Nitrate-responsive miR393/*AFB3* regulatory module controls root system architecture in *Arabidopsis thaliana*

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One of the most striking examples of plant developmental plasticity to changing environmental conditions is the modulation of root system architecture (RSA) in response to nitrate supply. Despite the fundamental and applied significance of understanding this process, the molecular mechanisms behind nitrate-regulated changes in developmental programs are still largely unknown. Small RNAs (sRNAs) have emerged as master regulators of gene expression in plants and other organisms. To evaluate the role of sRNAs in the nitrate response, we sequenced sRNAs from control and nitrate-treated *Arabidopsis* seedlings using the 454 sequencing technology. miR393 was induced by nitrate in these experiments. miR393 targets transcripts that code for a basic helix-loop-helix (bHLH) transcription factor and for the auxin receptors TIR1, AFB1, AFB2, and AFB3. However, only *AFB3* was regulated by nitrate in roots under our experimental conditions. Analysis of the expression of this miR393/*AFB3* module, revealed an incoherent feed-forward mechanism that is induced by nitrate and repressed by N metabolites generated by nitrate reduction and assimilation. To understand the functional role of this N-regulatory module for plant development, we analyzed the RSA response to nitrate in *AFB3* insertional mutant plants and in miR393 overexpressors. RSA analysis in these plants revealed that both primary and lateral root growth responses to nitrate were altered. Interestingly, regulation of RSA by nitrate was specifically mediated by *AFB3*, indicating that miR393/*AFB3* is a unique N-responsive module that controls root system architecture in response to external and internal N availability in *Arabidopsis*.

nitrogen | microRNA | auxin | feed-forward mechanism

Nitrate is one of the major forms of inorganic nitrogen in the biosphere and nitrate availability is the most limiting factor for plant growth and agricultural productivity. Besides its role as a nutrient, nitrate (as well as other N metabolites) has been shown to act as a signal that regulates global gene expression (1–5). Although genomic data show that the nitrate response is global, little is known about the molecular basis of nitrate sensing and signaling and how the transcriptomic changes can result in developmental responses. In the last years, microRNAs (miRNAs) have emerged as master regulators of gene expression in plants and other systems (6–8). miRNAs are small ~21–22 nt molecules, that play critical roles in various developmental, stress, and signaling responses (reviewed in refs. 9, 10). Microarray analysis showed that target transcripts of miRNAs are regulated by nitrate and/or sucrose treatments in *Arabidopsis* roots (4), suggesting that posttranscriptional gene expression control by miRNAs can be a general mechanism integrating nitrate signals into developmental changes. miRNAs have been known for years to be important for phosphate and sulfate deprivation responses in plants (11–13). More recently, miR167 and its target *ARF8* were shown to be part of an organic N-responsive regulatory network that controls lateral root initiation in *Arabidopsis* (14) and N- and P-limitation regulated miRNAs have been identified in *Arabidopsis* seedlings (15). In this work, we used 454 sequencing to detect N-regulated miRNAs and we identified a nitrate responsive miRNA/target regulatory module that integrates N and auxin signaling to control root system architecture (RSA) in response to changes in nitrate availability.

Results

Identification of Nitrate-Responsive sRNAs in *Arabidopsis* Roots. As a first approximation to evaluate the contribution of sRNAs to the nitrate response in *Arabidopsis*, we used the 454 sequencing technology to identify nitrate responsive sRNAs (16). Sequencing approaches have been shown to provide accurate estimates of transcript levels (17, 18) and to allow for the discovery of miRNAs [or other sRNAs such as small interfering RNAs (siRNAs) not previously identified (19–24)]. Previous genomic analyses of the nitrate and sucrose response in *Arabidopsis* roots have shown that most of the previously identified nitrate-responding genes are in fact regulated by some type of carbon (C)/nitrogen (N) interaction (4). Therefore, we chose to perform a combined nitrate/sucrose treatment to maximize N-responding sRNA discovery. *Arabidopsis* plants were grown hydroponically in basal MS media without N, supplemented with 1 mM ammonium as sole N source and 3 mM sucrose for 2 weeks, and were then treated with 5 mM KNO_3 and 30 mM sucrose (treatment) or with 5 mM KCl and 30 mM mannitol (control) for 20 min and for 2 h. These experimental conditions have been shown to elicit a robust transcriptomic response in previous studies (1, 4). Total RNA was extracted from seedlings and the small RNA fraction was isolated for 454 sequencing (16, 25). In a pilot experiment, we pooled the two time points and obtained ~16,000 sequences from the treatment and control samples. The raw sequence data were processed with custom made PERL scripts, mapped to the *Arabidopsis* genome, and a list of sRNAs was generated with the normalized frequency of occurrence in the control and treated samples. Using a fourfold difference cutoff between the normalized frequency in the treatment and control samples, we identified miR393 as the only sRNA induced by the treatment in these experiments. miR393 targets the transcripts that code for a basic helix-loop-helix (bHLH) transcription factor (bHLH77, ref. 26) and the auxin receptors TIR1, AFB1, AFB2, and AFB3 (13, 27, 28). Auxin is a key phytohormone, mediating growth and

