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Molecular framework integrating nitrate sensing in root and auxin-guided shoot adaptive responses

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Mineral nutrition is one of the key environmental factors determining plant development and growth. Nitrate is the major form of macronutrient nitrogen that plants take up from the soil. Fluctuating availability or deficiency of this element severely limits plant growth and negatively affects crop production in the agricultural system. To cope with the heterogeneity of nitrate distribution in soil, plants evolved a complex regulatory mechanism that allows rapid adjustment of physiological and developmental processes to the status of this nutrient. The root, as a major exploitation organ that controls the uptake of nitrate to the plant body, acts as a regulatory hub that, according to nitrate availability, coordinates the growth and development of other plant organs. Here, we identified a regulatory framework, where cytokinin response factors (CRFs) play a central role as a molecular readout of the nitrate status in roots to guide shoot adaptive developmental response. We show that nitrate-driven activation of NLP7, a master regulator of nitrate response in plants, fine tunes biosynthesis of cytokinin in roots and its translocation to shoots where it enhances expression of *CRFs*. CRFs, through direct transcriptional regulation of *PIN* auxin transporters, promote the flow of auxin and thereby stimulate the development of shoot organs.

nitrate | plant development | macronutrient

Nitrogen, a building block of organic macromolecules such as nucleic acids and proteins, constitutes one of the essential chemical elements determining the growth and development of all organisms (1). Plants, as sessile organisms, rely on absorption of this macroelement from the soil, where it is available in different forms such as nitrate, ammonium, urea, or amino acids. While nitrate represents one of the major inorganic forms of nitrogen in aerobic soil, its availability might dramatically fluctuate in both time and space (2). To face these constraints, plants developed a wide range of adaptive mechanisms triggered by sensing systems to balance nitrate uptake with internal homeostasis (3, 4). At the molecular level, balancing nutrients acquisition with the plant's requirements implies a close communication between pathways controlling uptake, distribution, and homeostasis of nutrients and the pathways coordinating plant growth and development. Over the last few years, mechanisms of nitrate acquisition, sensing, and signaling have been dissected and key components including the nitrate transceptor NPF6.3/NRT1.1/CHL1 (5–8) and transcriptional regulators of the nitrate response such as NLP6/7, TCP20, LBD37/38/39, SPL9, and TGA1/TGA4 identified. They mediate primary nitrate responses including feedback on the expression of nitrate transport and assimilation genes (9–15).

Flexible adjustment of developmental programs is an essential part of plant adaptation to nitrate availability and a key foraging strategy to optimize its uptake and overcome temporal deficiency of this nutrient. Plant responses to nitrate availability can occur locally, confined to the root organ where nitrate is sensed and absorbed and systemically, involving regulatory signals that elicit responses in distant plant organs (16). The root–shoot–root systemic signaling theme appears to be central in communicating N status and in the coordination of root and shoot organ growth and development according to resources of nitrate (17–20). Mobile signaling molecules such as hormones and peptides have been identified as key components of systemic regulatory pathways (19–23). Among them, cytokinin has been proposed as a principal hormonal integrator, adjusting shoot development including apical meristem activity and leaf expansion to nitrate status sensed by the root system (20, 24–29). While cytokinin's role as an important component of the systemic nitrate response pathway is firmly established, little is known about the downstream effectors that coordinate the early shoot adaptive response to nitrate. Likewise, the role of auxin and polar auxin transport (PAT), as a core hormonal machinery coordinating plant growth and developmental processes, has been associated with plant adaptation to nitrate (9, 30). However, insights into molecular mechanisms

Significance

In our work, we address poorly understood mechanisms underlying root-to-shoot communication that lead to a rapid readjustment of shoot growth and development after nitrate provision. Applying a combination of genetics, cell and developmental biology approaches, grafting experiments, and hormonal analytics, we identified a molecular framework orchestrating shoot development with a root nitrate sensory system. We demonstrate that in response to nitrate supply, NLP7, a master regulator of nitrate response in plants, promotes the expression of cytokinin biosynthesis and facilitates translocation of this plant hormone to shoots. There, cytokinin response factors (CRFs) as direct regulators of *PINs*, stimulate the transport of auxin, and thereby promote shoot growth and development.

underlying regulation of PAT by nitrate and the physiological role of its components in plant adjustment to nitrate availability are scarce.

Here, we identified a molecular framework that integrates inputs of nitrate and cytokinin regulatory pathways to effectively adjust early shoot development according to the nitrate availability sensed by the root system. We demonstrate that nitrate, through NLP7-mediated signaling in roots, enhances biosynthesis and transport of cytokinins to shoots. Cytokinin response factors (CRFs), encoding APETALA2/ERF transcription factors, provide a readout of cytokinin levels in shoots, and through a fine-tuning expression of *PIN* auxin efflux carriers steer the flow of auxin, thereby promoting the growth of shoot organs.

Results

Developmental Adaptation to Nitrate Involves Adjustment of PIN Auxin Transporter Expression in the Shoot. Plants use nitrate (NO_3^-) and ammonium (NH_4^+) as major sources of inorganic nitrogen, although their preference for different forms might differ. Fluctuations in both concentrations and the form of nitrogen available in the soil have prominent effects on plant growth and development. To explore how *Arabidopsis* responds and adapts to different forms of nitrogen, seedlings were grown on ammonium succinate (AS) as an exclusive nitrogen source for 7 d and afterward transferred to media containing either AS or nitrate (day of transfer is indicated as D0). The growth of seedlings was monitored for 1, 4, and 14 d after transfer (DAT). As previously reported, nitrate promoted the growth of primary roots (PRs) and lateral roots (LRs), but not the density of LRs when compared to roots on media supplied with AS (*SI Appendix,* Fig. S1*A–C*) (31–33). To examine developmental responses of shoot organs, the surface area of cotyledons and fresh weight of shoots 1 and 14 DAT to nitrate, respectively, were quantified. Embryonic leaves of seedlings 1 DAT on medium supplied with nitrate expanded significantly more when compared to those supplemented with AS (Fig. 1*A* and *B*). Consistently, 14 DAT fresh weight of shoots was significantly higher in seedlings supplied with nitrate than with AS (*SI Appendix,* Fig. S1*D*). Hence, consistently with reports, nitrate provided to seedlings triggers overall modulation of plant development, including root and shoot organs . At the molecular level, the transfer of seedlings from AS to nitrate-supplemented medium resulted in rapid up-regulation (within 1 h) of the early nitrate responsive genes, including *HRS1* and *TGA1* in roots (9, 35, 36) (*SI Appendix,* Fig. S1*E*).

Auxin and its graded distribution play an instructive function in all plant growth and developmental processes (37–40). Thus, we asked whether and/or how the auxin transport mediated through PIN auxin efflux carriers contributes to the developmental adaptation of plant organs to nitrate provision. qRT-PCR analyses revealed relatively modest alterations of *PIN* transcription in roots after transfer of seedlings to nitrate-supplemented medium. Among tested *PINs*, *PIN3* expression was significantly increased in roots 1 and 6 h after seedlings were transferred on nitrate when compared to AS-containing medium, while *PIN7* transcription was attenuated 6 hours after transfer (HAT) (*SI Appendix,* Fig. S1*F*). Intriguingly, provision of nitrate triggered a profound response in shoots, in which transcription of several *PIN* genes, including *PIN1, PIN4, PIN6,* and *PIN7,* was enhanced 1 HAT and/or 6 HAT to nitrate when compared to AS (Fig. 1*C*). Analysis of *PIN::GUS* reporter lines corroborated the qRT-PCR results (Fig. 1*D* and *SI Appendix,* Fig. S1*G*). The nitrate-promoted expression of *PIN* genes correlated with the

abundance of PIN proteins quantified in *PIN1::PIN1-GFP* and *PIN7::PIN7-GFP* reporter lines. Significantly higher PIN1-GFP and PIN7-GFP signals at the plasma membrane (PM) of vasculature and epidermal cells at the adaxial side of cotyledons, respectively, were detected in seedlings provided by nitrate than by AS (Fig. 1*E–G*).

