light for 4h in an atmosphere containing 0 or 600ppm CO₂. The results confirmed (i) that both NRT2.1 and NRT2.4 are only induced by light in the presence of CO₂ and (ii) that NRT2.5 is not induced by light or photosynthesis as suggested by our previous experiment (Figure 1C and Supplemental Figure 1C).

Gene network for the regulation of root nitrate transporters by light and N starvation

The experiments performed above allowed us to reveal interesting interactions between C and N regulation of root NRT2 NO₃⁻ transporters. We took advantage of this experimental design to develop a systems biology approach aiming at inferring a gene regulatory network underlying the interactions between N and C signals in the regulation of root NO₃⁻ transporters. Due to the central position of NRT2.1 as regulatory target affecting N acquisition, we used it as a focus gene around which to find associated gene networks. We performed Affymetrix microarrays on selected combinations of light and N treatments, which were found discriminant for regulation of NRT2.1. Altogether, we chose 18 treatments labelled with numbered arrows in Figure 1. These 18 treatments correspond to 3 sets of conditions representative of (i) the light-dependent induction of NRT2.1 expression in response to N starvation, (ii) the light induction of NRT2.1 on 10mM NO₃⁻ and (iii) the specific regulation of NRT2.1 by photosynthesis and not by light itself. For each treatment, 2 independent biological replicates were generated and used for Affymetrix ATH1 microarray hybridization.

Regulation of NRT2.1, NRT2.2, NRT2.4 and NRT2.5 gene expression in response to N starvation and light/photosynthesis was similar on microarrays as compared to the results obtained by quantitative PCR in Figure 1 and Supplemental Figure 1 (Supplemental Figure 2). These results also confirmed that these four NO₃⁻ transporters are the main NRT2s.
expressed in roots. \textit{NRT2.3}, \textit{NRT2.6} and \textit{NRT2.7} showed very low expression levels on the microarrays under our experimental conditions. It is also noteworthy that \textit{NRT2.1} was the most highly expressed member of the family among the 7 \textit{NRT2}s (5 to 50 fold higher expression as compared to \textit{NRT2.2}, \textit{NRT2.4}, \textit{NRT2.5}) (Supplemental Figure 2).

To find gene regulatory networks that could integrate N and C signalling and thus control \textit{NRT2.1} expression, we defined 5 different subsets of conditions addressing the regulation by N on one side and by C on the other side, as described in Figure 2A. Genes defined as regulated by N-deprivation like \textit{NRT2.1} are differentially regulated by N provision in conditions 1 to 4 in experiment 1, where \textit{NRT2.4} is also found regulated and in conditions 7 to 14 in experiment 2, where \textit{NRT2.2} and \textit{NRT2.5} were also found regulated. To select the most robust genes regulated by N provision only the intersection between the 2 groups was isolated. In addition to \textit{NRT2.1}, the intersection defines a set of 33 genes including the 2 transcription factors \textit{TGA3} (At1g22070) and \textit{MYC1} (At4g00480). On another hand, genes considered as regulated by C provision like \textit{NRT2.1} are differentially regulated by light intensity in conditions 1, 3, 5, and 6 in experiment 1, by light time exposure in conditions 9, 11 and 13 in experiment 2 and by photosynthesis in conditions 15 to 18 in experiment 3. Similarly, to narrow down the specificity of gene regulation by C factor, only common genes to at least 2 experiments were isolated. This core set corresponds to 142 genes including \textit{NRT2.1} but also 2 others transcription factors \textit{bHLH093} (At5g65640) and \textit{ARR14} (At2g01760) (Figure 2A).

Next, we only focused on the 174 genes that showed a response to N starvation (34 genes) and/or C provision (142 genes); \textit{NRT2.1} being the common gene between the 2 responsive gene lists together with a \textit{Kinesin3} gene (At5g54670-ATK3) coding for a microtubule motor protein. The possible connection of the 4 transcription factors with \textit{NRT2.1} and the other genes was determined by a Gene Networks analysis performed on the VirtualPlant platform (Katari et al., 2010). The generated network contains 124 gene nodes. These genes are connected to each other by 260 edges, representing regulatory relationships such as predicted transcription factor-target gene interactions (Figure 2B). Regulatory interactions were proposed based on detection of at least one predicted binding site for a given transcription factor within the promoter region of the target gene as done previously (Gutierrez et al., 2008). According to the parameters used, 50 genes out of the 174 are not connected to any other genes in the network (See Material and Methods for details about the parameters). Among these 50 genes, the transcription factor \textit{ARR14} was excluded due, for instance, to a low level of correlation between this gene and \textit{NRT2.1} expression patterns. However, TGA3,
MYC1 and bHLH093 have all predicted regulatory interactions with NRT2.1 plus 40 other genes of the network (indicated in blue in Figure 2B). The network predicts also that only one or only two of these transcription factors putatively regulate the 79 remaining genes (one gene being connected to the network by predicted protein-protein interaction with 2 TGA3-targets).
Nevertheless, almost all sub-networks are interconnected through protein-protein interaction prediction, suggesting possible coordination within the network at the whole.

Regulation of \textit{MYC1}, \textit{TGA3} and \textit{bHLH093} in response to C and N

The gene regulatory network we obtained revealed 3 main transcription factors: MYC1 and TGA3 which were found to be co-regulated with \textit{NRT2.1} in response to N starvation and \textit{bHLH093} which was found to be co-regulated with \textit{NRT2.1} in response to light/photosynthesis. In order to validate their regulation, we measured gene expression by QPCR across all the conditions performed in experiment 1 and 2 (Figure 3A). The results confirmed that expression of \textit{TGA3} and \textit{MYC1} genes is induced 2- to 3-fold after transferring the plants to a N-free solution, especially under LL or HL conditions. Furthermore, similar to \textit{NRT2.1}, \textit{MYC1} regulation of gene expression requires the presence of light (Figure 3A and Supplemental Figure 3). The results also confirmed that \textit{bHLH093} gene expression is only induced by light (between 3- and 4-fold after 8h of HL), independent of N nutrition. This is supported by the fact that \textit{bHLH093} is not regulated by N starvation (Figure 3A and Supplemental Figure 3). On the contrary, \textit{MYC1} and \textit{TGA3} genes are not only regulated by N starvation, but their expression is also induced by light, especially in plants starved for N (Figure 3A and Supplemental Figure 3). Like for \textit{NRT2.1}, putative \textit{cis}-binding elements for TGA3, MYC1 and bHLH093 were also found in the promoters of \textit{NRT2.2}, \textit{NRT2.4} and \textit{NRT2.5} (Figure 3B). Furthermore, DNA affinity purification sequencing (DAP-seq) experiments recently performed by O’Malley et al. (2016) confirmed that TGA3 binds in silico to the promoter of \textit{NRT2.1}, \textit{NRT2.2} and \textit{NRT2.4} (Figure 3C). Unfortunately, no data are available for MYC1 and bHLH093 in this work. Altogether, these results suggest that the transcription factors we identified are involved in regulation of several root \textit{NRT2s}.