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developmental responses in plants (29). Auxin has been proposed as a long-range signal from shoot to root mediating root developmental responses to nitrate (30, 31) and is clearly important based on network analysis of nitrate-regulated genes (4). Thus miR393 was an attractive candidate to mediate developmental plant responses to nitrate. According to our sequencing results, miR393 was induced by the treatment with a $\log_2$ (treatment normalized expression/control normalized expression) of 3.3. To corroborate our results, and to better define the timing and organ regulation of miR393, we used a modified Northern blot procedure to analyze the regulation of this miRNA in shoot and root tissue after 20 min and and 2 h of nitrate plus sucrose treatment. We found miR393 to be induced by the treatment specifically in root tissue, after 2 h of treatment (Fig. S1). To define whether miR393 was responding to nitrate or to a combined nitrate/sucrose effect, we subjected the plants to an N-only treatment (5 mM KNO_3 or 5 mM KCl). miR393 was regulated similarly by nitrate treatments in the absence of sucrose, indicating that miR393 responds to nitrate independently of external sucrose levels (Fig. 1). These results prompted the hypothesis that nitrate regulation of miR393 in roots controls root auxin receptor levels, which is important for root morphological changes in response to nitrate.

The miR393/*AFB3* Module Is a Unique N-Regulatory Network Integrating External and Internal N Availability. To evaluate the effect of miR393 regulation over the transcript levels of its targets, we analyzed the expression of *bHLH77* and of the auxin receptors in *Arabidopsis* roots after nitrate treatments. Plants were grown for 2 weeks in ammonium as sole N source and were treated with 5 mM KNO_3 or 5 mM KCl as control for 1, 2, and 4 h. Transcript levels for *bHLH77*, *TIR1*, *AFB1*, *AFB2*, and *AFB3* were analyzed using real-time quantitative reverse transcription PCR (qRT-PCR). As shown in Fig. 1, nitrate treatment did not affect *bHLH77*, *TIR1*, *AFB1*, or *AFB2* transcript levels in roots. However, *AFB3* was induced in root organs with a peak 1 h after exposure to nitrate (Fig. 1). Interestingly, we found that *AFB3* mRNA levels decreased rapidly over time, suggesting active transcript degradation. As expected, miR393 shows a peak of accumulation 2 hours after nitrate treatment, just as the transcript levels of *AFB3* begin to decrease (Fig. 1). Analysis of *AFB3* expression in a miR393 overexpressor line (28) showed that *AFB3* levels are diminished in comparison with wild-type plants (Fig. 2*A*). In addition, we found a fragment corresponding to a miR393 *AFB3* cleavage product (Fig. 2*B*) 2 h after KNO_3 treatment using a modified RNA ligase-mediated 5′ rapid amplification of cDNA ends (RLM-RACE) procedure (28, 32). Moreover, we compared the kinetics of mRNA accumulation in *afb3* mutant plants expressing a miR393-resistant version of *AFB3* under the control of the endogenous *AFB3* promoter (*pAFB3:mAFB3-GUS*) (33) (Fig. 2*C*). In contrast to the rapid decrease of *AFB3* mRNA levels seen in wild-type plants, *AFB3* levels did not decrease over time in *pAFB3:mAFB3-GUS* plants after nitrate induction (Fig. 2*C*). These results indicate that miR393 specifically cleaves *AFB3* transcripts under our experimental conditions, controlling *AFB3* mRNA accumulation in roots in response to nitrate exposure.