Hence, early phases of seedling adaptation to nitrate provision might be accompanied by adjustment of polar auxin transport in shoots. To examine whether PIN-mediated auxin transport is essential for shoot growth enhancement triggered by nitrate, mutants in *PIN* genes were analyzed. To dissect alterations in adaptive responses to nitrate provision from general developmental defects that potentially might be caused by the lack of the respective PIN function, growth response was calculated as a difference in cotyledon size at D0 versus D1. Unlike wild type, no significant increase in the size of cotyledon surface area 1 DAT to nitrate when compared to AS was detected in the *pin4* and *pin7* mutants (Fig. 1*A* and *B*). Consistently, 14 DAT to nitrate, shoot fresh weight of *pin* mutants was significantly lower when compared to wild-type control (*SI Appendix,* Fig. S1*H–J*). Intriguingly, no pronounced shoot developmental defects in either *pin4* or *pin7* mutants have been reported previously (41–44), presumably because of the functional redundancies among PINs. Hence, to examine whether functionally redundant PINs mitigate deficiency in shoot growth at optimal nutrient conditions more efficiently than under nitrate deficiency, we performed an additional set of experiments. We grew wild-type and *pin* mutants for 21 d on 0.5×Murashige and Skoog (MS) medium. In parallel, to obtain a direct comparison, wild type and mutants were grown for 7 d on a medium supplemented with AS and transferred to nitrate for 14 d. Whereas no significant difference in shoot fresh weight was detected between wild-type and *pin4* and *pin7* grown on the MS medium, shoot fresh weight gain was significantly lower in *pin4* and *pin7* than in wild type when seedlings were transferred to nitrate (*SI Appendix,* Fig. S1*K* and *L*). This suggests that intact PIN-mediated transport is essential for plants to recover from the stress caused by the initial growth under suboptimal nitrogen supply (AS), and effectively adjust their development to nitrate provision.

Cytokinin Response Factors Mediate Shoot Developmental Adaptations to Nitrate. Up-regulation of the *PIN* transcription in shoots after the provision of nitrate motivated us to screen for upstream regulators involved in this response. CRFs, which have been identified as direct transcriptional regulators of *PINs* (45), were detected among the early nitrate responsive genes in several transcriptome profiling data (9, 14, 29). In agreement with these reports, qRT-PCR analyses confirmed up-regulation of *CRF2* and *CRF6* in shoots provided with nitrate, when compared to AS (Fig. 2*A* and *B*), whereas in roots no significant changes could be detected (*SI Appendix,* Fig. S2*A*). Consistently with qRT-PCR, staining of *CRFs::GUS* reporters corroborated nitrate responsive expression of *CRFs* in shoots (Fig. 2*C*).

NLP7, a NIN-like transcription factor, is a master regulator of plant adaptation to nitrate. Nitrate triggers nuclear retention of NLP7 protein, where it activates transcriptional responses (*SI Appendix,* Fig. S2*B* and *C*) (10, 12). To examine whether nitrate-promoted expression of *CRFs* is dependent on NLP7-mediated signaling we analyzed the *nlp7* loss-of-function mutant. Unlike wild type, neither *CRF2* nor *CRF6* transcription was enhanced in shoots or roots of *nlp7* seedlings 1 HAT to nitrate, when compared to AS (Fig. 2*D* and *SI Appendix,* Fig. S2*D*). These data suggest that transcriptional modulation of *CRFs* is part of the
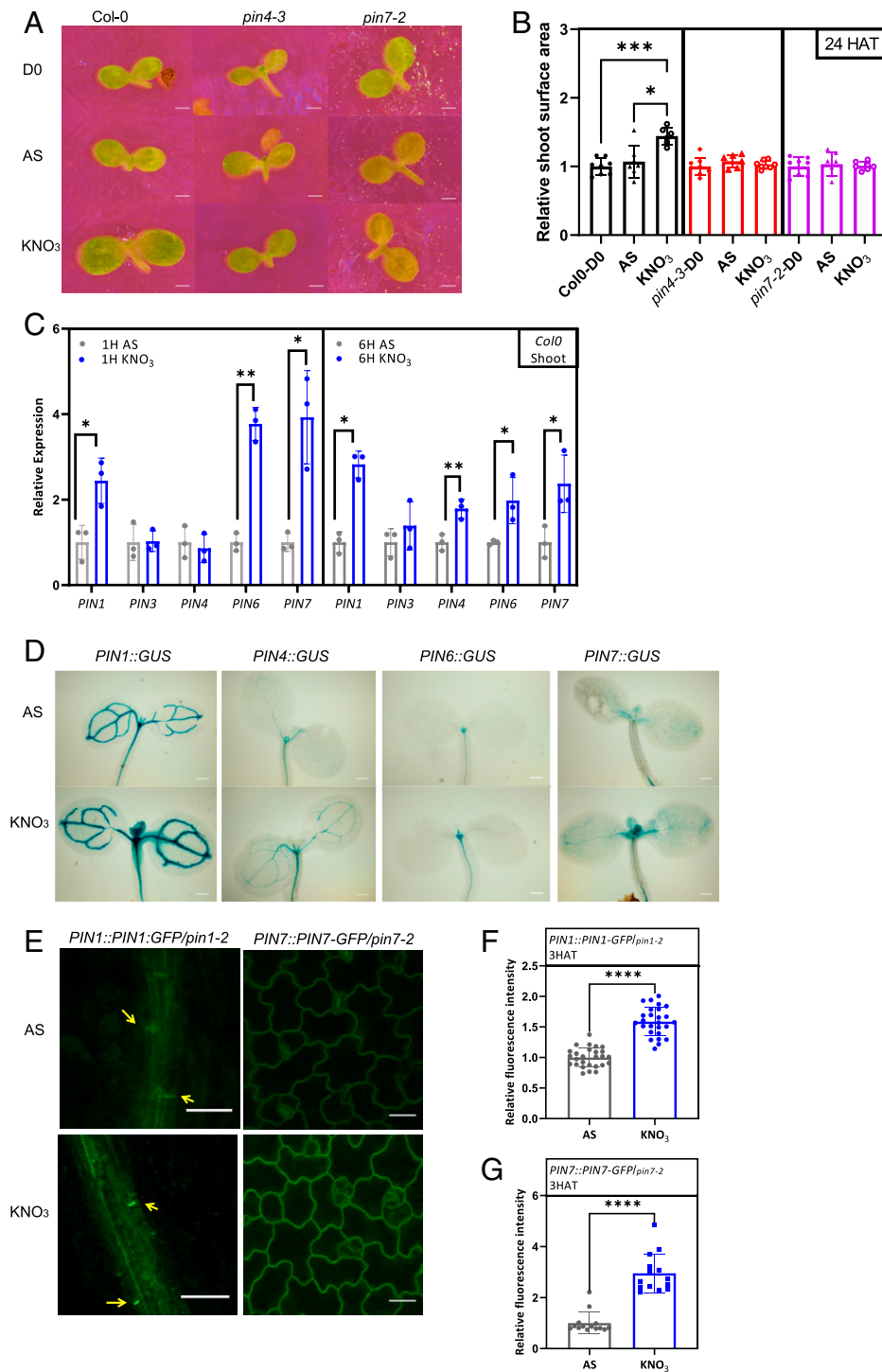
Fig. 1. Modulation of *PIN* expression is part of nitrate-mediated transcriptional reprogramming. (*A* and *B*) Imaging (*A*) and surface area quantification (*B*) of cotyledons from 7-d-old wild-type (Col 0), *pin4-3*, and *pin7-2* seedlings grown on AS (0.5 mM) (D0), and 24 HAT to either ammonium (AS)- or nitrate (5 mM KNO₃)-containing media. Relative size was quantified as mean surface area of cotyledons on AS or KNO₃ 24 HAT compared to D0 ($n = 5$ to 8 seedlings per treatment). The experiment was repeated three times; the result from one representative experiment is presented. Significant differences were determined by one-way ANOVA followed by a significant Tukey's multiple comparison test; *$P < 0.05$, ***$P < 0.001$. (*C*) qRT-PCR expression analysis of *PIN* genes normalized to *UBQ10* (*AT4G05320*) in Col 0 shoots, 1 and 6 HAT to AS- or KNO3-containing media. All qRT-PCR reactions were carried out with biological and technical triplicates. Statistical difference was determined by Student's *t* test; *$P < 0.05$, **$P < 0.01$. (*D*) Expression analyses of *PIN::GUS* reporters in shoots of 7-d-old seedlings, 6 HAT to AS and KNO₃. (*E–G*) Monitoring (*E*) and quantification of *PIN1::PIN1-GFP* (*F*) and *PIN7::PIN7-GFP* (*G*) membrane signal in vasculature (yellow arrows) and adaxial epidermal cells of cotyledons, respectively, 3 HAT on AS- or KNO₃-supplemented media. PIN-GFP signal measured in $n = 2$ epidermal cells, and at least two cells of vasculature in six to seven seedlings per treatment. Significant differences were determined by Student's *t* test; ****$P < 0.0001$ (*F* and *G*). (Scale bars in *A*, 400 µm; *D*, 200 µm; and *E*, 24 µm.)

