Systems approaches map regulatory networks downstream of the auxin receptor AFB3 in the nitrate response of *Arabidopsis thaliana* roots

Elena A. Vidal, Tomás C. Moyano, Eleodoro Riveras, Orlando Contreras-López, and Rodrigo A. Gutiérrez[1]

Fondo Nacional de Areas Prioritarias (FONDAP) Center for Genome Regulation, Millennium Nucleus Center for Plant Functional Genomics, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, 833101, Santiago, Chile

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Auxin is a key phytohormone regulating central processes in plants. Although the mechanism by which auxin triggers changes in gene expression is well understood, little is known about the specific role of the individual members of the TIR1/AFB auxin receptors, Aux/IAA repressors, and ARF transcription factors and/or molecular pathways acting downstream leading to plant responses to the environment. We previously reported a role for AFB3 in coordinating primary and lateral root growth to nitrate availability. In this work, we used an integrated genomics, bioinformatics, and molecular genetics approach to dissect regulatory networks acting downstream of AFB3 that are activated by nitrate in roots. We found that the NAC4 transcription factor is a key regulatory element controlling a nitrate-responsive network, and that *nac4* mutants have altered lateral root growth but normal primary root growth in response to nitrate. This finding suggests that AFB3 is able to activate two independent pathways to control root system architecture. Our systems approach has unraveled key components of the AFB3 regulatory network leading to changes in lateral root growth in response to nitrate.

systems biology | nitrogen | Sungear

Auxin is a key controller of root growth processes. The auxin response is initiated by binding of the hormone to TIR1/AFB receptors. Auxin receptors are part of the $SCF^{TIR1/AFB}$ E3 ubiquitin ligase complex (1). Auxin binding to TIR1/AFB receptors triggers the recognition and degradation by polyubiquitination of the Aux/IAA repressors, releasing the inhibition of the ARF transcription factors that mediate auxin-responsive gene transcription (2–5). The *Arabidopsis* genome encodes 29 Aux/IAA and 23 ARF factors. Genetics studies have shown that these factors may have both unique and redundant functions on plant growth and development, which likely depend on the specific patterns of expression of these factors on tissues or cells (6). The previous work on auxin signaling has focused mainly on development, and little is known about the specific role of the auxin receptors, ARF, and Aux/IAA proteins on plant responses to environmental cues.

Nitrogen (N) is an essential macronutrient and a limiting factor for agricultural productivity owing to its enormous impact on plant growth and development. In agricultural soils, the main N source available for plants is nitrate. Given the importance of nitrate as a plant nutrient, several studies have focused on the effect of nitrate treatment on gene expression. Nitrate is able to regulate more than 1,000 genes in roots and shoots, including genes involved in its transport, reduction, and assimilation as well as genes involved in hormone transduction pathways and diverse transcription factors, kinases, and phosphatases, among other genes (7, 8). A significant proportion of these genes are able to respond to nitrate directly, indicating that nitrate is the signal controlling their gene expression (9). However, the exact mechanisms by which nitrate is sensed and triggers changes in mRNA levels are not well understood. Auxin has been identified as an important player in the root response to nitrate. Nitrate is able to control lateral root growth by controlling the provision of auxin to lateral roots by the NRT1.1 nitrate transporter (10). In the absence of nitrate, NRT1.1 favors basipetal transport of auxin, preventing accumulation of auxin in the lateral root tip; in the presence of nitrate, this transport is inhibited, leading to accumulation of auxin and subsequent growth of laterals (10).

Lateral root growth in response to organic N has been shown to depend on miR167 and its target, the auxin response factor *ARF8* mRNA (11). This module acts specifically in the pericycle to control a connected network of genes, leading to induction of lateral root initiation and repression of lateral root elongation in response to N (11). We previously described another microRNA (miRNA)/target regulatory module consisting of miR393 and the auxin receptor AFB3 in root system architecture (RSA) control by nitrate (12). *AFB3* is induced by nitrate and repressed by miR393. miR393 is induced by N metabolites produced by nitrate reduction and assimilation (12). This regulatory network module is an example of a motif commonly present in regulatory networks of mammals, bacteria, and yeast known as the incoherent type I feed-forward loop (12).

In this work, we used integrated genomics, systems biology, and molecular genetics approaches to identify molecular mechanisms downstream of the miR393/*AFB3* module that lead to RSA modulation by nitrate. We found that AFB3 acts specifically in the context of the nitrate response regulating a connected gene network controlled by the NAC4 transcription factor. Phenotypical analysis of *nac4* mutants further implicates NAC4 in the lateral root response to nitrate in a pathway that requires AUX/IAA signaling.