To our knowledge, the transcription factors TGA3, MYC1 and bHLH093 have not been isolated in previous transcriptomic approach as candidates for regulation of root \textit{NO}\textsubscript{3}\textsuperscript{-} transporters. In order to understand why they have not been found before we looked at the expression pattern of the known regulatory elements for \textit{NRT2.1} in our experimental set up. The results show that the known regulators for \textit{NRT2.1} were not co-regulated with \textit{NRT2.1} expression in our conditions (Figure 4). This was also the case for \textit{HY5}, a transcription factor recently identified as involved in the regulation of \textit{NRT2.1} by light/photosynthesis (Chen et al., 2016). In our hands, this transcription factor was only induced by light independently of the presence of CO\textsubscript{2} and therefore not by photosynthesis like \textit{NRT2.1} (Figure 4). As most of the previous transcriptomic experiments were performed to study the signalling pathways
involved in short-term induction by NO\textsubscript{3}\textsuperscript{-}, we also looked at the regulation of \textit{TGA3}, \textit{MYC1}, and \textit{bHLH093} in those conditions (Supplemental Figure 4). We chose the transcriptomic experiments performed by Wang et al. (2004). In this study WT plants and the null mutant for nitrate reductase (NR) were treated with 5mM KNO\textsubscript{3} for 2h and compared to control plants.

Figure 3. \textit{TGA3}, \textit{MYC1} and \textit{bHLH093} are candidate transcription factors for the control of the expression of \textit{NRT2} gene family.

A. Gene expression analysis of the 3 candidate transcription factors in the extended set of Nitrogen/Carbon combinations confirms correlation with \textit{NRT2.1} regulation. Expression patterns have been determined by RT-QPCR (relative accumulation to \textit{Chlathrin} housekeeping gene).

B. \textit{NRT2.2}, \textit{NRT2.4} and \textit{NRT2.5} as well as \textit{NRT2.1} display putative cis-binding elements for the 3 transcription factors in their promoter region. The gene network has been done using the Gene Networks analysis tool in VirtualPlant software (http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/) (22); only Regulated Edges box and One Binding Site option has been selected in this case.

C. \textit{TGA3} binds \textit{in silico} with the promoter of \textit{NRT2.1}, \textit{NRT2.2} and \textit{NRT2.4}. The analysis has been done using the Plant Cistrome Database (http://ncomorph.salk.edu/PlantCistromeDB) (63).
treated with 5mM KCl for 2h. The data sets allowed the authors to determine the genes that respond specifically to NO$_3^-$ in both WT and NR-null plants. The results show that, as expected, NRT2.1, NRT2.2 and NRT2.4 are induced by NO$_3^-$ while NRT2.5 seems to be repressed (Supplemental Figure 4A). In the same time, most of the known regulators for
NRT2.1 are also induced by NO$_3^-$ except NLP7 and TCP20, two transcription factors which have not been isolated using transcriptomic approaches (Supplemental Figure 4B). On the contrary, in the same conditions, our three transcription factors, TGA3, MYC1 and bHLH093 were not regulated by NO$_3^-$ supply neither in WT nor NR-null plants. All these results reinforced the originality of our experimental set up and explain why we found new candidates that have never been isolated in previous transcriptomic experiments.

Role of MYC1, TGA3 and bHLH093 in the regulation of NRT2s root nitrate transporters

To determine if MYC1, TGA3 and bHLH093 are involved in regulation of NRT2 root NO$_3^-$ transporters we used two independent insertion mutants for each of the transcription factors: tga3.2, tga3.3 for TGA3, myc1.2, myc1.3 for MYC1 and bHLH093.1, bHLH093.5 for bHLH093. As both TGA3 and MYC1 were found to be regulated by N starvation, we also produced a double mutant, tga3.2/myc1.2, to test a potential additive effect of those transcription factors on the regulation of NRT2s. In addition, to reinforce our conclusions concerning the role of bHLH093, we also produced an overexpressing line by transforming the bhlh093.1 mutant with a 35S::bHLH093 construct. The measurement of MYC1, TGA3 and bHLH093 expression level confirmed an almost complete absence of their transcripts in their respective mutants and a strong overexpression of bHLH093 in the overexpressing line (Supplemental Figure 5A and B).

As expected for a role of TGA3 and MYC1 in the regulation of NRT2s by N starvation, the induction of both NRT2.4 and NRT2.5 is overall reduced in tga3 and myc1 mutants compared to wild type plants, especially after 72h of N starvation (Figure 5A). This lower induction in response to N starvation is stronger in the double mutant tga3.2/myc1.2 and is observed in that case consistently after 24h, 48h and 72h of N starvation for NRT2.4 and NRT2.5 and after 48h for NRT2.2. It suggests that TGA3 and MYC1 are not redundant and that both factors may function as transcriptional activators under low N conditions. This result is supported by the fact that neither the level of expression nor the regulation of MYC1 in tga3 mutants and of TGA3 in myc1 mutants are affected compared to wild type plants (Supplemental Figure 5A). However, surprisingly, MYC1 and TGA3 do not affect the regulation of NRT2.1 in the same conditions (Figure 5A). In agreement with a role of MYC1 and TGA3 in the regulation of NRT2.4 and NRT2.5, Y1H experiments show that both transcription factors are able to bind to the promoter of these two transporters (Figure 5B).
Out of the three NRT2s, which are induced by light, NRT2.4 and to a lower extent NRT2.1, have a significant lower induction after 4h and 8h of light in the bHLH093 mutants as compared to wild type plants (Figure 6A and 6B). Conversely, the induction by light of both NRT2.1 and NRT2.4 is higher in the 35S::bHLH093 plants (Figure 6B). Interestingly, this
phenotype seems to depend on the amount of NO$_3^-$ in the nutritive solution since the effect of bHLH093 is preferentially seen when plants are starved for N or on 1mM NO$_3^-$ and is almost absent when plants are grown on 10mM NO$_3^-$ (Figure 6A and 6B).