Nitrate in roots can be converted to other inorganic and organic N metabolites such as ammonium and the amino acids glutamate and glutamine. To test whether miR393 or *AFB3* were responding directly to nitrate or to N metabolites produced by nitrate reduction and/or assimilation, we used a nitrate reductase (NR)-null mutant of *Arabidopsis thaliana* (34). This mutant plant is unable to reduce nitrate, therefore genes responding to nitrate treatments in the NR-null mutant are controlled by nitrate and not by N metabolites produced after nitrate reduction. In the NR-null mutant, *AFB3* was induced by nitrate treatments with a peak of mRNA accumulation 1 h after the treatment (Fig. 3), similar to what was observed in wild-type plants (Fig. 1). However, *AFB3* levels did not decline over time in the NR-null mutant (Fig. 3) as compared to wild type (Fig. 1). As expected, this lack of repression over time, correlated with the lack of induction of miR393 in the NR-null mutant (Fig. 3). These results indicate that the induction of *AFB3* gene expression is caused by a nitrate signal (likely acting at the transcriptional level) but the downregulation seen at later times is caused by miR393 induction by an N metabolite downstream of nitrate reduction. To determine possible N signals controlling miR393, we tested the regulation of miR393 by ammonium and glutamate, N sources downstream of nitrate reduction and assimilation. Both N sources caused an increase in mature miR393 levels after 2 h of treatment (Fig. S2 *A* and *B*). These results

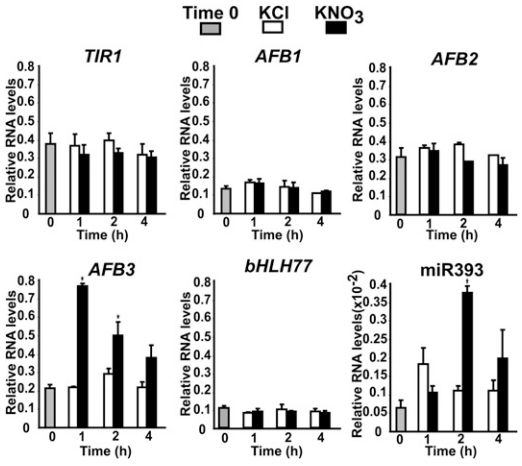

Fig. 1. Nitrate consistently regulates miR393 and its target *AFB3* in *Arabidopsis* roots. Plants were grown hydroponically for 14 days with ammonium as the sole N source and were treated with 5 mM KNO_3 or 5 mM KCl for the times indicated. Root transcript levels for *bHLH77*, *AFB1*, *AFB2*, *AFB3*, and mature miR393 were analyzed by real-time qPCR. We show the mean and standard error for three biological replicates. The asterisk indicates means that significantly differ between the control and treatment conditions ($P < 0.01$).

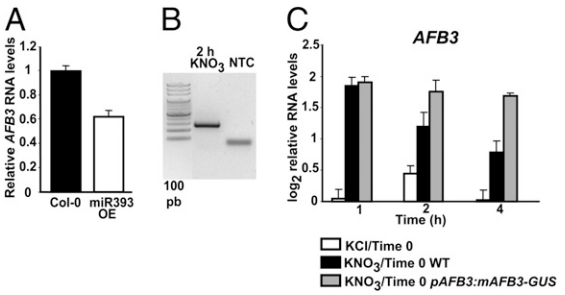

Fig. 2. *AFB3* transcript is cleaved by miR393 in response to nitrate. (*A*) Wild-type Col-0 plants and miR393 overexpressor plants (28) were grown in 0.5× MS salts supplemented with 30 mM sucrose in Petri dishes for 14 days. *AFB3* levels were analyzed in seedlings using qPCR. We show the mean and standard error for three biological replicates. (*B*) Plants were grown hydroponically for 14 days with ammonium as the sole N source and were treated with 5 mM KNO_3 for 2 h. Poly(A)$^+$ RNA was extracted from roots and a modified RLM-RACE procedure was used to amplify a miR393 cleavage product from *AFB3* (28). NTC, no template control. (*C*) *pAFB3:mAFB3-GUS* plants (33) were grown as described in *B* and were treated with 5 mM KNO_3 for the times indicated. *AFB3* transcript levels in roots were analyzed by real-time qPCR. Values are presented as the $\log_2$ ratio between the treatment level and the time 0 levels. As a reference, we also present the *AFB3* transcript levels in wild-type plants from Fig. 1.