specific plant adaptive responses to nitrate mediated by the core nitrate signaling machinery (9, 14, 46).

To further explore the function of CRFs in nitrate-regulated plant growth, seedlings with the modulated activity of CRFs were examined. Similar to *nlp7* mutants, which are defective in the shoot growth response to nitrate provision (Fig. 2*E* and *F* and *SI Appendix*, Fig. S2*E* and *F*) (10, 47), loss of CRF function affected seedling sensitivity to nitrate provision. The nitrate-promoting effects on cotyledon expansion 1 DAT were reduced in multiple *crf2*, *crf3*, *crf6*, and single *crf2*, *crf3*, and *crf6* mutants when
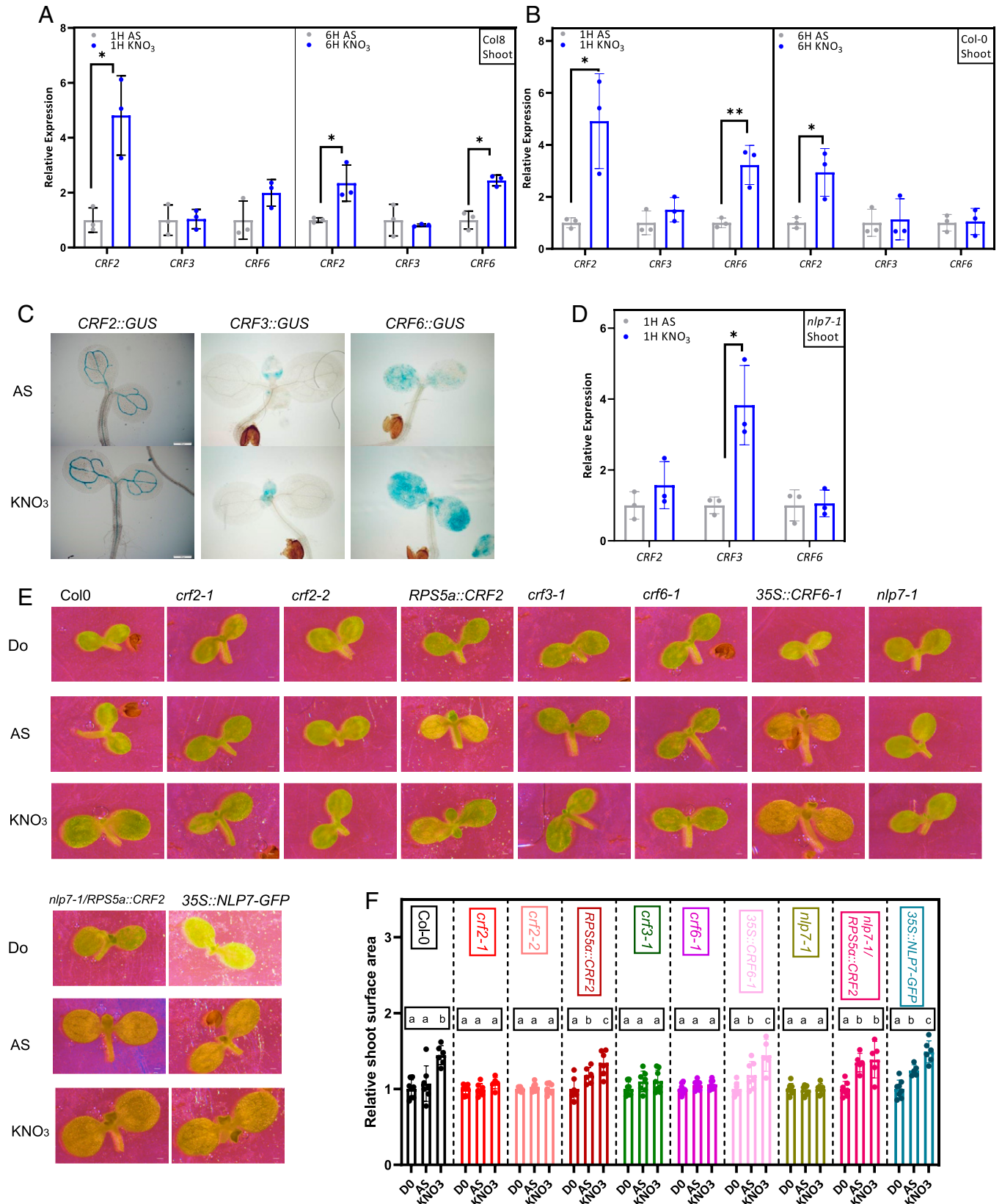
Fig. 2. Cytokinin response factors mediate shoot developmental adaptations to nitrate. (*A–D*) Expression analyses of *CRFs* using qRT-PCR (*A, B,* and *D*) and *CRF::GUS* reporters (*C*) in wild-type (Col 0) (*A*), (Col 8) (*B*), and *nlp7* (*D*) shoots of 7-d-old seedlings 1 HAT (*A–D*) and 6 HAT (*A* and *B*) to AS and KNO_3. Expression of *CRF* genes normalized to *UBQ10* (*AT4G05320*) 1 HAT and 6 HAT on AS- or KNO_3-containing media. All qRT-PCR reactions were carried out with biological and technical triplicates. Statistical difference was determined by Student's *t* test; *$P < 0.05$, **$P < 0.01$. (*E* and *F*) Imaging (*E*) and surface area quantification (*F*) of cotyledons from 7-d-old wild type (Col 0), mutants, and CRF overexpressors grown on AS (D0), and 24 HAT to AS-or KNO_3-containing media. Relative size was quantified as mean surface area of cotyledons on AS or KNO_3 24 HAT compared to D0 (*n* = 5 to 8 seedlings per treatment). The experiment was repeated twice; the result from one representative experiment is presented. Significant differences were determined by one-way ANOVA followed by Tukey's multiple comparison test; lowercase letters indicate significant differences of at least *$P < 0.05$. (Scale bars in C , 200 μm and E, 100 μm.)

compared to wild type (Fig. 2*E* and *F*). Shoot fresh weight 14 DAT was strongly reduced in *crf6* and *crf2, crf3, crf6* mutants when compared to wild type (*SI Appendix*, Fig. S2*G* and *H*). In contrast to the attenuated response of the *crf* mutants to nitrate, seedlings expressing *CRF2* and *CRF6* driven by constitutive *RPS5a* or *35S* promoter, respectively, displayed enhanced expansion of cotyledons and shoot growth irrespective of nitrate provision (Fig. 2*E* and *F* and *SI Appendix*, Fig. S2*I* and *J*). In addition, *RPS5a::CRF2* introduced into the *nlp7* background resulted in enhanced cotyledon expansion and shoot growth when compared to *nlp7* supplemented with either AS or nitrate (Fig. 2*E* and *F* and *SI Appendix*, Fig. S2*I* and *J*).