Figure 6. bHLH093 is required for NRT2.4 full induction by light in N-deprivation condition.

A. Characterization of the knock-out mutants for bHLH093 (bHLH093-1 and bHLH093-5) on 0N or 10mM NO$_3^-$: The plants were either starved for N for 48 hr (light gray bars) or supplied with NO$_3^-$ 10 mM one week ahead the experiment (black bars) and were kept in the dark 40 hr before transition to high light intensity (800 μmol m$^{-2}$ s$^{-1}$) during 1h, 2h, 4h and 8h. Roots have been collected to assess NRT2.1, NRT2.2 and NRT2.4 mRNA accumulation by RT-QPCR (relative accumulation to Clathrin housekeeping gene). Values are means of three biological replicates ± SD. Differences between WT (Col-0) and the KO mutants are significant at *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test).

B. Characterization of the knock-out (bHLH093-1 and bHLH093-5) and the over-expressor (35S:bHLH093) mutants for bHLH093 on 1mM NO$_3^-$: The plants were grown on 1mM NO$_3^-$ and were kept in the dark 40 hr before transition to intermediate light intensity (250 μmol m$^{-2}$ s$^{-1}$, IL) during 1h, 2h, 4h and 8h. Roots have been collected to assess NRT2.1 and NRT2.4 mRNA accumulation by RT-QPCR (relative accumulation to Clathrin housekeeping gene). Values are means of three biological replicates ± SD. Differences between WT (Col-0) and the mutants are significant at *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test).
Discussion

**Interaction between nitrogen and light provision affect regulation of NRT2.1 expression**

As part of its central physiological role, the root NO$_3^-$ HATS is a main target of the C/N regulatory networks ensuring the necessary integration of both, N acquisition by roots and C acquisition by shoots. The HATS regulation by N starvation has been well characterised in previous studies, especially through the study of NRT2.1 expression. Split-root experiments have demonstrated that this regulation relies on systemic signaling pathways (Gansel et al., 2001), and underlying molecular mechanisms have recently been unravelled (Ohkubo et al., 2017). On the other hand, NRT2.1 expression is also dramatically induced by light and sugars through an Oxidative Pentose Phosphate Pathway (OPPP)-dependent signaling mechanism (Lejay et al., 1999; Lejay et al., 2003; Lejay et al., 2008; de Jong et al., 2014). Over the past decade, the importance of signal interaction for the regulation of gene expression has become more and more obvious and especially for C/N regulation (Palenc harb et al., 2004; Gutierrez et al., 2007; Krouk et al., 2009). However, the details of how this interaction affects regulation of NRT2.1 expression in response to combined N/C treatments were unknown. Our results clearly show that the interplay of N and C signaling mechanisms has a major role as light conditions can totally suppress N regulation of NRT2.1 expression, and vice versa (Figure 1A and 1B). Similar to the case for inorganic N assimilation, it seems that low sugars inhibit NRT2.1 expression, overriding signals from N metabolism (Stitt et al., 2002; Nunes-Nesi et al., 2010). Surprisingly, the regulation of NRT2.1 by N starvation is not only abolished when plants are treated in the dark. It happens also under high light conditions (Figure 1A). However, in that case, the level of NRT2.1 expression is always high, even on normally repressive conditions like 10 mM NO$_3^-$, while in the dark the level of NRT2.1 stays low, independently of the level of N. One model to explain these results is that enhancement of growth due to combination of high light and high NO$_3^-$ supply results in a sustained high N demand for growth, relieving the feedback repression normally associated with high NO$_3^-$ supply. This model is supported by a recent metabolomics analysis performed on Arabidopsis thaliana under diverse C and N nutrient conditions (Sato and Yanagisawa, 2014). Taken together, these results clearly support the idea that the control of NRT2.1 expression involves a complex network of interactions between signals emanating from N and C metabolisms. However, this level of complexity seems to be rather specific for NRT2.1. In contrast to NRT2.1, expression of NRT2.2, NRT2.4 and NRT2.5 is always repressed on 10 mM NO$_3^-$, independent of light levels (Supplemental Figure 1A). It should be noted that in the N
starvation experiments plants are transferred on a media with no N. This leads to the variation
of two factors, the N status of the plants, which decreases when plants are starved for N, and
the presence of NO\textsubscript{3}\textsuperscript{−} in the nutritive solution, which is suppressed by the transfer to N-free
solution. Concerning the regulation of \textit{NRT2.2}, \textit{NRT2.4} and \textit{NRT2.5} it is not known which
one of these two factors is predominant since their expression was only measured in N
starvation experiments (Kiba et al., 2012; Lezhneva et al., 2014; Kotur and Glass, 2015). It is
thus possible that \textit{NRT2.2}, \textit{NRT2.4} and \textit{NRT2.5} are only regulated locally by NO\textsubscript{3}\textsuperscript{−} and not by
systemic signals of N demand. This idea is supported by the work of (Ma et al., 2015)
showing that the regulation of \textit{NRT2.4} and \textit{NRT2.5} by N starvation depends on CBL7, which
is specifically induced by NO\textsubscript{3}\textsuperscript{−} deficiency. Moreover, NIGT/HRS\textsubscript{1}s have been shown to act
as transcriptional repressor of \textit{NRT2.4} and \textit{NRT2.5} upon NO\textsubscript{3}\textsuperscript{−} treatment (Kiba et al.,
2018)Safi et al. 2018). Local regulation by NO\textsubscript{3}\textsuperscript{−} would explain why these transporters, unlike
\textit{NRT2.1}, are always repressed when plants are on 10 mM NO\textsubscript{3}\textsuperscript{−}, regardless of the light
conditions (Supplemental Figure 1A).