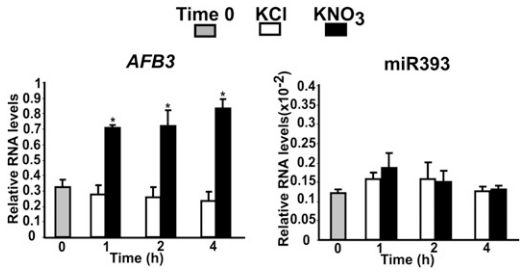

Fig. 3. *AFB3* is directly induced by nitrate and is under posttranscriptional regulation by N metabolites produced after nitrate reduction by a pathway involving miR393. Nitrate reductase-null mutant plants (34) were grown hydroponically as described before and were treated with 5 mM KNO_3 or 5 mM KCl for the times indicated. *AFB3* transcript levels and mature miR393 levels were analyzed by real-time qPCR in roots. We show the mean and standard error for three biological replicates. The asterisk indicates means that significantly differ between control and treatment conditions ($P < 0.01$).

indicate that miR393 responds to N signals produced after nitrate reduction and assimilation and acts as a negative feedback loop regulating *AFB3* levels over time according to external and internal N availability.

Nitrate Regulates Primary Root Growth by a Pathway Involving the AFB3 Auxin Receptor. To understand the function of the miR393/*AFB3* regulatory module in the nitrate response, we first analyzed the expression of this auxin receptor in roots after nitrate treatments. We used a previously described reporter line expressing the β-glucuronidase (GUS) reporter gene fused to a 1,800-bp sequence upstream of the *AFB3* transcription initiation site, *pAFB3::GUS* (35). We treated the reporter lines with 5 mM KNO_3 or 5 mM KCl for 1 h and we stained for GUS activity. We found that the *AFB3* promoter is able to drive expression of GUS throughout the root in both KNO_3- and KCl-treated roots, indicating root expression of the *AFB3* gene as previously described (35). However, the nitrate treatment increased GUS activity preferentially in the root tip area (Fig. 4*A*), indicating that *AFB3* mRNA accumulation by nitrate is due to transcriptional activation. To confirm these qualitative GUS results, we analyzed *AFB3* RNA levels in the root tip using qRT-PCR. *AFB3* was induced after 1 h of treatment in root tips (Fig. 4*B*). To evaluate whether this *AFB3* induction correlated with increased auxin activity in the root tip, we analyzed GUS activity in the DR5::GUS reporter line (36). We found that GUS activity was also increased in the DR5::GUS line, indicating increased auxin response in the root tip in the nitrate-treated plants (Fig. 4*C*). Consistent with this result, we found that nitrate is able to regulate auxin-responsive genes in wild-type roots in our experimental conditions (Fig. S3*A*). In addition, we also found regulation of auxin-related genes that are not reported to be auxin responsive, such as the auxin response factors *ARF9* and *ARF18* and an auxin efflux carrier (*At2g17500*) (Fig. S3*B*). These results suggest that nitrate is able to modulate auxin signaling and responses at multiple levels as previously described (4).

Auxin is known to control primary root growth in a concentration-dependent manner (37). Thus, the observed nitrate-induced auxin activity through AFB3 may lead to a repression in primary root growth in the nitrate condition. To test this hypothesis, we subjected wild-type plants and the *AFB3* T-DNA insertional mutant *afb3-1* (35) to a 3-day KNO_3 or KCl treatment. We measured the primary root length of plants grown for 2 weeks on ammonium as sole N source and after 3 days of 5 mM KNO_3 or KCl treatment. At the end of the 3-day treatment, we found that nitrate-treated wild-type plants have shorter primary roots as compared with control-treated plants (Fig. 4*D*), indicating that nitrate availability inhibits primary root elongation.