Results

Transcriptomic Analysis of the Nitrate Response in *afb3-1* Mutant Plants Uncovers a Specific Role of AFB3 in the Nitrate Response of *Arabidopsis* Roots. AFB3 has been identified as an important regulator of primary root and lateral root modulation by nitrate (12). AFB3 controls auxin-responsive gene transcription by promoting protein degradation of the Aux/IAA transcriptional repressors in the presence of auxin (2, 13, 14). Thus, loss of AFB3 function would lead to changes in transcript abundance of its direct and indirect targets. To identify downstream targets of AFB3 in the context of the nitrate response, and to find molecular factors involved in primary and/or lateral root responses that are dependent on AFB3 regulation, we analyzed the transcriptome of the *afb3-1* mutant (1) in response to nitrate treatments and compared it with the transcriptome of WT plants. Plants were grown

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[1]To whom correspondence should be addressed. E-mail: rgutierrez@bio.puc.cl.

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with ammonium succinate as the only N source for 14 d and were treated at the beginning of the light period on day 15 with 5 mM KNO_3 or 5 mM KCl. Under these experimental conditions, AFB3 demonstrated a rapid and transient response to nitrate, with a peak of induction at 1 h after nitrate exposure, as described previously (12).

Because we anticipated that changes in transcript abundance in response to nitrate of downstream target genes would be delayed compared with AFB3, we treated plants with nitrate for 2 h for transcriptome analysis. Total RNA was isolated from roots and prepared for Affymetrix ATH1 GeneChip hybridization. Gene expression data were normalized using robust multiarray analysis (RMA) (15), and differential gene expression was determined using two-way ANOVA as described previously (16), considering the treatment (T) and genotype (G) as factors and controlling type I error using the false discovery rate (17). Our analysis identified 445 genes responding in our experiments, including 442 genes with a significant T factor, 38 genes with a significant G factor, and 39 genes with a significant TG interaction factor.

To simplify the analysis of our results and to provide an initial insight into how AFB3 regulates gene expression in response to nitrate, we analyzed our data using the Sungear tool (18) available at the VirtualPlant webpage (http://www.virtualplant.org) (19). Sungear allows for the visualization and analysis of multiple datasets to identify genes that are unique or are shared by different gene lists (18, 20). We used Sungear to generate a triangle representing the 445 genes with significant T, G, or TG factors (Fig. S1). Each vertex in the triangle represents a factor of the ANOVA model, and the circles inside the triangle (vessels) represent the number of genes controlled by the different factors, as indicated by the arrows around the vessels.

Using Sungear, we found that treatment was the sole significant factor for 390 genes (87.6% of the total genes regulated), indicating that the nitrate response of these genes was not altered by the *afb3* mutation under our experimental conditions. Analysis of the genes in this group suggested that basic N metabolic functions are not affected in the *afb3* mutant, given that several genes in the nitrate transport, reduction, and assimilation pathways responded similarly to the treatments in WT and *afb3* mutant plants (e.g., nitrate reductase genes *NIA1* and *NIA2*; nitrite reductase gene *NiR*; NADH-dependent glutamate synthase gene *GLT1*; glutamine synthetase genes *GSR2, GLN2*, and *GLN1;4*; and nitrate transporters *NRT1.1*, *NRT2.1*, and *NRT3.1*). This result suggests that modulation of RSA by AFB3 in response to nitrate does not depend on alterations of N transport or metabolism. Interestingly, we found no genes with G as the sole significant factor, indicating that under our experimental conditions, the effects of the *afb3-1* mutation were evident only in the context of the nitrate response. These results are consistent with a specific role of AFB3 in the root nitrate response of *Arabidopsis thaliana*.

Network Analysis Identifies a Highly Connected Nitrate-Responsive Regulatory Module Controlled by AFB3. To identify targets of AFB3, we focused on the 39 genes that showed an altered response to nitrate in the *afb3-1* mutant (i.e, significant TG factor). Altered expression of these genes could account for the altered root phenotype seen in the *afb3-1* mutants in response to nitrate (12). To predict possible regulatory relationships between these differentially regulated genes, we generated a network view using the Gene Networks tool available at the VirtualPlant Web page. This integrative network bioinformatics approach has been used and validated previously (21, 22). Cytoscape software (23) was used to visualize the network, in which genes are represented as nodes and the edges linking these nodes represent the functional relationships between nodes. Twenty-eight genes with a significant TG factor have predicted regulatory interactions and were included in our analysis. The genes were grouped into two gene networks: a small network containing six genes, basic helix–loop–helix transcription factor bHLH64 (24), five target genes, and a miRNA (Fig. S2) and a larger network including 22 genes, NAM/ATAF/CUC transcription factor NAC4, basic helix–loop–helix transcription factor bHLH128 (24), and zinc finger transcription factors OBP4 and SZF1, along with their predicted target genes (Fig. 1). We focused our analysis in the larger network because it contained most of the TG genes. Interestingly, NAC4 is predicted to target all of the genes in the large network by direct binding to their promoters or indirectly by controlling the OBP4 or bHLH128 transcription factors (Fig. 1); thus, NAC4 might be an important component of a coordinated regulatory network controlling root nitrate response downstream of AFB3.