**Identification of three new candidates for regulation of \textit{NRT2} genes using a systems
biology approach**

Over the past few years, transcriptomic approach and systems biology have been powerful
tools to identify new regulatory elements involved in N signaling (For review (Medici and
Krouk, 2014; Vidal et al., 2015). For root \textit{NRT2s} genes and HATS activity in \textit{Arabidopsis}, it
enabled the identification of CIPK23 and CIPK8 in response to NO\textsubscript{3}\textsuperscript{−}, LBDs transcription
factors in response to high N and BT2, a negative regulator of \textit{NRT2.1} and \textit{NRT2.4} under low
N conditions (Figure 7) (Ho et al., 2009; Hu et al., 2009; Rubin et al., 2009; Araus et al.,
2016). For C and N signaling, previous microarray studies in response to transient treatments
with NO\textsubscript{3}\textsuperscript{−}, sucrose or NO\textsubscript{3}\textsuperscript{−} plus sucrose have been used to reveal, at the level of the genome,
the existence of interaction between C and N signaling (Wang et al., 2003; Price et al., 2004;
Scheible et al., 2004; Wang et al., 2004; Gutierrez et al., 2007; Huang et al., 2016). In
\textit{Arabidopsis}, over 300 genes have been found differentially expressed by combined C:N
treatments compared to C or N treatments (Palenchar et al., 2004). However, because of the
number of genes affected by C and/or N regulation and the complex interactions between the
signalling pathways, none of these studies have led so far to the identification and the
validation of new regulatory elements. The unexpected regulations of root \textit{NRT2s} and
especially of \textit{NRT2.1} in our experimental set-up offer an interesting opportunity to find genes
more specifically involved in the regulation of root NO\textsubscript{3}\textsuperscript{−} transporters by C and/or N, and to
build a gene network model integrating regulators responding to N and/or C signals. As compared to previous transcriptomic approaches on N and C signaling in plants, we were able to narrow down the number of candidate genes by (i) using \textit{NRT2.1} as a specific target and (ii) integrating the data from several Affymetrix microarrays to find gene networks co-

Figure 7. Schematic representation of the known regulatory elements for the regulation of root high-affinity NO\textsubscript{3}\textsuperscript{-} transporters in response to external NO\textsubscript{3}\textsuperscript{-}; the N status of the plant and light/photosynthesis.

Purple circles represent the transcription factors identified in previous studies while red circles represent the transcription factors identified in our study.
regulated with the expression of NRT2.1 in response to different combinations of light and N treatments. Therefore, the gene regulatory network includes only three transcription factors, bHLH093, MYC1 and TGA3 (Figure 2B). bHLH093 was found co-regulated with NRT2.1 in response to light through photosynthesis because, like NRT2.1, it is not induced by light in the absence of CO₂ (Supplemental Figure 3). MYC1 and TGA3 were found co-regulated with NRT2.1 in response to N starvation. The analysis of their level of expression across all the experiments revealed that TGA3 and MYC1 are induced by N starvation but especially in LL and HL conditions, while bHLH093 seems overall induced by light no matter what the level of N (Figure 3A). Furthermore, MYC1 is also clearly induced by light (Figure 3A and Supplemental Figure 3). Taken together these results support the validity of our approach to find regulatory elements affected by C and/or N signalling and which are thus candidates for the regulation by C/N interaction. Interestingly, none of these three transcription factors was found involved in the regulation of root NO₃⁻ transporters by previous studies. One explanation to this relates to the fact that the expression of bHLH093, MYC1 and TGA3 is not responsive to the induction by NO₃⁻, which was by far the major environmental change investigated by previous studies (Supplemental Figure 4A). Conversely, none of the regulatory genes identified in previous studies was found with our approach. Indeed, most of them are not affected by N starvation and/or by light (Figure 4). The only exception is HY5, which encodes a recently identified mobile transcription factor involved in the regulation of NRT2.1 by sugar signals (Chen et al., 2016) and that is not found co-regulated with NRT2.1 in our analysis. This is explained by the fact that, unlike NRT2.1, we found HY5 induced by light even in the absence of CO₂ in our dataset (Figure 4). It indicates that expression of HY5 does not depend on the production of sugars through photosynthesis and is directly regulated by light. The role of HY5 in light signalling and not in C signalling is supported by previous studies showing that HY5 works downstream phytochrome signalling (Quail, 2002; Li et al., 2010). Taken together, these results suggest that NRT2.1 would be induced by both a light component dependent on HY5 and a C component dependent on the OPPP (Lejay et al., 2008; de Jong et al., 2014; Chen et al., 2016). Accordingly, both Lejay et al. (2008) and Chen et al. (2016) found that induction of NRT2.1 by light is higher as compared to the addition of sucrose in the dark. Furthermore, there is still an induction of NRT2.1 expression by increasing supply of sucrose in the mutant hy5 (Chen et al., 2016).
The use of mutants validated our approach and showed that bHLH093 has mainly a role in the induction by light of NRT2.4, while MYC1 and TGA3 affect induction by N starvation of both NRT2.4 and NRT2.5 and in a more modest way NRT2.2 (Figure 5A and Figure 6). Furthermore, Y1H experiments support the fact that MYC1 and TGA3 are direct regulators of NRT2.4 and NRT2.5 as already suggested for TGA3 and NRT2.4 by the results obtained by O’Malley et al. (2016) (Figure 3 and Figure 5B). Conversely, Chromatin Immunoprecipitation (ChIP) experiments, using plants expressing bHLH093 fused to GFP, failed to reveal a robust interaction with the promoter of NRT2.4 (data not shown). It suggests that bHLH093 is an indirect regulator and that it is rather involved in the signalling pathway governing the regulation of NRT2.4 and in a lesser extend NRT2.1 by photosynthesis.