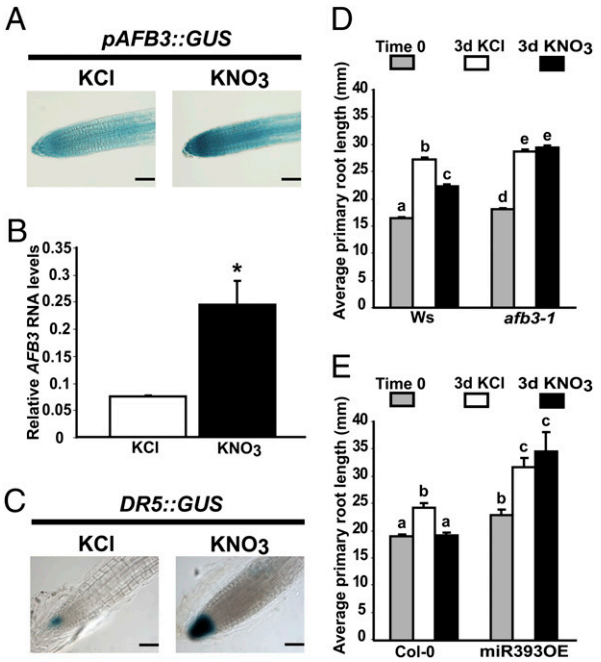

Fig. 4. Nitrate regulates primary root growth by a pathway involving *AFB3*. Plants were grown hydroponically as described before and were treated for the times indicated. (*A*) *pAFB3::GUS* plants (35) were treated for 1 h with 5 mM KNO_3 or KCl and were then stained for GUS activity for 4 h. Qualitative GUS staining was analyzed using DIC optics. Photographs are representative of at least 15 stained plants. (Scale bar, 100 μm.) (*B*) Wild-type plants were treated for 1 h with 5 mM KNO_3 or KCl. Root tips were excised from nitrate-treated or control-treated plants and *AFB3* RNA levels were measured using qPCR. Bars represent SE. The asterisk represents means that significantly differ ($P < 0.01$). (*C*) Auxin reporter DR5::GUS plants (36) were treated for 1 h with 5 mM KNO_3 or KCl and were then stained for GUS activity for 12 h. Qualitative GUS staining was analyzed using DIC optics. Photographs are representative of at least 15 stained plants. (Scale bar, 100 μm.) (*D*) Primary root length of Ws wild-type plants or *afb3-1* mutant plants was measured using the ImageJ program after 3 days of 5 mM KNO_3 or KCl treatment. Bars represent standard errors. Different letters represent significantly different means ($P < 0.01$). (*E*) Primary root length of Col-0 wild-type plants or 35S::miR393 overexpressor plants (28) was measured using the ImageJ program after 3 days of 5-mM KNO_3 or KCl treatment. Bars represent standard errors. Different letters represent statistically different means ($P < 0.01$).

However, the primary roots of *afb3-1* plants were not inhibited by nitrate as observed in wild-type plants or in the other individual auxin receptor mutants *tir1-1*, *afb1-1*, and *afb2-1* (Fig. 4*D* and Fig. S4 *A* and *B*). These results suggest that *AFB3* plays a specific role in modulating primary root growth in response to nitrate. We also analyzed the response of the primary root to KNO_3 treatments in the miR393 overexpressor line that shows reduced levels of *AFB3* (Fig. 2*A*). Consistent with the results in the *afb3-1* mutant line, the miR393 overexpressor line is completely insensitive to the KNO_3 treatments as compared with the wild-type plants (Fig. 4*E*).

Our results show that *AFB3* is involved in primary root growth inhibition in response to nitrate and that this effect is likely mediated by nitrate regulation of *AFB3* levels in root tips.