NAC4 Transcription Factor Acts Downstream of AFB3 to Control Root Nitrate Responses. NAC4 is a member of a family of transcription factors present only in plants (25). Although NAC4 has no reported function, the closely related NAC1 and NAC2 factors are known to be involved in lateral root development in *Arabidopsis* (26, 27); thus, NAC4 represented an attractive candidate for mediating the effects of nitrate over RSA downstream of AFB3.

NAC4 is predicted to both positively or negatively regulate different genes in the network directly or indirectly by regulating expression of the *bHLH128* and *OBP4* transcription factors. bHLH128 and OBP4 target the remaining genes in the network, possibly accounting for most of the changes seen in our microarray experiment (Fig. 1). To validate our network predictions, we analyzed mRNA levels of *NAC4*, *bHLH128*, and *OBP4* over time after nitrate treatment in WT plants and in the *afb3-1* mutant using quantitative RT-PCR (qRT-PCR).

NAC4 exhibited an early peak of induction at 1 h after nitrate treatment in WT plants (Fig. 2). *NAC4* response over time was similar to the transient response of *AFB3* (12), as expected for an AFB3 target. No changes in RNA levels were detected in the *afb3-1* mutant even after 4 h of nitrate treatment. This result clearly indicates that AFB3 function is required for nitrate regulation of *NAC4*.

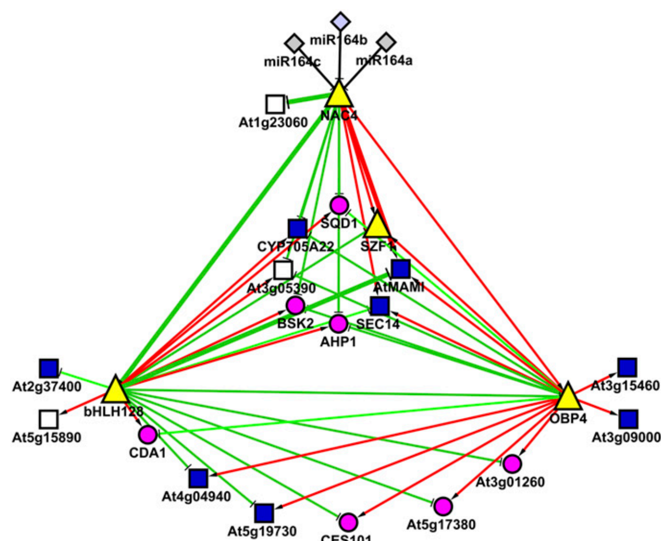

Fig. 1. A connected network of regulatory factors and their potential targets is differentially regulated in *afb3-1*. The nodes represent genes (gray squares, miRNA; purple circles, enzyme- coding genes; blue squares, protein-coding genes; white squares, unknown protein-coding genes; yellow triangles, transcription factor-coding genes), and the edges represent miRNA/*TARGET* regulation or predicted regulatory interactions based on the occurrence of a transcription factor-binding site on the gene promoter. Green edges represent repression, and red edges represent induction based on correlation analysis of our Affymetrix data.