As represented in Figure 7, most of the regulatory elements identified to date concern the primary NO₃⁻ response (PNR), with only three elements involved in the repression by high N or high NO₃⁻ and one in the induction by light. Along with CBL7, MYC1 and TGA3 seem thus to be part of an independent signalling pathway involved in the induction of root NO₃⁻ transporters in response to low N, while bHLH093 is, to our knowledge, the first element involved in a regulatory mechanism linked to photosynthesis (Ma et al., 2015). As discussed above, the role of these transcription factors in the regulatory mechanisms involved in C/N interactions is also supported by our results. Indeed the role of bHLH093 in the regulation by light seems to be dependent of the level of N and the role of MYC1 and TGA3 seems to be stronger in high light conditions (Figure 5A and Figure 6).

However, surprisingly, none of these 3 transcription factors affect strongly the regulation of NRT2.1, that we used as a target gene in our systems biology approach. This result could indicate that the regulatory mechanisms differ between the four NRT2 genes involved in the HATS. Indeed, NRT2.1 is regulated by at least 4 different mechanisms (local induction by NO₃⁻ and repression by high NO₃⁻, systemic repression by N metabolites and induction by C), while NRT2.4 is regulated by C and N starvation and NRT2.5 only by N starvation. Furthermore, our experimental setup revealed obvious complex interactions between N and C signalling for NRT2.1, which do not exist for NRT2.2, NRT2.4 and NRT2.5. As discussed above, if NRT2.2, NRT2.4 and NRT2.5 are only repressed by NO₃⁻ and not by N metabolites, MYC1 and TGA3 could be involved in a NO₃⁻–specific signalling pathway upregulating the very high-affinity transporters (NRT2.4 and NRT2.5) when the external NO₃⁻ concentration becomes too low to be efficiently taken up by NRT2.1. Previous results support a role, for at least TGA3, in a NO₃⁻–specific signalling pathway. Indeed, TGA3 is part of a family of 7 genes in Arabidopsis thaliana and two of them, TGA1 and TGA4, have already been...
involved in the induction of NRT2.1 and NRT2.2 in response to NO$_3^-$ (Alvarez et al., 2014). Taken together these results and our findings suggest that this family of transcription factors could participate in a more general way to the regulation of root NO$_3^-$ transporters by NO$_3^-$. Concerning MYC1 there is no direct evidence to support its role in a NO$_3^-$ signalling pathway (Bruex et al., 2012).

Since NRT2.1 and NRT2.4 are both regulated by C through OPPP, it was even more surprising to find that the absence of bHLH093 affects mainly the induction by light of NRT2.4 compared to NRT2.1 (Figure 6) (Lejay et al., 2008). However, the role of bHLH093 seems to be dependent on the level of N since it plays a significant role in the regulation of NRT2.4 only under low N conditions, whereas the induction of NRT2.1 by light is mostly seen in this experiment under high N conditions (10mM NO$_3^-$). It could explain why, in those conditions, bHLH093 mutation does not affect the regulation of NRT2.1 by light, while in the second experiment, where plants were grown on a moderate level of NO$_3^-$ (1mM), NRT2.1 is well induced by light and the NO$_3^-$ concentration could be low enough to reveal the impact of bHLH093 on NRT2.1 regulation (Figure 6B). To our knowledge, the role of bHLH093 in the roots and in response to light has never been characterised before. The only information concerns a role in flowering promotion under non-inductive short-day conditions through the gibberellin pathway (Sharma et al., 2016).

Materials and Methods

Plant Material

Arabidopsis thaliana genotypes used in this study were the wild-type Col-0 ecotype and mutants obtained from the Salk Institute: tga3.2 (Salk_081158), tga3.3 (Salk_088114), myc1.2 (Salk_057388), myc1.3 (Salk_006354), bHLH093.1 (Salk_121082) and bHLH093.5 (Salk_104582).

In all experiments plants were grown hydroponically under non sterile conditions as described by Lejay et al. (1999). Briefly, the seeds were germinated directly on top of modified Eppendorf tubes filled with pre-wetted sand. The tubes were then positioned on floating rafts and transferred to tap water in a growth chamber under the following environmental conditions: light/dark cycle of 8 h/16 h, light intensity of 250 µmol·m$^{-2}$·s$^{-1}$, temperature of 22/20°C, and RH of 70%. After 1 week, the tap water was replaced with a complete nutrient solution. The experiments were performed on plants grown on 1 mM NO$_3^-$ as N source. The other nutrients were added as described by Lejay et al. (1999). The plants were allowed to
grow for 3 additional weeks before the experiments. Nutrient solutions were renewed weekly and on the day before the experiments.

Treatments

Two different sets of experiments were performed to (i) study the impact of light on the regulation of NO$_3^-$ transporter genes in the roots by N starvation, and (ii) study the impact of the N status of the plants on the regulation of these genes by light.

In the first set of experiments 4 weeks old plants were transferred on a solution containing 10 mM NO$_3^-$. After one week the plants were transferred in the morning either in continuous dark or in a light/dark cycle at three different light intensities (50, 250 and 800 μmoles.h$^{-1}.m^{-2}$) and starved for N during 24h, 48h and 72h, by replacing NO$_3^-$ with CaCl$_2$ 2.5 mM and K$_2$SO$_4$ 2.5 mM.

In the second set of experiments 4 weeks old plants were transferred on a solution containing 10 mM NO$_3^-$. They were then pre-treated during 3 days on nutrient solution containing contrasted level of N: (i) no N, (ii) 1 mM NO$_3^-$ or (iii) 10 mM NO$_3^-$. After 32h in the dark the plants were transferred to light for 1h, 2h, 4h and 8h under three different light intensities (50, 250 and 800 μmoles.h$^{-1}.m^{-2}$).

The dependence of the expression of NO$_3^-$ transporter genes on photosynthesis was investigated by modifying the CO$_2$ concentration in the atmosphere. After a pretreatment of 40 h in the dark, plants grown on 1mM NO$_3^-$ were placed for 4 h in the light (150 μmol·m$^{-2}$·s$^{-1}$) or in the dark in a 240-L, airtight plexiglass chamber connected to a computerized device for controlling temperature, humidity, and CO$_2$ concentration in the atmosphere (Atellience Instruments; see Delhon et al. (1996) for details). The CO$_2$ concentration in the atmosphere was held constant during the treatments at 0 or 600 μL L$^{-1}$.