Nitrate Regulates Lateral Root Growth by a Pathway Involving the Auxin Receptor AFB3. Analysis of GUS activity in the *AFB3*::GUS reporter lines indicated that *AFB3* can be also induced by nitrate in the central vascular area, including the pericycle area (Fig. 5*A*). It is known that lateral roots are initiated from pericycle founder cells opposite to the xylem poles (38, 39). Therefore nitrate regulation of *AFB3* in the pericycle may lead to changes

in lateral root development. To quantify expression of *AFB3* in the pericycle, we isolated KNO_3 or KCl GFP-tagged pericycle cells using a fluorescence activated cell sorter (FACS) and extracted total RNA as described previously (9). KNO_3 treatment induced *AFB3* expression in the pericycle (Fig. 5*B*), suggesting that nitrate can also regulate auxin signaling in pericycle cells. Because lateral roots are produced from pericycle cells, we analyzed the number of initiating (stages I, II, III, IV, Va, Vb, VIa, VIb, and VII, after ref. 39) and emerging lateral roots using DIC microscopy in wild-type plants and in the *afb3-1* mutant after 3 days of 5 mM KNO_3 or 5 mM KCl treatment (Fig. 5*C*). In wild-type plants, nitrate treatments increased the density of lateral roots (both initiating and emerging) as compared with the KCl control condition. Most of the emerging laterals were short (<0.5 mm; Table S1), which can be related to an inhibitory effect of KNO_3 on root elongation as described previously (40). On the other hand, the increased density of initiating laterals (Table S1) can be related to a positive KNO_3 effect on root initiation, as described previously (14). The lateral root response was altered in the *afb3-1* mutant, which showed decreased density of emerging and initiating lateral roots as compared to wild type (Fig. 5*C*). However, none of the other individual auxin receptor mutants showed altered lateral root response to nitrate treatments (Fig. S5 *A* and *B*). This result shows that, as we have seen for primary root, *AFB3* has also a specific role in lateral root response to nitrate. We also analyzed lateral root density in the miR393 overexpressor line (Fig. 5*D*). Lateral root density after 3 days of KNO_3 treatment was no different from lateral root density after 3 days of control treatment, indicating that this response was also absent in this line (Fig. 5*D*). These results show that nitrate regulates lateral root growth by a pathway involving *AFB3*.

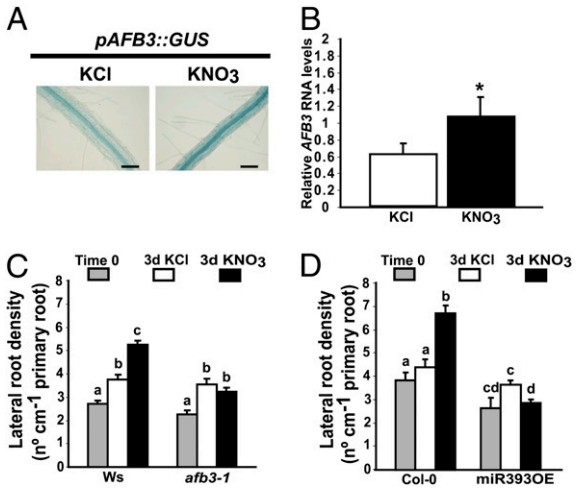

Fig. 5. Nitrate regulates lateral root growth by a pathway mediated by *AFB3*. Plants were grown hydroponically as described before and were treated for the times indicated. (*A*) *pAFB3::GUS* plants (35) were treated for 1 h with 5 mM KNO_3 or KCl and were then stained for GUS activity for 4 h. Qualitative GUS staining was analyzed using DIC optics. Photographs are representative of at least 15 stained plants. (Scale bar, 100 μm.) (*B*) Pericycle marker line plants were treated for 1.5 h with 5 mM KNO_3 or KCl. Protoplast were prepared from roots and pericycle cells expressing GFP were sorted by FACS. RNA levels for *AFB3* were measured using qPCR. Bars represent standard errors. The asterisk represents statistically different means ($P < 0.05$). (*C*) The number of initiating and emerging lateral roots of *afb3-1* mutants (35) or Ws wild-type plants treated for 3 days with 5 mM KNO_3 or KCl was counted using DIC optics. Bars represent standard errors. Different letters indicate statistically different means ($P < 0.01$). (*D*) The number of initiating and emerging lateral roots of 35S::miR393 overexpressor plants (28) or Col-0 wild-type plants treated for 3 days with 5 mM KNO_3 or KCl was counted using DIC optics. Bars represent SE. Different letters indicate statistically different means ($P < 0.01$).