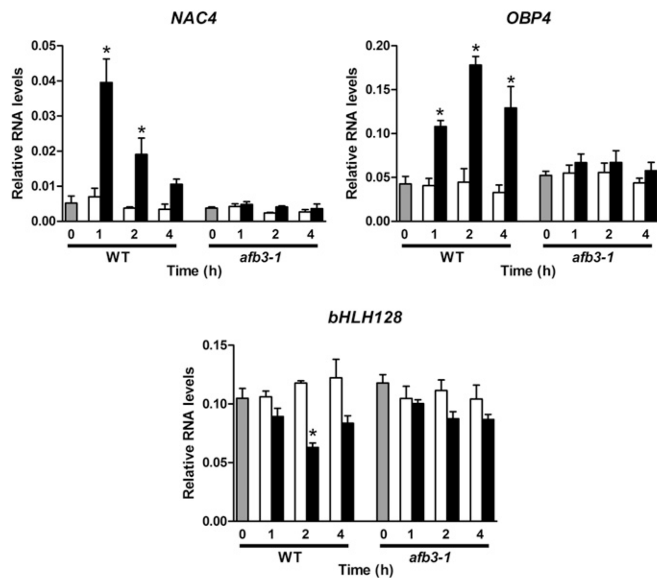

Fig. 2. Nitrate response of the *NAC4*, *OBP4*, and *bHLH128* transcription factors is altered in the *afb3-1* mutant. WT Ws and *afb3-1* mutant plants were grown in ammonium succinate for 2 wk and then treated with 5 mM KNO_3 or 5 mM KCl for 1, 2, and 4 h. RNA levels of the *NAC4*, *OBP4*, and *bHLH128* transcription factors were measured using qRT-PCR. Values are mean ± SE of three biological replicates. Gray bars represent time 0 (before treatment), white bars represent KCl treatment, and black bars represent KNO_3 treatment. Asterisks denote means that are statistically different between control and treatment ($P < 0.05$).

OBP4 and *bHLH128* exhibited transient responses to nitrate similar to *AFB3* and *NAC4* but delayed, with a peak of regulation at 2 h after treatment in WT plants (Fig. 2). However, in *NAC4*, the nitrate response of these transcription factors was seriously compromised in the *afb3-1* mutant (Fig. 2). Our qRT-PCR data are consistent with a model in which nitrate induction of *NAC4* triggers changes in the levels of *OBP4* and *bHLH128*.

We previously reported that *AFB3* is regulated directly by nitrate, given that *AFB3* is induced by nitrate in a nitrate reductase (NR)-null *A thaliana* mutant, *nia1*/*nia2* (12). Considering that our network predicts that *NAC4*, *OBP4*, and *bHLH128* are acting downstream of AFB3, we expected these transcription factors to also be regulated by nitrate directly as a signal. We analyzed the expression of NAC4, OBP4 and bHLH128 in *nia1*/*nia2* plants after nitrate treatments using qRT-PCR. These transcription factors were still regulated by nitrate in the NR-null mutant, indicating that, as AFB3, they respond to nitrate and not to N metabolites generated by nitrate reduction or assimilation (Fig. S3*A*). Accordingly, nitrite or ammonium treatments had no effect on *NAC4*, *OBP4*, and *bHLH128* mRNA levels (Fig. S3 *B* and *C*). *NAC4*, *OBP4*, and *bHLH128* mRNA levels in the NR-null mutant differed from those described in the WT plant after 4 h of treatment (compare Fig. 2 and Fig. S3*A*), suggesting a complex regulation of these transcription factor levels by both nitrate and N metabolites other than nitrate. We found a similar regulation for AFB3, which is transcriptionally induced by nitrate and posttranscriptionally repressed by N metabolites produced by N reduction/assimilation.

To validate *OBP4* and/or *bHLH128* as NAC4 targets in the context of the nitrate response, we analyzed two *nac4* T-DNA insertion mutant lines obtained from the *Arabidopsis* Biological Resource Center, *nac4-1* (SALK_040204) and *nac4-2* (SALK_006735). We measured *OBP4* and *bHLH128* mRNA levels in WT plants and in the *nac4-1* and *nac4-2* mutants over time after nitrate treatment. *bHLH128* response was not affected in the mutants, indicating that this gene is repressed by AFB3 in a NAC4-independent manner; however, *OBP4* response was altered in *nac4* mutants, indicating that this transcription factor acts downstream of NAC4 (Fig. 3).

NAC4 Regulates Lateral Root Responses to Nitrate Downstream of AFB3. Based on our network analysis predicting NAC4 as an important regulator of the AFB3 regulatory network, we wished to determine whether this transcription factor is involved in primary and/or lateral root responses to nitrate as AFB3. To evaluate the impact of NAC4 in RSA modulation, we grew plants under the experimental conditions used to analyze the effect of AFB3 in RSA (12). Because in our previous work we used a medium containing 1× Murashige and Skoog (MS) salts without N, and because under some experimental conditions the salt concentration in this medium can inhibit root growth (28), we also performed the experiments using 0.5× and 0.2× MS salts (Fig. S4). We found no major differences between plants grown on 1× MS salt and those grown on 0.5× MS salt. However, plant growth was affected in 0.2× MS salt (Fig. S4 *A* and *B*), likely owing to the limiting nutrient concentration in this medium under our experimental conditions.

We measured primary root length and lateral root density, two parameters affected by the *afb3* mutation (12), after KNO_3 or KCl treatment in WT Col-0 and *nac4* mutants. Given the previously reported effects of nitrate on lateral root elongation (29–32), we also measured lateral root length after KNO_3 or KCl treatment. We found no significant differences between the KCl and KNO_3 conditions in WT or *nac4* plants (Fig. S4 *E* and *F*); thus, nitrate treatment had no effect on visible lateral root length under our experimental conditions. The inhibitory effect of nitrate on primary root elongation was not affected by the *nac4* mutation (Fig. 4*A*), indicating that this response occurs through an AFB3-dependent signaling pathway that does not involve NAC4.