These data strongly support the role of CRFs as molecular components of the pathway that, downstream of NLP7-mediated signaling, regulate plant growth adaptation to nitrate provision.

NLP7 Fine Tunes Expression of *PINs* and Shoot Developmental Adaptation to Nitrate through CRFs. To examine whether nitrate promotes the expression of *PINs* in shoots through NLP7-mediated regulation of CRFs, *crf* and *nlp7* mutants were examined closely. Unlike wild type, in which supply of nitrate led to a significant increase in shoot expression of several *PINs*, in *nlp7* and *crf2* mutants, transcription of *PINs* was largely unaltered 1 HAT to nitrate when compared to AS (*SI Appendix*, Fig. S3*A* and *B* compared to Fig. 1*C*). PIN7-GFP and PIN1-GFP signals at the PM of epidermal and vascular cells in cotyledons in *nlp7*, *crf6*, and *crf2* mutants, respectively, remained unaffected after transfer to nitrate, thus further corroborating the role of NLP7 and CRFs in the adjustment of PIN-mediated transport during shoot response to nitrate provision (Fig. 3*A–D* and *SI Appendix*, Fig. S3*C–G*). Conversely, in *RPS5a::CRF2* seedlings, an elevated PIN7-GFP abundance at the PM of epidermal cells in cotyledons was detected irrespectively of the nitrogen source. The strength of the PIN7-GFP signal in cotyledons of the *RPS5a::CRF2* seedlings was significantly higher than that detected in wild-type seedlings supplemented with nitrate (Fig. 3*E* and *F*). CRFs control *PIN* transcription through binding to cytokinin response elements (*PCRE*) in their promoters (45). Notably, nitrate provision did not enhance the expression of *PIN7-GFP* driven by the *ΔPIN7* promoter, which lacks the CRF binding domain in cotyledons of wild-type as well as *RPS5a::CRF2* seedlings (Fig. 3*E* and *F*). In line with the role of CRF-mediated regulation of *PINs* in developmental adaptation of shoots to nitrate provision, neither *ΔPIN7::PIN7:GFP* nor *ΔPIN1::PIN1:GFP* was able to recover cotyledon growth of *pin7* and *pin1* mutants in response to nitrate provision (*SI Appendix*, Fig. S3*H* and *I*).

To gain further insights into the mechanism underlying nitrate-promoting effect on the cotyledon expansion, we examined growth at the cellular level. For these purposes, we observed pavement cells at the adaxial side of cotyledons at D0 and D1 after transfer to either AS- or nitrate-supplemented medium. Pavement cells of wild-type seedlings supplied with nitrate displayed a pronounced jigsaw puzzle–like cell pattern and expanded significantly more when compared to those supplemented with AS. Mutants in *NLP7*, *CRF2*, *PIN4*, and *PIN7* were severely affected in expansion and patterning of pavement cells in response to nitrate, when compared to wild type (*SI Appendix*, Fig. S4).

Altogether, these results support a role of the NLP7-CRF-PIN module in shoot adaptation to nitrate and indicate that adjustment of the PIN-mediated auxin transport is important for the auxin-driven expansion and patterning of pavement cells.

NLP7-Mediated Signaling in Roots Controls Cytokinin Levels in Shoots. Our analyses indicated that enhanced growth of shoots in response to nitrate is dependent on *NLP7/CRF*-mediated

regulation of PIN-directed auxin transport. Intriguingly, while the root is the principal organ for nitrate uptake, significant alterations in the transcription of *PINs* by nitrate supply were detected in shoots. This raised a question as to whether adjustment of PIN-mediated auxin transport and shoot growth in response to nitrate might involve some mobile signal translocated from roots. NLP7 is a core molecular component of the nitrate signaling pathway, whose loss of activity severely affects shoot growth in response to nitrate (10, 47) (Fig. 2*E* and *F* and *SI Appendix*, Fig. S2*E* and *F*). To examine whether proper uptake and sensing of nitrate in roots can recover shoot development of *nlp7*, we implemented grafting experiments. Roots and shoots of 7-d-old wild-type and *nlp7-1* seedlings germinated on media supplemented with AS were separated, and using a sealing tube, various grafting combinations were performed. Grafted seedlings were transferred to nitrate-containing medium and monitored for 14 d. As expected, shoots of Col 8 wild-type grafted on Col 8 roots (ColR/ColS) displayed enhanced development and bigger size when compared to shoots of *nlp7* grafted on the mutant roots (*nlp7*R/*nlp7*S). Notably, the partial rescue of the *nlp7* shoot development was detected in grafts with Col 8 roots, while Col 8 shoots grafted on roots of *nlp7* mutant displayed retarded development when compared to ColR/ColS grafts (*SI Appendix*, Fig. S5*A*). This suggested that nitrate, in an NLP7-dependent manner, promotes translocation of some mobile signal from roots to above-ground organs where it coordinates shoot development.

Cytokinin is an established positive hormonal regulator of shoot growth, strongly associated with adaptive responses to nitrate (20–22, 48). Mutants defective either in cytokinin perception (*ahk2* and *ahk3*), long-distance root-to-shoot cytokinin transport (*abcg14*), or biosynthesis (*ipt3,5,7*) are strongly affected in shoot growth adaptation to nitrate provision (Fig. 4*A* and *B* and *SI Appendix*, Fig. S5*B* and *C*). Analysis of the early cytokinin response gene *ARR5* revealed that 6 HAT of seedlings from AS to nitrate-containing medium expression of *ARR5::GUS* reporter in shoots is enhanced (*SI Appendix*, Fig. S5*D*). To examine whether nitrate promotes the expression of *ARR5* in an organ-autonomous manner or through the root-derived hormonal signal, the *ARR5::GUS* shoots were grafted on roots of *ARR5::GUS* as well as on the roots of *abcg14* and *ipt3,5,7* mutants affected in cytokinin long-distance transport and biosynthesis, respectively. *ARR5::GUS* reporter expression was stronger in shoots grafted on wild-type roots when compared to shoots grafted on either *abcg14* or *ipt3,5,7* roots (*SI Appendix*, Fig. S5*E*). Consistently with previous studies (20–22), these results suggest that also in our system cytokinin acts as a mobile signal that is transported to shoots, upon transfer of seedlings to nitrate. Recently, measurements of cytokinins in the *nlp7,nlp6* seedlings revealed reduced levels of the trans-Zeatin (tZ) type of cytokinins when compared to wild type after nitrate supply hinting at their role in regulation of cytokinin biosynthesis (41). To explore whether a reduced amount of cytokinin transported from roots to shoots might underlie the retarded development of *nlp7* shoots despite sufficient nitrate availability, *ARR5::GUS* shoots were grafted on *nlp7* roots. Expression of *ARR5::GUS* remained low in shoots grafted on roots of the *nlp7* mutants when compared to those grafted on control roots (Fig. 4*C*). These results suggest that NLP7 signaling activated by nitrate in roots is involved in the control of cytokinin translocation to shoots where it promotes their growth.