Discussion

RSA modulation in response to nutrients is a classical example of plant plasticity to changing environmental conditions (41–43). Given their importance in controlling growth and developmental programs, phytohormones have arisen as the missing links between nutrient availability and plant developmental responses. Because of its central role in root development (44, 45) a role for auxin has been proposed in the modulation of RSA in response to nutrients (46–51). Here, we showed that nitrate treatments modulate both primary and lateral root growth by a pathway involving the N-responsive miR393/*AFB3* regulatory module. Recently, another auxin-related regulatory module, miR167/*ARF8*, has been identified in *Arabidopsis* to regulate the ratio between initiating and emerging lateral roots (14). miR393/*AFB3* regulatory module is unique as nitrate can regulate auxin responses by direct regulation of an auxin receptor, modifying auxin perception in the roots and thus affecting both primary and lateral root growth. The regulatory mechanism of miR393/*AFB3* regulation by nitrate involves at least transcriptional and posttranscriptional mechanisms. We showed that nitrate is able to transcriptionally induce expression of *AFB3* in roots (Figs. 1, 4*A*, and 5*A*) and that N metabolites produced after nitrate reduction and assimilation lead to a downregulation of *AFB3* levels due to the induction of miR393 (Figs. 1–3). This mechanism (Fig. S6) is consistent with the type I incoherent feed-forward loop (FFL) motif described for transcriptional networks in yeast, bacteria, and mammals (52–54). In this model, a transcription factor A can activate both a target gene *Z* and also a repressor of *Z* in response to a signal, leading to a transient activation of *Z*. This regulatory design allows for *Z* to be rapidly responsive to the input signal (55) and can also produce a nonmonotonic response, where the output of *Z* is first increased with the input signal but decreases when the signal is high (56–58). The observed regulation of *AFB3* expression by nitrate and metabolites produced downstream of nitrate reduction might constitute a mechanism to rapidly and precisely adjust root growth depending on external and internal nitrate availability. Incoherent and coherent FFL involving miRNA-target pairs are recurrent motifs in mammalian gene regulatory networks (54, 59–62). Because most miRNA targets encode transcription factors in plants, incoherent FFLs are probably also a common feature of plant gene networks. Besides the FFL reported here, a coherent feed-forward loop involving miR164 and its target *ORE1*/*NAC2* has been recently described in *Arabidopsis* that regulates age-dependent cell death (63).

AFB3 is part of the ubiquitin protein ligase $SCF^{TIR1/AFB}$ complex that targets and mediates the polyubiquitination and proteasomal degradation of the Aux/IAA transcriptional repressors to promote transcription of auxin-responsive genes (64–66). The finding that both primary and lateral root responses to nitrate are altered in the *afb3-1* mutant (Fig. 4*D* and Fig. 5*C*) suggests that for this response to occur, a functional *AFB3* is required. Moreover, *AFB3* is induced by nitrate in primary root tips (Fig. 4*C*) and in pericycle cells (Fig. 5*B*). Therefore, we propose that the increase in the expression of *AFB3* is responsible for the changes observed in RSA in response to nitrate.

We found that nitrate treatments were able to regulate the levels of auxin-responsive and auxin-related genes in *Arabidopsis* roots (Fig. S3), as previously demonstrated by network analysis of C- and N-regulated genes (4). However, we did not find misregulation of any of these genes in the *afb3-1* mutant, suggesting that nitrate can modulate auxin signaling and responses at multiple levels and in an AFB3-dependent and AFB3-independent manner.

RSA modulation by auxin can depend on three main factors: changes in auxin homeostasis, auxin transport, and auxin sig-

naling. Here we show that N regulation of a hormone receptor can lead to changes in hormonal signaling causing RSA changes. Recently, the auxin receptor TIR1 has been involved in lateral root formation in response to phosphate starvation in *Arabidopsis*; however, the other family members might also play a role in this response (49). Our evidence shows that RSA modulation in response to nitrate is a specific function of AFB3. Moreover, AFB3 has a dual role in primary and in lateral root development in response to nitrate whereas TIR1 seems to have a role only in lateral root formation (49).