We have previously shown that repression of root growth by nitrate correlates with an induction of AFB3 in root tips (12). Our analysis of *AFB3* regulation in the meristematic zone and in the elongation zone of the primary root revealed that after 2 h of nitrate treatment, *AFB3* was induced in the meristematic zone but not in the elongation zone (Fig. S5). However, our analysis of the nitrate response of NAC4 and OBP4 in meristematic and elongation zones found that these factors did not respond to the treatments (Fig. S5), in accordance with our results showing that *nac4-1* has no primary root phenotype in response to nitrate. However, the effect of nitrate over lateral root density was altered in the *nac* mutants (Fig. 4*B*).

We evaluated the density of initiating and emerging lateral roots using differential interference contrast (DIC) microscopy as described previously (12). We found that in WT plants, nitrate

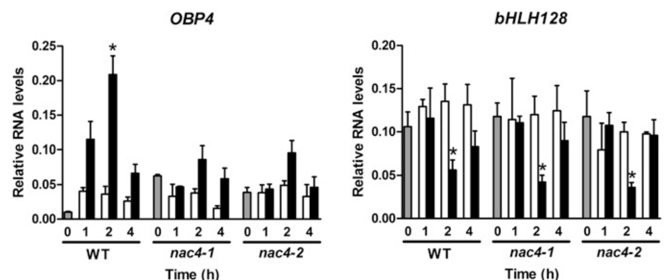

Fig. 3. Nitrate response of the *OBP4* transcription factor is altered in *nac4* mutants. WT Col-0, *nac4-1*, and *nac4-2* mutant plants were grown in ammonium succinate for 2 wk and then treated with 5 mM KNO_3 or 5 mM KCl for 1, 2, and 4 h. RNA levels of the *OBP4* and the *bHLH128* transcription factors were measured using qRT-PCR. Values are mean ± SE of three biological replicates. Gray bars represent time 0 (before treatment), white bars represent KCl treatment, and black bars represent KNO_3 treatment. Asterisks denote means that are statistically different between the KCl and KNO_3 treatments ($P < 0.05$).

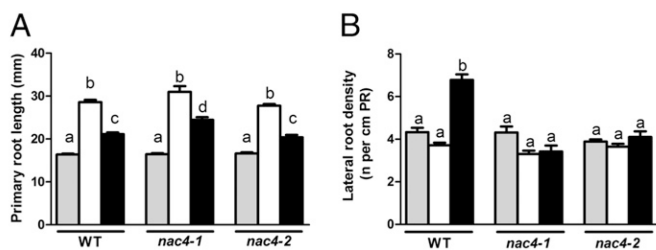

Fig. 4. NAC4 is involved in lateral root development, but not in primary root development, in response to nitrate treatment. (*A*) Primary root lengths of Col-0 WT plants and *nac4-1* and *nac4-2* mutant plants were measured using ImageJ at day 0 (gray bars) and after 3 d of treatment with 5 mM KCl (white bars) or 5 mM KNO_3 (black bars). (*B*) The number of lateral roots (initiating and emerging) of Col-0 WT and *nac4-1* and *nac4-2* mutant plants at day 0 (gray bars), treated for 3 d with 5 mM KCl (white bars) or 5 mM KNO_3 (black bars), was counted using DIC optics. Values are mean ± SE of three biological replicates (n = 15). Different letters represent means that are statistically different ($P < 0.05$).

treatment increased the density of initiating and emerging lateral roots, but this response was altered in the *nac4* mutants (Fig. S6), suggesting a specific role for NAC4 in controlling lateral root initiation and emergence in response to nitrate. This root phenotype is similar to the *afb3-1* phenotype that we described previously (12), further supporting NAC4's function downstream of the AFB3 auxin receptor.

Nitrate treatment regulates *AFB3* expression in pericycle cells in accordance with AFB3's role in mediating changes in lateral root growth in response to nitrate (12). Considering that *NAC4* and *OBP4* are regulated by AFB3, we expected to find these factors spatially coregulated with AFB3. We analyzed the expression of AFB3, NAC4, and OBP4 in different cell types of the root, using GFP-expressing lines and fluorescence-activated cell sorting after 2 h of nitrate treatment. AFB3 was induced in pericycle cells after 2 h of nitrate treatment (Fig. S7). This result complements our previous findings using a pAFB3:GUS line (12). We found a similar nitrate induction in pericycle cells for NAC4 and OBP4 (Fig. S7), suggesting that AFB3-NAC4-OBP4 might represent a regulatory module that acts specifically in the pericycle to control lateral root growth in response to changes in nitrate availability.