All experiments were repeated two or three times.

RNA Extraction and Gene Expression Analysis

Root samples were frozen in liquid N$_2$ in 2-mL tubes containing one steel bead (2.5 mm diameter). Tissues were disrupted for 1 min at 30 s$^{-1}$ in a Retsch mixer mill MM301 homogenizer (Retsch, Haan, Germany). Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently 4 μg of RNA were treated with DNase (DNase I, SIGMA-ALDRICH, USA) following the manufacturer’s instructions. Reverse transcription was achieved in the presence of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) after annealing with an anchored oligo(dT)$_{18}$
primer as described by Wirth et al. (2007). The quality of the cDNA was verified by PCR using specific primers spanning an intron in the gene APTR (At1g27450) forward 5'-CGCTTCTTTCTCGACACTGAG-3'; reverse 5'-CAGGTAGCTTCTTGCGGCTTC-3'. Gene expression was determined by quantitative real-time PCR (LightCycler; Roche Diagnostics, Basel, Switzerland) with the kit LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions with 1 µl of cDNA in a total volume of 10 µl. The amplifications were performed as described previously by Wirth et al. (2007). All the results presented were standardized using the housekeeping gene Clathrin (At4g24550). Gene-specific primer sequences were: NRT2.1 forward, 5'-AACAAAGGGCTAACGTGGATG-3'; NRT2.1 reverse, 5'-CTGCTTCTCTGCTCATCC-3'; NRT2.2 forward, 5'-GCAGCAGATGGCATGCATT-3'; NRT2.2 reverse, 5'-AAGCATTGTTGGTGCGTCC-3'; NRT2.4 forward, 5'-GAACAAAGGGCTGACATGGAT-3'; NRT2.4 reverse, 5'-GCTTCTCGGTCTCTGACC-3'; NRT2.5 forward, 5'-TGTGGACCCTCTTTCCAAA-3'; NRT2.5 reverse, 5'-TTGGGGATGAGTCGTGTTTG-3'; MYC1 forward, 5'-AACCTTAACGACTCTGTG-3'; MYC1 reverse, 5'-CCGCAACTATGTAGTCTCG-3'; TGA3 forward, 5'-CTCTCAGAAAGTTGCGC-3'; TGA3 reverse, 5'-CATATACGAGGAGATGTC-3'; bHLH093 forward, 5'-AGCTTGAGGCCAAC-3'; bHLH093 reverse, 5'-GCTTCTCATGTAATCTATGGCA-3'; Clathrin forward, 5'-AGCATACTCGTGCAAAAG-3'; Clathrin reverse, 5'-TCGCTGTGTCACATATCTC-3'.

**Acquisition of Genome-Wide Expression and Statistical Analysis**

Genome-wide expression was determined using Affymetrix ATH1 GeneChip expression microarrays according to manufacturer's instructions. To do so, biotinylated cRNA was synthesized from 200 ng of total RNA from Arabidopsis roots. Affymetrix data were normalized in R (http://www.r-project.org/) using MAS5. Then, normalized data were subjected to different statistical analyses, all centered on NRT2.1 expression pattern but including various sets of microarray data among the whole data set. As a first approach to build a gene network involved in the regulation of root NO₃⁻ transporters, we examined genes displaying expression pattern correlated to NRT2.1 expression pattern across the entire dataset. A R² coefficient cut-off above 0.8 or below -0.8 led to the identification of 79 AGIs displaying an expression pattern correlated to NRT2.1, including 77 genes positively correlated with NRT2.1 (Table S1). Among these 79 genes, none of them...
displays a function related to gene regulation but rather related to metabolic activity and more precisely to carboxylic acid metabolic process as, for example, the Glutamate synthase 2 gene (Supplemental Figure 6A). Moreover, a hierarchical clustering of the treatments according to the expression pattern of these genes clearly revealed that their response is largely driven only by the light/carbon factor, putting aside any possible regulation by N provision (Supplemental Figure 6B). Therefore, we determined that a global analysis of the entire data set was not relevant to identify regulators of NO₃⁻ transport integrating C and N availability and that a finest analysis of gene expression in different subsets of treatments will be more powerful.

The list of genes regulated by N-deprivation specifically under low light regime was determined by a t.test analysis (p.value<0.05) between conditions 3 and 4. All genes also found regulated between conditions 1 and 2 based on the same analysis are removed from this list (Figure 1, Table S1). Genes regulated by N-deprivation during light induction are determined by a 2 ways ANOVA using Nitrogen as one factor (presence = conditions 7,9,11,13 / absence = conditions 8,10,12,14) and Light as the second factor (no Light = conditions 7,8 / 1hr-light = conditions 9,10 / 2hr-light = conditions 11,12 / 4hr-light = conditions 13,14). Genes of interest are regulated by the interaction of the 2 factors (p.value<0.05) and display a similar regulation by N from dark to 2hr-light as observed for NRT2.1 (Figure 1, Table S2). Genes regulated by light intensity under high N-provision and by light time exposure under high N-provision are both determined by a linear modeling of gene expression across light intensity (conditions 1,3,5,6) or time exposure (conditions 7,9,11,13) using a R² above 0.9 (p.value is below 0.003) (Figure 1, Tables S3 and S4). Finally, genes regulated by photosynthesis activity are determined by a 2 ways ANOVA using CO₂ level as one factor (0ppm = conditions 15,17 / 600ppm = conditions 16,18) and Light as the second factor (Dark = conditions 15,16 / Light = conditions 17,18). To narrow down the list of NRT2.1-like genes, only those passing post-hoc Tukey tests comparing conditions 18 to all 3 others (p.value<0.05) and displaying a ratio >2 or <0.5 are selected (Figure 1, Table S5).