NLP7-Mediated Nitrate Signaling Controls Cytokinin Levels in Shoot. Weaker *ARR5::GUS* expression in shoots grafted on *nlp7* roots when compared to those on control roots indicates that
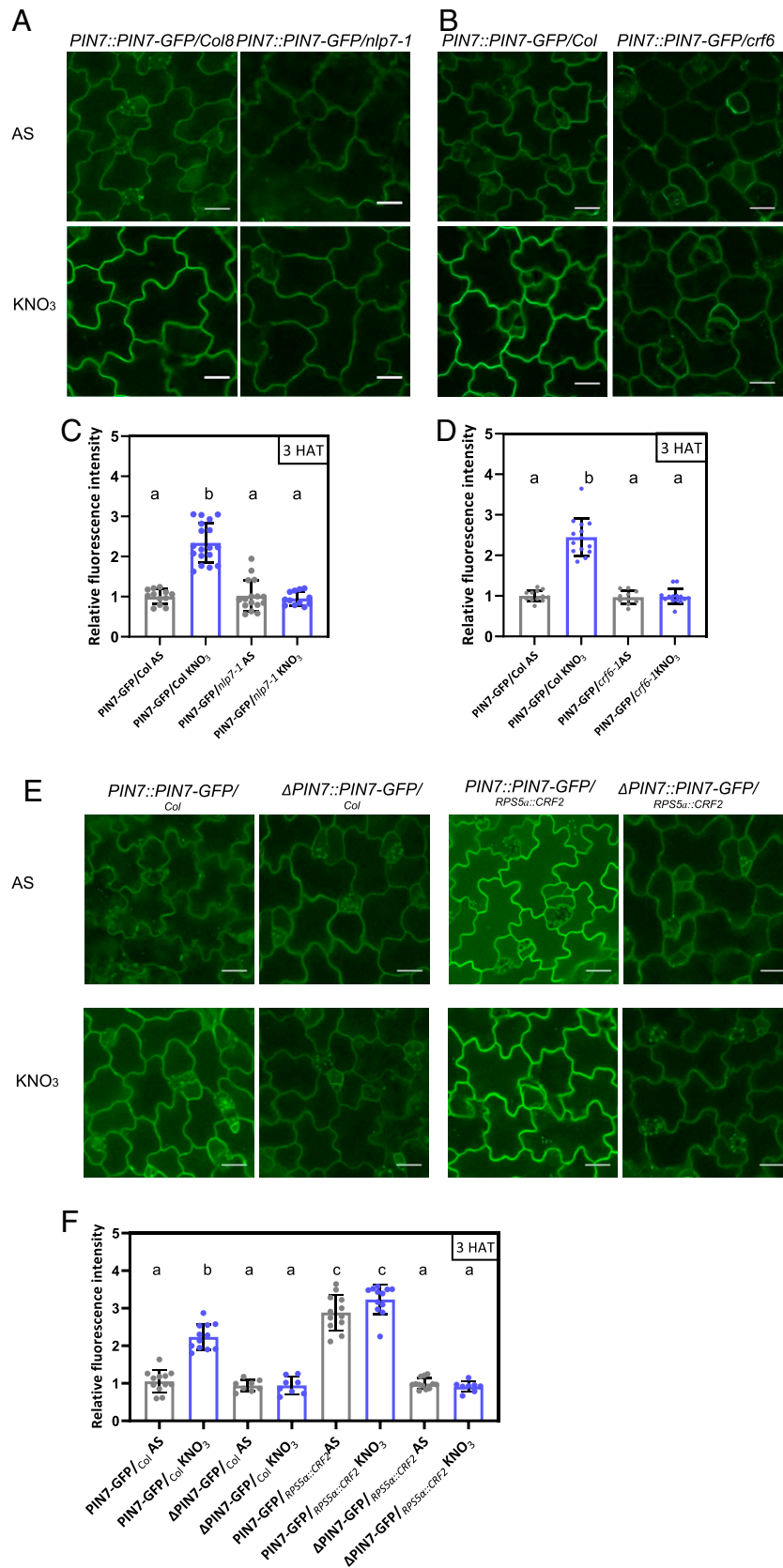
Fig. 3. NLP7 fine tunes expression of *PINs* and shoot developmental adaptation to nitrate through CRFs. (*A–F*) Imaging (*A, B,* and *E*) and quantification (*C, D,* and *F*) of PIN7-GFP membrane signal in adaxial epidermal cells of cotyledons 3 HAT to medium supplemented with AS or KNO_3 in *nlp7-1* (*A* and *C*), *crf6-1* (*B* and *D*), and *PIN7::PIN7-GFP*, Col 0 and *ΔPIN7::PIN7-GFP,RPS5α::CRF2* (*E*) lines. Different lowercase letters indicate significant difference at one-way ANOVA followed by Tukey's multiple comparison test (*P* < 0.05). PIN7-GFP signal measured in *n* = 2 epidermal cells, in at least five seedlings per treatment. Experiments were repeated at least twice; the result from one experiment is presented (*C, D,* and *F*). (Scale bars in *A, B,* and *E*, 24 μm.)
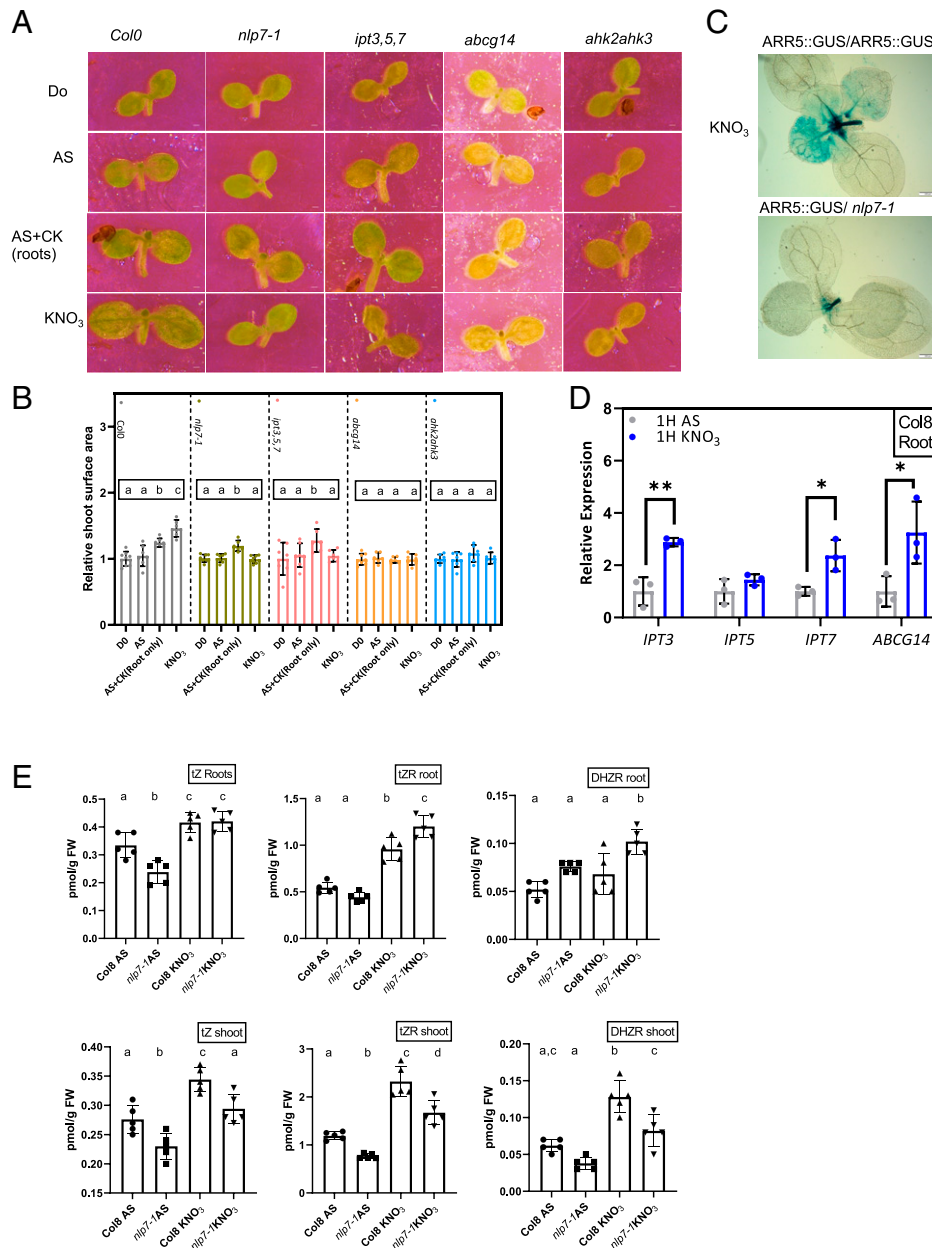
Fig. 4. NLP7-mediated signaling in roots controls cytokinin levels in shoots. (*A* and *B*) Imaging (*A*) and surface area quantification (*B*) of cotyledons from 7-d-old wild type (Col 0), *nlp7-1*, *ipt3,5,7*, *abcg14*, and *ahk2ahk3* seedlings grown on AS (D0), and 24 HAT to either AS, AS supplemented with cytokinin (CK, 100 nM 6-benzyla-minopurine), and KNO$_3$-containing media. Relative size was quantified as mean cotyledon surface area on AS, AS plus CK, or KNO$_3$ 24 HAT compared to D0 (*n* = 6 to 8 seedlings per treatment). The xperiment was repeated at least twice; results from one representative experiment are presented. Significant differences were determined by one-way ANOVA followed by Tukey's multiple comparison test; different lowercase letters indicate *P* < 0.05 (*B*). (*C*) Representative images of *ARR5::-GUS* expression in shoots of 7-d-old seedlings grafted on Col 0 or *nlp7-1* roots. Grafted seedlings after 72 h of healing on AS medium were then transferred to KNO$_3$ for 24 h. (*D*) qRT-PCR analysis of *IPT* genes and *ABCG14* expression normalized to *UBQ10* (*AT4G05320*) in Col 8 roots 1 HAT to AS- or KNO$_3$-containing media. All qRT-PCR reactions were carried out with biological and technical triplicates. Statistical difference was determined with a *t* test; *P < 0.05,**P < 0.01 . (*E*) Quantification of *tZ*, *tZR*, and *DHZR* in roots and shoots of Col 8 and *nlp7-1* seedlings 6 HAT to AS- or KNO$_3$-containing medium. Different lowercase letters indicate significant one-way ANOVA followed by Tukey's multiple comparison test (*P* < 0.05) (*n* = 5 biological replica/genotype/treatment). (Scale bars in *A*, 100 μm and *C*, 200 μm.)