Nitrate Regulation of NAC4 Depends on AUX/IAA Signaling Function. Nitrate-specific induction of *AFB3* in roots might control a specific combination of Aux/IAA and ARF factors that control *NAC4* induction and lateral root growth. In *Arabidopsis*, lateral root development depends on multiple Aux/IAA-ARF modules that act in sequential steps to generate new lateral roots. Lateral root founder cell specification occurs in a zone between the meristem and elongation zone called the basal meristem and depends on IAA28 and ARF proteins that might include ARF5, ARF6, ARF7, ARF8, and ARF19 (33-35). The IAA14(SLR)-ARF7-ARF19 module operates in the zone above the basal meristem and regulates the coordinated nuclear migration and posterior asymmetric cell division of the founder cells for lateral root initiation (36–38). IAA12(BDL)-ARF5(MP) also activates lateral root formation, acting after the IAA14(SLR)-ARF7-ARF19 module (39).

To determine whether these IAA-ARF modules participate upstream of NAC4, we analyzed the *NAC4* response to nitrate in plants with altered function of these factors. We found that only the IAA14/SLR gain-of-function mutation altered NAC4 response to nitrate (Fig. 5 and Fig. S8). Accordingly, IAA14 is expressed in the xylem pole pericycle cells (38). This suggests that normal AUX/IAA-mediated auxin signaling in pericycle cells is required for induction of NAC4 by nitrate. We found that the OBP4 response was also altered in the *slr-1* mutant, supporting its role as an NAC4 target acting in the AFB3-NAC4 pathway in the pericycle (Fig. 5). As expected, the nitrate response of *bHLH128*, which is not a NAC4 target, was not altered in this mutant (Fig. 5).

In summary, our work has identified important components of an AFB3-dependent network that controls lateral root growth in response to changes in nitrate availability (Fig. S9). These factors are specifically regulated in pericycle cells and act downstream of AUX/IAAs for lateral root development.

Discussion

Auxin controls key aspects of plant growth and development and is specifically important for the control of root growth and development (5, 40, 41). These changes in root morphology are caused mainly by changes in gene expression triggered by auxin (41). Although the mechanism by which auxin is able to increase or decrease mRNA levels of auxin-responsive genes has been characterized, the specific combinations of auxin receptors, Aux/IAAs, ARFs, and underlying regulatory networks controlling auxin dependent processes remain incompletely understood. In this work, we used an integrated approach including genomics, bioinformatics, and molecular genetics to identify the molecular networks acting downstream of the miR393/*AFB3* nitrate-responsive regulatory module in RSA modulation in response to nitrate.

To determine which nitrate-responsive genes were affected by the *afb3-1* mutation, leading to the changes in RSA response observed in this mutant (12), we performed a transcriptomic analysis of WT and *afb3-1* nitrate-treated and control plants. Most of the genes responded to nitrate independent of *AFB3*, indicating that the *afb3-1* mutation affects only a small proportion of root genes regulated by nitrate. Genes with a T-only model included those involved in nitrate transport, reduction, and assimilation, suggesting that the RSA response to nitrate is related to a signaling effect, not to a metabolic effect of nitrate. The absence of genes with a G-only model indicates that there are no genes whose basal expression depends on *AFB3*, and that under our experimental conditions gene expression alterations are detectable only in a nitrate-dependent context. This observation is in agreement with a previous phenotypic analysis of *afb3-1*