Visualization of gene connectivity by clustering and gene network analysis

Heat map hierarchical cluster of gene expression and samples was generated with the MeV software using Pearson correlation as distance metric and Average as linkage method (www.tm4.org) (Saeed et al., 2003). The Gene Network was generated with the VirtualPlant 1.3 software (http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/) (Katari et al., 2010). The connectivity of the nodes is based on 5 categories corresponding to literature data, post-
transcriptional regulation, protein:protein interactions, transcriptional regulation and regulated edges meaning transcription factor - target relationship based at least on one binding site in the promoter of the target gene. Two nodes are linked by an edge if they fall in any of these categories combined to an expression pattern correlated at a $R^2 > 0.7$ or $< -0.7$. Visualization of the gene regulatory network has been performed with Cytoscape (http://www.cytoscape.org/) (Shannon et al., 2003). Node properties have been modified to reveal connectivity with the 3 transcription factors and highlight $NRT2.1$ position within the network.

**Y1H Assays**

For the generations of the plasmids for promoter analysis by Y1H, particular promoter fragments of NRT2.4 (1968bp), NRT2.5 (1692bp) were first amplified by PCR with overlapping ends as described by Gibson et al. (2009). For the bait, the pMW2 and pMW3 vectors were used (Deplancke et al., 2006). pMW vectors were amplified by PCR with overlapping ends as a single sequence (pMW2) or as 2 independent sections (pMW2). Final vectors were made as described by Gibson et al., 2009. The Y1H prey vectors for TGA3 and MYC1 transcriptions factors were a kind gift from Franziska Turck (Castrillo et al., 2011). All the fragments generated for all constructs were validated by DNA sequencing. The Y1H assay was performed according to protocol described by Grefen (2014) with minor modifications. Briefly, the vectors pMW2-NRT2.4, pMW3-NRT2.4, pMW2-NRT2.5, pMW3-NRT2.5 were first linearized with restriction enzymes. For pMW2 vectors BamH1 (NEB) was used and for pMW3 vectors Xho1 (NEB). The resulting linearized constructs were subsequently co-integrated into the yeast strain: YM4271 as described by Grefen (2014). The transformed yeast strains were tested for autoactivation and the selected colonies with the higher sensitivity to 3-AT were then transformed with the construct pDEST-AD-TGA3 or pDEST-AD-MYC1 or pDEST-AD (Empty vector). Empty vector was included as a negative control. Resulting yeast were dropped on selection media (SD –His–Ura–Trp) supplemented with increasing concentrations of 3-AT (0, 15, 30, 50, 80, 100 mM). Yeast growth was verified after 48h.

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**Figure legends**

**Figure 1.** Interaction between Nitrogen and Light/Carbon provision modulates NRT2.1 mRNA accumulation in roots. A, Different light regimes modulate NRT2 regulation in roots of plants experiencing from high NO₃⁻ provision (10 mM) to N deprivation (-N). The light regimes encompass dark, low light intensity (50 μmol m⁻² s⁻¹; LL), intermediate light intensity (250 μmol m⁻² s⁻¹; IL) and high light intensity (800 μmol m⁻² s⁻¹; HL). Plants were supplied with NO₃⁻ 10 mM one week ahead the experiment and acclimated for 24 hours in the different light regimes before applying the N deprivation for 24, 48 or 72 hr. B, Different N provisions modulate NRT2 regulation in roots of plants experiencing a dark to light transition. The N provisions encompass 10mM NO₃⁻, 1mM NO₃⁻ (for 72 hr) and N deprivation for 48 hr (-N). Plants are kept in the dark (i.e., 40hr) before transition to high light intensity (800 μmol m⁻² s⁻¹; HL) and roots are collected at time 0 (Dark) and 1, 2, 4 and 8 hr after light transition. C, Regulation of NRT2 by photosynthesis activity. Plants are grown in regular NO₃⁻ regime (1mM) and intermediate light intensity until they are transferred for 4 hr in a CO₂-deprived atmosphere (0ppm) or in high CO₂-supplied atmosphere (600ppm), either in the dark or in the light. In these 3 experimental conditions, roots have been collected to assess NRT2.1 mRNA accumulation by RT-QPCR (relative accumulation to Clathrin housekeeping gene). Expression pattern of NRT2.1 across the 35 conditions tested (16 in A, 15 in B and 4 in C) has driven the choice of 18 conditions to investigate gene reprogramming associated to the regulation of NO₃⁻ transport. These 18 conditions are indicated with arrows and numbers on the x-axis of the 3 NRT2.1 bar graphs (Each arrow corresponds to one condition with 2 independent biological repeats constituted of a pool of approx. 10 plants each).

**Figure 2.** Gene expression multi-analysis driven by NRT2.1 expression pattern combined to an integrative analysis identified a candidate gene regulatory network connected to the NO₃⁻ transport system. A, Venn diagrams identifying common genes regulated by N provision on low light condition and dark to light transition (34 genes) or regulated by light/carbon (142 genes). The union of these gene lists defines a population of 174 genes, including 4 transcription factors. B, The core set of 174 genes differentially expressed has been structured into a Gene Regulatory Network using the Gene Networks analysis tool in VirtualPlant software (http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/) (Katari et al., 2010). The network includes 124 nodes (genes) and 260 edges connecting genes. The nodes have been organized according to their connection to the 3 transcription factors MYC1, TGA3 and bHLH093 and...
Figure 3. TGA3, MYC1 and bHLH093 are candidate transcription factors for the control of the expression of NRT2 gene family. A, Gene expression analysis of the 3 candidate transcription factors in the extended set of Nitrogen/Carbon combinations confirms correlation with NRT2.1 regulation. Expression patterns have been determined by RT-QPCR (relative accumulation to Clathrin housekeeping gene). B, NRT2.2, NRT2.4 and NRT2.5 as well as NRT2.1 display putative cis-binding elements for the 3 transcription factors in their promoter region. The gene network has been done using the Gene Networks analysis tool in VirtualPlant software (http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/) (Katari et al., 2010); only Regulated Edges box and One Binding Site option has been selected in this case. C, TGA3 bounds in silico with the promoter of NRT2.1, NRT2.2 and NRT2.4. The analysis has been done using the Plant Cistrome Database (http://neomorph.salk.edu/PlantCistromeDB) (O'Malley et al., 2016).