NLP7-mediated nitrate signaling in roots plays a critical role in regulating cytokinin provision to the shoot.

In agreement with previous reports (20–22, 29, 41, 49, 50), qRT-PCR analyses showed that in wild-type seedlings, root expression of *IPT3*, *IPT7*, and *ABCG14* is enhanced 1 HAT to nitrate. In shoots, no major changes in expression of either cytokinin biosynthesis or transport genes were observed 1 HAT to nitrate, relative to AS transfer, in line with a note that cytokinin acts as a mobile signal to mediate root-to-shoot communication (Fig. 4*D* and *SI Appendix*, Fig. S4*F*). Unlike wild type, no increase in *IPT3* and *ABCG14* expression by nitrate was detected in *nlp7* roots or shoots, supporting NLP7 function in

fine tuning cytokinin biosynthesis and transport in roots supplied with nitrate (*SI Appendix*, Fig. S4*G* and *H*).

To further investigate the role of NLP7 in cytokinin biosynthesis and/or transport to shoots, we examined whether the provision of cytokinins locally to roots can recover retarded shoot development in the *nlp7* mutant. To validate the experimental setup we first monitored cytokinin effects on shoot recovery in *ipt3,5,7*, *abgc14*, and *ahk2,ahk3* mutant. Cytokinin applied to roots promoted shoot growth in the *ipt3,5,7* mutant defective in cytokinin biosynthesis, while no enhancement could be detected in *abcg14* or *ahk2,ahk3* mutants in transport and perception, respectively. Notably, cytokinin applied to roots promoted shoot growth in

nlp7, thus hinting at the role of NLP7 in the regulation of root-derived cytokinins to coordinate shoot growth (Fig. 4*A* and *B*).

In summary, NLP7-mediated regulation of cytokinin biosynthesis and transport derived from roots is an important part of the shoot developmental adaptation to nitrate provision.

Nitrate-Dependent Cytokinin Biosynthesis and Distribution Are Affected in *nlp7*.

To examine the role of NLP7 in the adjustment of cytokinin levels to nitrate provision to shoots we performed hormonal analyses in wild type and *nlp7* mutant 6 HAT to either AS- or nitrate-supplemented medium. In agreement with previous reports (20, 41, 49, 50) we found that provision of nitrate resulted in significant increase of biologically active cytokinin tZ, as well as its transport form transzeatin riboside (tZR) and dihydrozeatin riboside (DHZR) in both roots and shoots of wild-type seedlings, when compared to those supplemented with AS (Fig. 4*E*). Provision of nitrate also enhanced accumulation of isopentenyladenosine-5′-monophosphate (iPRMP) and isopentenyladenosine (iPR), but not isopentenyladenine (iP) in shoots when compared to those on AS (*SI Appendix*, Fig. S4*I* and Table S1). Levels of cis-zeatin (cZ) and its derivatives were not altered by nitrate provision, indicating that the pathway encompassing cytokinin synthesis from the tRNA-IPT pathway is not significantly contributing to the adjustment of the cytokinin pool to nitrate availability (*SI Appendix*, Table S1). Likewise, levels of biologically inactive or storage forms of cytokinins such as O-, N7-, N9-glucosides remained largely unaffected by nitrate provision, suggesting that increased levels of active cytokinins after transfer to nitrate do not originate from the conversion of their storage counterparts (*SI Appendix*, Table S1). Overall, measurements of cytokinin derivatives in wild-type seedlings are consistent with previous reports demonstrating that provision of nitrate is accompanied by enhanced biosynthesis of cytokinins, and in particular tZ types of cytokinins, which are translocated to shoots via the xylem (20, 49, 51).

Unlike wild type, transfer of *nlp7* to nitrate resulted in significantly lower accumulation of tZ, tZR, and DHZR in shoots (Fig. 4*E*). Noteworthy, levels of tZR and DHZR in roots of *nlp7* mutants 6 HAT to nitrate were higher than in wild-type roots, suggesting that their transport from roots to shoots might also be affected in the mutant background (Fig. 4*E*). Surprisingly, in *nlp7* mutants supplemented with AS, increased levels of iP in both roots and shoots were detected when compared to wild type, hinting at activation of a mechanism compensating for low levels of tZ cytokinins in the *nlp7* mutant (*SI Appendix*, Fig. S4*I* and Table S1). Provision of nitrate led to reduction of the iP forms in *nlp7* presumably due to their conversion of tZ triggered by nitrate. No dramatic alterations in amounts of cytokinin conjugates in *nlp7* mutant on AS versus nitrate could be detected, in line with the assumption that modulation of cytokinin levels in response to nitrate mediated by NLP7 does not involve reactivation of storage forms of cytokinins (*SI Appendix*, Table S1). Overall, these data support the previously established role of cytokinin as signaling molecule involved in plant adaptation to nitrate availability (20–22, 26, 41, 49, 52) and show that NLP7, in addition to fine tuning cytokinin metabolism (41), might also play an important role in its distribution according to nitrate levels.

Adjustment of *PIN* Expression and Shoot Growth by Nitrate Is Coordinated by Cytokinin Derived from Roots.

To test whether shoot expression of *PIN* genes in seedlings transferred to nitrate is dependent on cytokinin transported from roots, we grafted shoots of the *PIN7::GUS* reporter line on roots of wild type, *ipt3,5,7*, or *abcg14*, respectively. Expression of *PIN7::GUS* in grafts on control roots was stronger when compared to shoots grafted on *ipt3,5,7*, *abcg14*, or *nlp7* roots, in line with the assumption that cytokinin is a mobile signal produced in roots supplied with nitrate and transported to shoots where it might activate expression of *PINs* (Fig. 5*A*). Consistently, expression of *PIN7::PIN7-GFP* was enhanced in cotyledons of seedlings that were transferred to media supplemented with AS and had cytokinin applied locally to roots. In the *nlp7* mutant, the cytokinin treatment enhanced PIN7-GFP signal in epidermal cells of cotyledons (Fig. 5*B* and *C*), whereas in the *abcg14* mutant no enhancement of PIN7-GFP abundance was observed under this condition (Fig. 5*D* and *E*).

