Supplementary Information for

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

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Supplementary text

Figures S1 to S5

Tables S1 to S2

SI References

Other supplementary materials for this manuscript include the following:

None

Supplementary Information text

Supplementary Material and Methods

Plant material

All *A. thaliana* lines used in this study are in the Columbia ecotype. The mutant lines in this study are; *pin7-2*[1], *pin4-3*[2], *crf2-1*[3], *crf2-2*[3], *crf3-1*[3], *crf6-1*[3], *crf2,3,6*[3] , *RPS5a::CRF2*[4], *35S::CRF6*[3], *35S::CRF3*[3], *nlp7-1*[5], *nlp7-2*[5], *ipt3,5,7*[6], *abcg14*[7], *ahk2ahk3*[8]. The following previously described GUS marker lines were used*; PIN1-GUS*[1], *PIN3-GUS*[9], *PIN4-GUS*[10], *PIN6-GUS*[1], *PIN7-GUS*[1], *CRF2-GUS* [3], *CRF3-GUS*[11], *CRF6-GUS*[11], and *NLP7-GUS*[5], *ARR5-GUS*[12] all in Col0 background. The green fluorescent protein marker lines used in this work were previously described; *PIN1::PIN1-GFP*[3], *ΔPIN1::PIN1-GFP*[3] in Col0, their expression in *pin1-2* was obtained by crossing *PIN1::PIN1-GFP*[3], *ΔPIN1::PIN1-GFP*[3] with heterozygote *pin1-201* after crossing selection was based on the presence of GFP signal in seedlings, and are homozygote for T-DNA insertion of *pin1-201* using genotyping primers reported in the table below. *PIN7::PIN7-GFP*[3]*, ΔPIN7::PIN7-GFP in pin7-2*[3], *CRF2::CRF2-GFP*[3] in Col0, *NLP7::NLP7-GFP*[5], *35S:: GFP-NLP7*[5].

Growth conditions

Seeds of *A. thaliana* were surface-sterilized with 70% ethanol and sown on a modified Murashige and Skoog (MS) medium; Boric Acid 6.2 mg/L, Calcium Chloride (anhydrous) 332.2 mg/L, Cobalt Chloride ($6H_2O$) 0.025 mg/L, Cupric Sulfate ($5H_2O$) 0.025 mg/L, Na_2EDTA ($2H_2O$) 37.26 mg/L, Ferrous Sulfate ($7H_2O$) 27.8 mg/L, Magnesium Sulfate (anhydrous)180.7 mg/L, Molybdic Acid (disodium salt $2H_2O$) 0.25 mg/L, Potassium Iodide 0.83 mg/L, Potassium Phosphate (monobasic, anhydrous) 170 mg/L, Zinc Sulfate ($7H_2O$) 8.6 mg/L; supplemented with 0.1% sucrose and 1% agar (Type E, Sigma A4675), pH=5,8. As a nitrogen source 0.5mM Ammonium Succinate (AS, Santa Cruz Biotechnology) (76 mg/L) or 5mM Potassium Nitrate KNO_3 (505 mg/L) was added. Seeds were stratified at least for 2 days and grown for 7-21 days at 21 ^oC in a 16 h light/8 h dark cycle.

Hormonal treatment such as application of cytokinin to roots only was performed as follows: The MS medium (see above) supplemented with 0.5mM Ammonium Succinate and cytokinin (100nM 6-Benzylaminopurine; BAP) was poured in square petri-dishes and after

solidification the upper ~2cm large band of medium was removed. 7-day-old seedlings were carefully placed on the medium so that only roots were in contact with the agar.

Primary root growth

7-day-old seedlings grown on 0.5mM AS containing medium were transferred on media supplemented with either 0.5mM AS, or 5mM KNO_3. Seedlings were imaged using Epson Perfection V700 flatbed scanner daily for 4 consecutive days. The length of roots was measured manually using Fiji (V1.52) and root growth rate calculated as an increase in root length per day. Statistical evaluation was performed using Tukey's comparison test following a one-way ANOVA.

Lateral root density and developmental stage analyses

7-day-old seedlings grown on AS were transferred on media supplemented with either AS or KNO_3. After 4 days seedlings were cleared according to the protocol as previously published[13]. Briefly, seedlings were incubated in 4% HCl, 20% methanol at 67°C for 5 min, in 7% (w/v) NaOH, 60% ethanol at room temperature (RT) for 15 min, followed by incubation in a series of ethanol dilutions from 75% to 10% at RT. Seedlings were mounted on glass slides in drop of mounting medium (5%ethanol, 50%glycerol)[13,14]. The developmental stages of LRs and LRP in 8 seedlings per treatment were observed using Olympus BX53 microscope. The length of roots was measured manually using Fiji (V1.52) and LRP density calculated. Significant differences between LRP densities at each stage were determined using Tukey's multiple comparisons test following a one-way ANOVA.