Figure 4. Most of the genes previously determined as NRT2s regulators do not display expression patterns similar to the patterns of the 3 candidate transcription factors in the set of Nitrogen and Light/Carbon combinations. Graphs display the expression pattern of the 20 genes extracted from the whole transcriptomic dataset. Data are organized according to the multi-analysis (i.e., S1 to S5, Figure 2). LBD37, LBD38, LBD39 repress the expression of genes involved in NO₃⁻ uptake (NRT2.1 and NRT2.5) and assimilation, likely mimicking the effects of N organic compounds (Rubin et al., 2009). TGA1, TGA4, NLP6, NLP7, NRG2, NRT1.1, CIPK8, CIPK23 are required for the NO₃⁻-dependent induction of NRT2.1 (Munos et al., 2004; Castaings et al., 2009; Ho et al., 2009; Hu et al., 2009; Konishi and Yanagisawa, 2013; Marchive et al., 2013; Alvarez et al., 2014; Xu et al., 2016). TCP20 and HN19/IWS1 are involved into NRT2.1 regulation controlled by systemic signaling (Widiez et al., 2011; Guan et al., 2014). BT2 represses expression of NRT2.1 and NRT2.4 genes under low NO₃⁻ conditions (Araus et al., 2016). CBL7 regulates NRT2.4 and NRT2.5 expression under N-starvation conditions (Ma et al., 2015). HY5 has been recently identified as a regulator of NRT2.1 by mediating light promotion of NO₃⁻ uptake (Chen et al., 2016). HRS1, HHO1, HHO2 and HHO3 are repressors of NRT2.4 and NRT2.5 expression under high N conditions (Kiba et al., 2018).
Figure 5. TGA3 and MYC1 are required for NRT2.4 and NRT2.5 full induction during N-deprivation. A, Characterization of the knock-out mutants for TGA3 (tga3.2 and tga3.3), MYC1 (myc1.2 and myc1.3) and the TGA3/MYC1 double mutants (tga3.2 myc1.2). The plants were supplied with NO$_3^-$ 10 mM one week ahead the experiment and acclimated for 24 hr in high light conditions (800 μmol m$^{-2}$ s$^{-1}$) before applying the N deprivation for 24, 48 or 72 hr. Roots have been collected to assess NRT2.1, NRT2.2, NRT2.4 and NRT2.5 mRNA accumulation by RT-QPCR (relative accumulation to Clathrin housekeeping gene). Values are means of three biological replicates ± SD. Differences between WT and the KO mutants are significant at *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test). B, Characterization of TGA3 and MYC1 interaction with NRT2.4 and NRT2.5 promoters in a Y1H assay. Yeast cells were grown on SD-H-U-T minimal media without histidine (H), uracil (U), tryptophan (T) and containing 3- amino-1,2,4-triazole (3AT) at 0, 15, 30 and 50 mM. Interaction between the transcription factors and the promoters results in HIS3 reporter activation in contrast to the empty vector that does not interact.

Figure 6. bHLH093 is required for NRT2.4 full induction by light in N-deprivation condition. A, Characterization of the knock-out mutants for bHLH093 (bHLH093-1 and bHLH093-5) on 0N or 10mM NO$_3^-$. The plants were either starved for N for 48 hr (light gray bars) or supplied with NO$_3^-$ 10 mM one week ahead the experiment (black bars) and were kept in the dark 40 hr before transition to high light intensity (800 μmol m$^{-2}$ s$^{-1}$) during 1h, 2h, 4h and 8h. Roots have been collected to assess NRT2.1, NRT2.2 and NRT2.4 mRNA accumulation by RT-QPCR (relative accumulation to Clathrin housekeeping gene). Values are means of three biological replicates ± SD. Differences between WT (Col-0) and the KO mutants are significant at *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test). B, Characterization of the knock-out (bHLH093-1 and bHLH093-5) and the over-expressor (35S::bHLH093) mutants for bHLH093 on 1mM NO$_3^-$. The plants were grown on 1mM NO$_3^-$ and were kept in the dark 40 hr before transition to intermediate light intensity (250 μmol m$^{-2}$ s$^{-1}$; IL) during 1h, 2h, 4h and 8h. Roots have been collected to assess NRT2.1 and NRT2.4 mRNA accumulation by RT-QPCR (relative accumulation to Clathrin housekeeping gene). Values are means of three biological replicates ± SD. Differences between WT (Col-0) and the mutants are significant at *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test).

Figure 7. Schematic representation of the known regulatory elements for the regulation of root high-affinity NO$_3^-$ transporters in response to external NO$_3^-$, the N status of the plant and
light/photosynthesis. Purple circles represent the transcription factors identified in previous studies while red circles represent the transcription factors identified in our study.

**Supplemental Figure 1.** Interaction between Nitrogen and Light/Carbon provision modulates mRNA accumulation in roots of most of the *NRT2* family members.

**Supplemental Figure 2.** Expression pattern of *NRT2* family genes in the set of Nitrogen and Carbon/Light combinations as determined by Arabidopsis Affymetrix ATH1 microarray hybridization.

**Supplemental Figure 3.** Expression pattern of *TGA3, MYC1* and *bHLH093* transcription factors in the set of Nitrogen and Carbon/Light combination as determined by *Arabidopsis* Affymetrix ATH1 microarrays hybridization.

**Supplemental Figure 4.** *TGA3, MYC1* and *bHLH093* display expression pattern different than most of the known regulators of *NRT2* genes in response to NO$_3^-$. 

**Supplemental Figure 5.** Expression pattern of *TGA3, MYC1* and *bHLH093*.

**Supplemental Figure 6.** Biomaps and hierarchical clustering of the 79 most correlated genes to *NRT2.1* expression across all experiments.

**Supplemental Table 1.** List of 430 probes regulated by N deprivation under low light regime only.

**Supplemental Table 2.** List of 573 probes regulated by the interaction between nitrogen and light.

**Supplemental Table 3.** List of 128 probes linearly regulated by light intensity.

**Supplemental Table 4.** List of 985 probes linearly regulated during light induction.

**Supplemental Table 5.** List of 509 probes regulated by the interaction between light and CO$_2$. 
Supplemental Table 6. List of 80 probes coregulated based on \textit{NRT2.1} expression and pearson correlation.

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