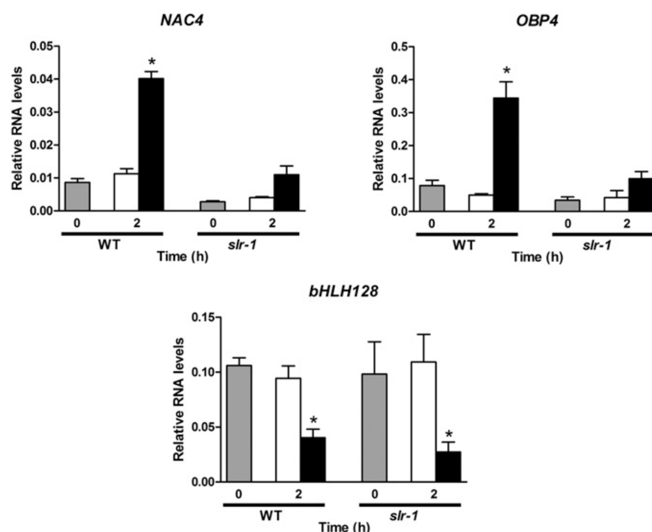

Fig. 5. Nitrate response of *NAC4* and its target OBP4 is altered in *IAA14/SLR* gain-of-function mutant *slr-1*. WT Col-0 and *slr-1* mutant plants were grown in ammonium succinate for 2 wk and then treated with 5 mM KNO_3 or 5 mM KCl for 2 h. RNA levels of the *NAC4*, *OBP*, and *bHLH128* transcription factors were measured using qRT-PCR. Values are mean ± SE of three biological replicates. Gray bars represent time 0 (before treatment), white bars represent KCl treatment, and black bars represent KNO_3 treatment. Asterisks represents means that are statistically significantly different between control and treatment ($P < 0.05$).

showing that this mutant has no visible root phenotype when grown under standard conditions (1). This is likely related to functional redundancy between the TIR1/AFB auxin receptors in plant development. Consistently, a RSA phenotype was evident under our experimental conditions only when *afb3-1*, and not the other auxin receptor mutants, was treated with 5 mM KNO_3 (12), supporting an *AFB3*-specific role in root nitrate response.

Given our focus on identifying nitrate regulatory networks affected in the *afb3-1* mutant that might explain the altered lateral and/or primary root response to nitrate, we centered our analysis on genes exhibiting an altered nitrate response in this mutant. Network analysis of the TG genes showed that the genes formed two networks composed of transcription factors and their putative targets. The larger network, containing most of the altered genes, is predicted to have a main regulator, *NAC4*. This transcription factor is part of the plant-specific NAM/ATAF/CUC family of transcription factors but has no reported function on plant development. Our network predicted that NAC4 could regulate the genes included in its network either directly or by regulating the OBP4 or bHLH128 transcription factors. We found that *NAC4*, *OBP4*, and *bHLH128* are regulated directly by nitrate as a signal. This was expected based on our previous finding that AFB3 is also induced directly by nitrate (12). Continuous exposure to nitrate in the *nia1*/*nia2* mutant caused altered expression of *NAC4*, *OBP4*, and *bHLH128* compared with their response over time in WT plants. This suggests that N metabolites other than nitrate modulate the expression of these transcripts after their initial nitrate response, integrating signals from both external and internal N availability. This is similar to the regulation of *AFB3* by miR393, in which N metabolites induce this miRNA to decrease AFB3 levels (12).

We found that NAC4 was necessary for the nitrate response of *OBP4* gene, but not for the response of the *bHLH128* transcription factor. Considering we verified that AFB3 controls *bHLH128* nitrate response, a possible explanation for this finding is that AFB3 controls *bHLH128* directly through an AuxIAA/ARF mediated pathway independent of NAC4. Consistently, *bHLH128* has two ARF-binding sites in its promoter according to the Arabidopsis Gene Regulatory Information Server (AGRIS) (http://arabidopsis.med.ohio-state.edu).

We found that NAC4 acts downstream of AFB3 to mediate the lateral root response to nitrate. NAC1 and NAC2 also have been implicated in lateral root responses to auxin (26, 27). Thus, NAC4 also works in an auxin-related pathway by regulating lateral root growth, but in the context of the nitrate response. As we reported previously, NAC4 is necessary for the nitrate response of *OBP4*. Similar to NAC4, OBP4 does not have a reported function in plant development. Other members of the OBP family—the OBP1, OBP2, and OBP3 proteins—are able to interact with the OBF4 protein (also known as TGA4), and have been identified as salicylic acid- and auxin-responsive (42). Lateral root initiation is known to be controlled by auxin through activation of the asymmetrical division of precursor xylem-pole pericycle cells (43). Thus, the nitrate-AFB3-NAC4-OBP4 pathway might transduce the auxin signal into activation of lateral root initiation by controlling cell cycle progression in the pericycle. Auxin control of the cell cycle involving changes in TIR1 auxin receptor levels in the pericycle has been reported in lateral root initiation in response to low phosphate (44). Furthermore, OBP1 is involved in cell cycle regulation (45). Our mechanism does not preclude other possible mechanisms contributing to the developmental effects of NAC4, such as control of other hormonal signaling pathways. We have found that components of the cytokinin and brassinosteroid pathways have an altered nitrate response in the *afb3-1* mutant, and these hormones are known to modulate RSA in *Arabidopsis* (46–51).