Enhanced shoot growth was observed in *PIN1::PIN1-GFP/pin1* and *PIN7::PIN7-GFP/pin7* seedlings provided with AS and with cytokinin applied to roots. In contrast, PIN-GFP driven by promoters lacking elements recognized by CRFs, such ΔPIN1 and ΔPIN7, were unable to recover *pin1* and *pin7* shoot growth when cytokinin was added to AS-containing medium (*SI Appendix*, Fig. S3*G* and *H*). These data support a model where modulation of PIN expression, driven by cytokinins translocated from roots, play an important role in controlling shoot growth adaptation to nitrate.

Discussion

Over the last few years, nitrate-dependent regulation of several components of PAT, including Aux/LAX, PINs, and ABCBs efflux transporter has been reported (29, 33, 53–55). However, insights into molecular mechanisms underlying regulation of PAT by nitrate and its function in plant developmental adaptations to nitrate availability and nitrogen status are scarce. Interestingly, although the root is the primary organ for nitrate uptake and sensing, we detected a profound nitrate-promoted transcription reprogramming of *PINs* in shoot organs, pointing at the role of the PIN-mediated auxin transport in shoot adaptive responses to nitrate provision. Consistently with this assumption, interference with the activity of *PIN* genes significantly attenuated stimulatory effects of nitrate on shoot development when compared to wild-type seedlings.

CRFs, a subgroup of transcription factors from the AP2/ERF family, emerged as potential molecular components underlying convergence of nitrate signaling and the PAT from the overlap between nitrate responsive transcriptomes (9, 12, 14, 29, 56) and transcriptional regulators of *PIN* genes identified by the yeast one-hybrid screen (45). Nitrate-sensitive transcription of *CRF* genes in shoots, deficiency of the respective mutants to respond to nitrate provision by enhanced shoot growth, and promoting effects of *CRF2* overexpression on shoot growth irrespective of nitrate supply, collectively, support a role of these transcription factors in the regulation of shoot developmental responses to nitrate. Importantly, nitrate-stimulated expression of *PINs* is attenuated in *crf* mutants, or when the *PIN* promoters are deprived of the cis-elements targeted by CRFs in the wild type as well as *RPS5a::CRF2* background, pointing at CRF-PINs as a regulatory module coordinating shoot developmental adaptation to nitrate.

The CRF-mediated regulation of *PIN* expression in shoots needs to be tightly coordinated with processes such as nitrate absorption, distribution, and signaling occurring primarily in the root. Cytokinins have been identified as one of the principal long-distance signaling molecules that mediate root-to-shoot communication about the nitrate status (20–22, 26, 49). Nitrate provision has been reported to induce expression of cytokinin biosynthesis genes, such as *IPT3*, and root-to-shoot translocation
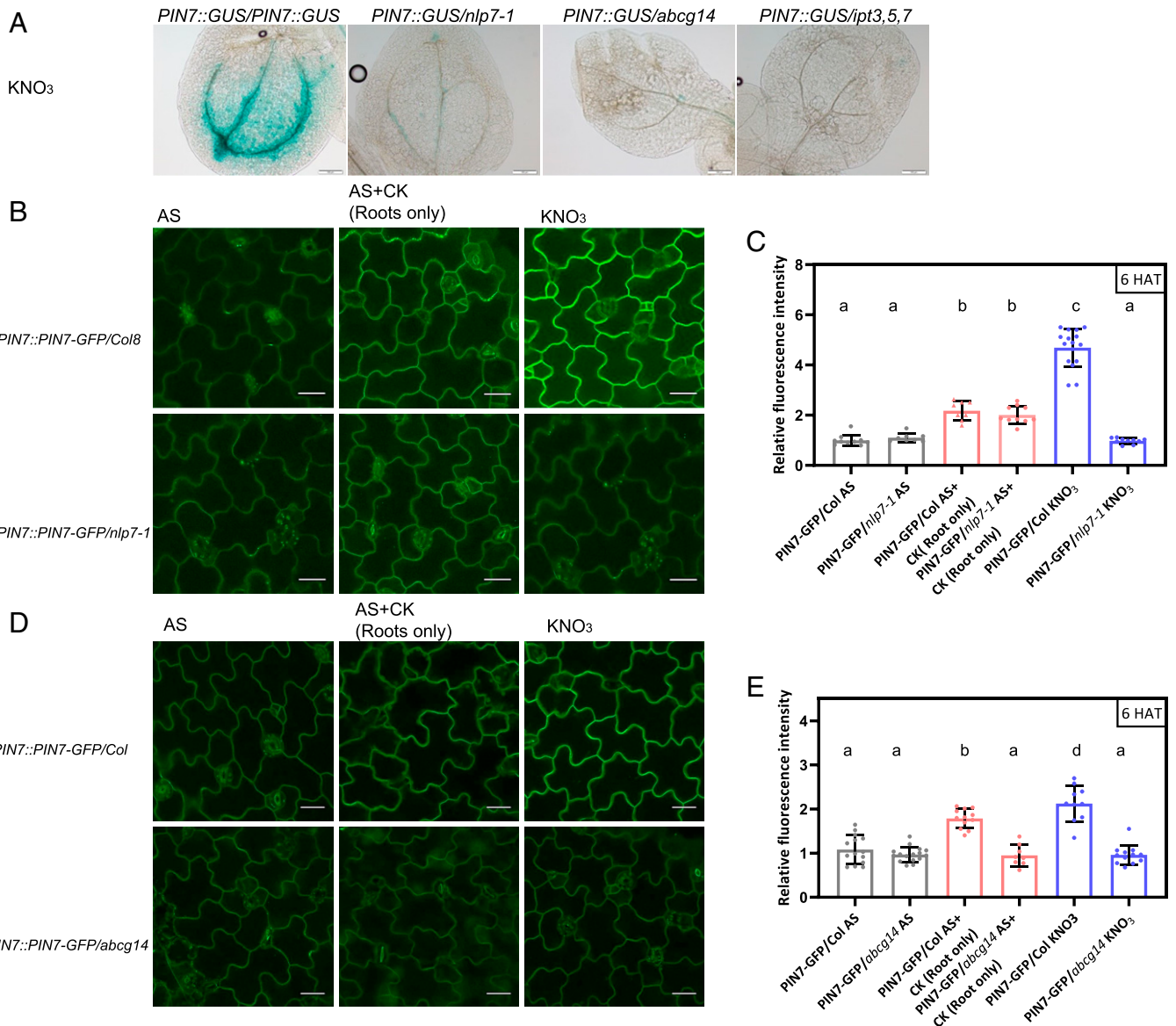
Fig. 5. Nitrate-dependent fine tuning of *PIN* expression in shoots is driven by cytokinin translocated from roots. (*A*) Representative images of *PIN7::GUS* expression in shoots grafted on roots of *PIN7::GUS*, *nlp7*, *abcg14*, or *ipt3,5,7* roots. Grafted seedlings after 48 h of healing on AS, then transferred to KNO$_3$-containing medium for 24 h. (*B–E*) Imaging (*B* and *D*) and quantification (*C* and *E*) of PIN7-GFP membrane signal in adaxial epidermal cells of cotyledons 6 HAT on AS, AS plus cytokinin (CK, 100 nM 6-benzylaminopurine), or KNO$_3$-supplemented media in *nlp7-1* (*B* and *C*) and *abcg14* (*D* and *E*). PIN7-GFP signal was measured in $n = 2$ epidermal cells, four different seedlings per treatment. Experiments were repeated at least twice; results from one experiment are presented. Different lowercase letters indicate significant one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$) (*C* and *E*). (Scale bars in *A*, 100 μm and *B* and *D*, 24 μm.)

of cytokinins (21, 22, 29, 41, 49). Our expression analysis and measurements of cytokinins revealing up-regulation of cytokinin biosynthesis and transport genes after transfer to nitrate in comparison with AS, and enhanced accumulation of cytokinin derivatives such as tZ, tZR, and DHZR, are fully consistent with these reports (20, 43, 49). In light of these findings, CRFs as transcription factors whose expression is stimulated by cytokinins (46), are plausible candidates for regulators that, in response to cytokinins translocated to shoots, adjust the activity of the PAT.