N effects on RSA are complex and have been shown to depend on the N source and concentration available for plants as well as on other environmental conditions. In *Arabidopsis*, these effects can include changes in primary root growth (67), lateral root initiation (14, 68, 69), and elongation (40, 70). Under our experimental conditions, we found that supplementing 5 mM KNO_3 to wild-type ammonium-fed plants causes an inhibition of primary root growth (Fig. 4 *D* and *E*) and an increase in initiating and emerging lateral root density as compared with the control KCl condition (Fig. 5 *C* and *D*). This observation contrasts the previous observation by Walch-Liu et al. (67), where it was seen that a 5-mM KNO_3 treatment stimulated primary root growth when compared with a 5-mM KCl control. This discrepancy may arise from a higher apparent nitrate availability in our hydroponic condition, in which roots are surrounded by media, compared to the vertical plate condition in which roots are superimposed over the media-containing agar. This assumption is supported by findings by Tian et al. (71) where incubation of maize in solutions with KNO_3 concentrations of 5 mM or more caused inhibition of root elongation. The inhibitory effect of KNO_3 on primary root length was also seen after 18 days of continuous growth in vertical plates containing high nitrate concentrations in *Arabidopsis* (72). Our results are also similar to the ones reported by Gifford et al. (14), where the same KNO_3 treatment caused an increase in the number of initiating lateral roots relative to the number of emerging lateral roots (14) although there is no mention of the effect of nitrate on primary root growth.

Our results are consistent with a model in which a high nitrate provision would prevent further rooting (as it would not significantly enhance N acquisition) by an interaction with the auxin signaling pathway mediated by miR393/*AFB3*, adjusting plant growth and development to external and internal N availability (Fig. S6).

Materials and Methods

Plant Material. *Arabidopsis* (*A. thaliana*) plants were of Columbia (Col-0) ecotype or Wassilewskija (Ws) ecotype as indicated. *tir1-1*, *afb1-1*, *afb2-1*, *afb3-1*, *pAFB3::GUS*, and *pAFB3:mAFB3-GUS* lines were kindly donated by Mark Estelle, University of California San Diego, La Jolla, CA (33, 35). miR393 overexpressor lines were kindly donated by Jonathan Jones, the Sainsbury Laboratory, John Innes Centre, Norwich, UK (28). Nitrate reductase-NULL mutant lines were kindly provided by Nigel Crawford, University of California San Diego, La Jolla, CA (34). The GFP line that marks the pericycle (E374) was obtained from http://enhancertraps.bio.upenn.edu.

Growth and Treatment Conditions. Approximately 1,500 *Arabidopsis* seedlings were grown hydroponically on Phytatrays on MS-modified basal salt media without N (Phytotechnology Laboratories, M531) supplemented with 0.5 mM ammonium succinate and 3 mM sucrose under a photoperiod of 16 h of light and 8 h of darkness and a temperature of 22 °C using a plant growth incubator (Percival Scientific, Inc.). After 2 weeks, plants were treated with 5 mM KNO_3 with or without 30 mM sucrose or 5 mM KCl with or without 30 mM mannitol as control for different time periods as indicated. For the phenotypic analysis of the root response to nitrate treatment, seedlings were grown as described above and were treated with 5 mM KNO_3 or 5 mM KCl for 3 days.

Histochemical Analysis of GUS Activity. For histochemical analysis of GUS activity, *Arabidopsis* seedlings were incubated at 37 °C in a GUS reaction buffer (100 mM sodium phosphate buffer, pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% (vol/vol) Triton X-100, 0.1% (wt/vol) sodium lauroyl sarcosine) plus 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc). The seedlings were cleared according to the protocol described in ref. 39 and were imaged using DIC optics on a Nikon Eclipse 80i microscope. For each marker line and treatment, at least 15 plants were analyzed.

Analysis of Root Architecture Traits. Initiating and emerging lateral roots (stages I, II, III, IV, Va, Vb, VIa, VIb, and VII, according to ref. 39) were counted using DIC optics on a Nikon Eclipse 80i microscope. For primary root measures, plants were scanned using an Epson Perfection V700 Photo scanner, and roots were measured using the National Institutes of Health program ImageJ. The data were statistically analyzed in the Graph Pad Prism 5 Program.

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