IAA14/SLR is considered a master regulator of lateral root initiation, controlling the initial pericycle cell divisions that give birth to new roots (36, 52). We found that a IAA14/SLR gain-of-function mutation altered NAC4's response to nitrate in *Arabidopsis* roots. IAA14 is known to interact with the ARF factors ARF7 and ARF19 to regulate lateral root initiation (39, 52); however, we found no altered NAC4 response in *arf7-1*, *arf19-1*, or *arf7-1*/*arf19-1* mutants. This may suggest that in the context of the nitrate response, IAA14 is able to interact with a different set of ARF factors to control NAC4 expression. Alternatively, the accumulation of IAA14 protein caused by the lack of repression by the auxin receptors might lead to an unspecific binding to ARF factors that normally would not interact with IAA14. In either case, we can conclude that normal AUX/IAA-dependent signaling, possibly IAA14 itself, is required for the proper regulation of NAC4 and its target OBP4.

AFB3 is also able to repress primary root growth in response to nitrate. We found that NAC4 was not involved in this response, with the *nac4* primary root demonstrating a normal nitrate response. Consistently, we did not find regulation of *NAC4* or *OBP4* in root tips, although *AFB3* is regulated in the meristematic zone. Given that IAA14 is expressed primarily in xylem pole pericycle cells (53), the lack of Aux/IAA expression in root tips might explain the absent nitrate response of *NAC4* and *OBP4* even when *AFB3* is induced in this root zone. Thus, AFB3 regulation of different regulatory networks in root tips and pericycle might independently control the growth of lateral and primary roots, finely modulating RSA in response to external and internal N availability.

Methods

Plant Material and Growth Conditions. WT *A thaliana* ecotypes Ws and Col-0 were used in these experiments. The *afb3-1* mutant (1), *arf7-1*, *arf7-1*/*arf19-1* double mutant (54), *arf6-2*, *arf8-3* (55), and *slr-1* gain-of-function mutant (36) were kindly donated by Dr. Mark Estelle, Indiana University, Bloomington, IN. The *iaa28-1* (35) mutant was kindly donated by Dr. Bonnie Bartel, Rice University, Houston. *arf19-1* (CS24617) (54), *iaa12-1*(CS25213) (56), *mp-S319* (SALK_021319) (57), *nac4-1* (SALK_040204), and *nac4-2* (SALK_006735) were obtained from the *Arabidopsis* Biological Resource Center. GFP lines that mark the epidermis (pWER:GFP), cortex (pAT1G09750:GFP), endodermis (pSCR:GFP), and stele (pWOL:GFP) were kindly donated by Dr. Phil Benfey, Duke University, Durham, NC. The GFP line that marks the pericycle (E3754) was obtained from http://enhancertraps.bio.upenn.edu. The *nia1*/*nia2* mutant line (58) was kindly donated by Dr. Nigel Crawford, University of California at San Diego, La Jolla, CA.

Plants were grown in hydroponic cultures using modified MS basal salt medium without N (M531; Phytotechnology Laboratories), supplemented with 0.5 mM ammonium succinate and 0.1% sucrose. The plants were grown for 14 d under long-day conditions at 22 °C in Percival incubators. The plants were treated at the onset of the light cycle of the 15th day as indicated in the figure legends. For primary and lateral root length measurements, plant images were acquired using an Epson Perfection V700 scanner, and roots were measured using the ImageJ program (http://rsbweb.nih.gov/ij/). Lateral roots were counted using DIC optics on a Nikon Eclipse 80i microscope.

Gene Expression Using Affymetrix ATH1 Microarrays and Network Data Analysis. Biotinylated cRNA was synthesized from 5 μg of total RNA from *Arabidopsis* roots using the Affymetrix IVT Kit according to the manufacturer's instructions. cRNA was used to hybridize ATH1 GeneChip expression microarrays. Affymetrix data were normalized in R (http://www.r-project.org/) using RMA (15). Normalized data were subjected to two-way ANOVA, with a false discovery rate of 5%. For the ANOVA, we used a model considering the expression of a given gene Y as $Yi = \beta 0 + \beta 1T + \beta 2G + \beta 3TG + \varepsilon$, where $\beta 0$ is the global mean; $\beta 1$, $\beta 2$, and $\beta 3$ are the effects of T, G, and the TG interaction; and the variable ε is the unexplained variance. The data were analyzed with bioinformatics tools available at the VirtualPlant Web site (http://www.virtualplant.org).

Genes containing significant T, G, or TG factors were analyzed using the Sungear tool, and a molecular network for genes with a significant TG factor was created using the Gene Networks tool. The network includes miRNA–RNA interactions from miRBase (http://www.mirbase.org) and the protein/DNA regulatory interactions from AGRIS (http://arabidopsis.med.ohio-state.edu). To improve the regulatory predictions, the protein–DNA interactions were filtered to include only transcription factor/target pairs with significantly correlated ($P \leq 0.05$) expression values of ≥0.7 or ≤−0.7 in our data. Network modeling was performed using Cytoscape software (23).

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