However, a question arises about the molecular components and pathways underlying adjustment of cytokinins to nitrate availability. Our analyses of the mutant lacking functional *NLP7*, a master regulator of the nitrate response, revealed that deficient shoot response of *nlp7* to nitrate provision can be partially recovered by either its grafting on wild-type roots, cytokinin supply to roots, or expression of *CRF2* under a constitutive promoter. Altogether, these observations suggest that NLP7

impinges on the regulation of cytokinin metabolism in roots or transport of the hormone to shoots. In line with these assumptions, expression of the cytokinin biosynthetic gene *IPT3* in roots is stimulated by nitrate in an NLP7-dependent manner. Interestingly, loss of *NLP7* did not significantly interfere with nitrate-induced transcription of *IPT7*, thus hinting at potential redundancies in the regulation of the cytokinin biosynthetic genes by NLP7 homologs (12, 56). Furthermore, enhanced transcription of *ABCG14* in response to nitrate might be part of a positive feedback loop to reinforce the transport of cytokinins to the shoot that seems to be dependent on NLP7 function. Analyses of cytokinins in *nlp7* mutants when compared to wild type further strongly support the role of NLP7 in the adjustment of cytokinin biosynthesis and distribution according to nitrate availability. Thus, our findings extend the previously recognized link between nitrate and cytokinins with NLP7 as a molecular component (20–22, 41), which converges both

regulatory pathways and coordinate distribution of this hormone between roots and shoots.

Recently, chromatin immunoprecipitation sequencing (ChIP-seq) experiments predicted several *CRF* promoters to be directly bound by NLP7 upon its translocation into the nucleus (12, 56). Additionally, the *PIN7* promoter has been detected among the potential genes directly regulated by NLP7 in the protoplast system (56, 57). Thus, alternatively, NLP7 might regulate *PIN7* locally in tissues and organs where their expression coincides. Our findings do not rule out a contribution of such a direct interaction in addition to NLP7-mediated enhancement of cytokinin activity and transport from root to shoot, which might provide an efficient way for the robust *CRF-PIN* activation in the early phases of the shoot adaptation to nitrate.

Altogether, our study reveals a molecular framework orchestrating shoot developmental processes with a root nitrate sensory system. In response to nitrate supply, NLP7 promotes the expression of cytokinin biosynthesis and facilitates cytokinin translocation to shoots. There, CRFs as direct regulators of PINs, stimulate the transport of auxin and thereby promote shoot growth and development.

Materials and Methods

Plant Material. All *Arabidopsis thaliana* lines used in this study are in the Columbia ecotype, detailed information of which is provided in *SI Appendix, Materials and Methods*.

Growth Conditions. Seeds of *A. thaliana* sterilization and seedling growth conditions used in this study and external hormonal treatment with cytokinin (100 nM 6-benzylaminopurine [BAP]) are detailed in *SI Appendix, Materials and Methods*.

Phenotype Analyses of Primary Roots, Lateral Roots, and Cotyledon Surface Area. Seven-day-old seedlings grown on 0.5 mM AS containing medium were transferred on media supplemented with either 0.5 mM AS, or 5 mM KNO_3. Seedlings were imaged using an Epson Perfection V700 flatbed scanner on respective time points for primary root length. For lateral root density and cotyledon surface area measurements an Olympus BX53 microscope and the Leica EZ4HD were used, respectively. Detailed descriptions are provided in *SI Appendix, Materials and Methods*.

Pavement Cell Imaging and Cell Size Measurements. For observation of cotyledon pavement cells, the adaxial side of the cotyledon surface was placed on melted 2% (wt/vol) low-melt agar containing 0.01% (wt/vol) bromophenol blue on a glass slide according to Horiguchi et al. (58). For details refer to *SI Appendix, Materials and Methods*.

Shoot Fresh Weight Measurement. Seven-day-old seedlings grown on AS were transferred to KNO_3 containing medium and grown for 14 d vertically under the previously reported growth conditions. The experiments consisted of three biological replicates, each replicate containing eight seedlings. On the 14th day the shoots were excised and weighed on a VWR analytical balance (series no. IT1301517). Presented are average shoot fresh weights per seedling. Significant differences were calculated by Tukey's test following a one-way ANOVA. Details are in *SI Appendix, Materials and Methods*.

GUS (β-Glucuronidase) Staining. *GUS* expression was analyzed in 7-d-old seedlings transferred for 6 h on AS or KNO_3 supplemented media. Seedlings were incubated in staining buffer containing 1 mM of ferro- and ferricyanide, 150 mM sodium phosphate buffer (pH 7) and 1 mg/mL of X-Gluc (dissolved in Dimethyl sulfoxide (DMSO)) at 37 °C for 4 to 24 h (time adjusted to the strength of *GUS* expression in individual lines). For more details refer to *SI Appendix, Materials and Methods*.

qRT-PCR Analysis. Total RNA was extracted from excised 7-d-old seedling's roots and shoots 1 and 6 HAT to AS- or KNO_3-containing plates using the RNeasy Plant Mini kit from (Qiagen) according to the manufacturer's protocol. A total of

1 and 0.5 μg of RNA was used to synthesize cDNA (shoot and root, respectively) using the iScriptTM cDNA synthesis kit (Bio-Rad). The analysis was carried out on a LightCycler 480 II (SW1.5.1 version; Roche Diagnostics) with the SYBR Green I Master kit (Roche Diagnostics) according to the manufacturer's instructions. All PCR reactions were carried out with three biological and technical triplicates. The levels of expression of each gene were first measured relative to *AT4G05320* (*UBQ10*) and then to respective mock treatment (AS). For more details refer to *SI Appendix, Materials and Methods*.

Grafting. The 7-d-old seedlings were grafted according to the hypocotyl-grafting procedure provided in ref. 59. For detailed description of grafting refer to *SI Appendix, Materials and Methods*.

Imaging and Image Analysis of PIN-GFP Expression. The 7-d-old seedlings were transferred onto the AS- or KNO_3-containing media for 3 h, then mounted onto slides in a droplet of water and imaged with a Zeiss, LSM800 vertical confocal microscope equipped with a 20×/0.8 Plan-Apochromat M27 objective. Fluorescence signals for GFP (excitation 488 nm, emission 507 nm) and chlorophyll A signal for autofluorescence (excitation 640 nm, emission 645 to 700 nm) to verify the authenticity of membrane-bound signal were detected. Maximum intensity Z-stack projections of confocal images of at least four cotyledons from four different seedlings per treatment were used. To monitor *PIN7::PIN7-GFP* expression pattern, cotyledons in their middle part were imaged. The membrane PIN-GFP signal of two epidermal cells at the adaxial side per cotyledon was measured using the segmented line function to mark the cell membrane to obtain a quantification of mean gray value using Fiji (v. 152). Significant differences were calculated by Tukey's test following one-way ANOVA. Experiments were conducted two to three times; representative images and quantification from one experiment are shown.

For monitoring *PIN1::PIN1-GFP*, a clearing method using Clearsee solution was utilized to diminish chlorophyll autofluorescence and to enhance deep imaging and optical sectioning of the vasculature extending between the petiole and the first vasculature branching junction in the cotyledon. The clearing process was performed according to manufacturer protocols (60). Maximum intensity Z-stack projection images of at least four different cotyledons from four different seedlings per treatment were obtained. The plasma membrane of cells within the vasculature were marked using the segmented line function in Fiji (v. 152) and a mean gray value as a quantification of PIN1-GFP intensity was obtained. Experiments were repeated a total of two to three times; representative images and quantification from one repetition are shown. Significant differences were calculated by Tukey's test following a one-way ANOVA.

Measurements of Endogenous Cytokinins. Quantification of cytokinin metabolites was performed according to the method described in ref. 61, including modifications described in ref. 62. For more information refer to *SI Appendix, Materials and Methods*.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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