Cotyledons surface area analysis

7-day-old seedlings grown on medium containing AS were either collected (D0), their shoots excised and mounted on slides in a drop of 25% glycerol, or transferred to AS or KNO_3 containing media for 24h and afterword shoots collected and processed as described for D0. The Leica EZ4HD stereomicroscope was used to obtain images of cotyledons and their surface area was measured manually using Fiji (v1.52). The growth of cotyledons was evaluated as the relative increase in surface area 24HAT on AS or KNO_3 containing media when compared to surface area of cotyledons at D0. 6-8 seedlings per treatment analyzed,

experiment repeated 2-3 times. Results from one representative experiment are presented. Significant differences were calculated by Tukey's Test following one-way ANOVA followed.

Pavement cell imaging and cell size measurements

For observation of cotyledon pavement cells, the adaxial side of cotyledon surface was placed on melted 2% (w/v) low-melt agar containing 0.01% (w/v) bromophenol blue on a slide glass according to (Horiguchi *et al.,*2006)[15]. The cotyledon was peeled off when the agar began to dry. Completely dried agar was observed under Olympus BX53 microscope equipped with Olympus DP26 digital camera controlled by cellSense Entry software. The digital images of cotyledons surfaces were processed using CellSeT program to extract automated map of pavement cells [16]. The surface area of each cell in the map obtained by CellSeT program was measured automatically using the analyze particle function in Fiji (v1.52) program excluding the edges.

Shoot fresh weight measurement

7-day-old seedlings grown on AS were transferred to KNO_3 containing medium and grown for 14 days vertically under the previously reported growth conditions. The experiments consisted of three biological replicates, each replicate containing 8 seedlings. On the 14th day the shoots were excised and weighed on VWR analytical balance (series no.IT1301517). Presented are average shoot fresh weight per a seedling. Significant differences were calculated by Tukey's Test following a one-way ANOVA.

For *pin1-201* phenotype analysis, a heterozygote T-DNA insertion line was used. A total of 74 seedlings were genotyped of which 17 homozygote seedlings were identified. A representative image of *pin1-201* and Col0 seedlings grown on the same plates presented.

For shoot fresh weight (SI Apendix, Fig.S1. K, L) seedlings were grown (0.5×) Murashige and Skoog (MS) medium (Duchefa) with 1% (w/v) sucrose and 1% (w/v) agar (pH 5.9) for 21 days, then handled as above.

GUS (β-Glucuronidase) staining

GUS expression was analyzed in 7-day-old seedlings transferred for 6 hours on AS or KNO_3 supplemented media. Seedlings were incubated in staining buffer containing 1mM of Ferro-

Ferricyanide, 150mM sodium phosphate buffer (pH 7) and 1mg/ml of X-Gluc (dissolved in DMSO) at 37°C for 4-24 hours (time adjusted to the strength of GUS expression in individual lines). Seedlings were cleared as described above and mounted on slides in droplets of 5%ethanol-50%glycerol mounting solution. The GUS histochemical staining was analyzed using Olympus BX53 microscope equipped with Olympus DP26 digital camera controlled by cellSense Entry software.

RT-qPCR analysis

Total RNA was extracted from excised 7-day-old seedling's roots and shoots 1 and 6 HAT to ammonium or nitrate containing plates using RNeasy® Plant Mini kit from (QIAGEN) according to the manufacturer's protocol. 1 and 0.5µg of RNA was used to synthesize cDNA (shoot and root respectively) using 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. Expression levels of target genes were quantified by specific primers that were designed using Quant Prime[17], and validated by performing primer efficiency for each primers pair. The levels of expression of each gene were first measured relative to *AT4G05320* (*UBQ10*) and then to respective mock treatment (AS). Significant differences between expression level of a certain gene on nitrate and AS were calculated using two tailed t test. Full list of qPCR primers provided in the supplementary information section (SI Appendix, Supplementary Table 2).

Grafting

7-day-old seedlings were grafted according to the hypocotyl-grafting procedure by [18]. After a transverse cut at the hypocotyl, root and shoot parts were aligned within the silicon collar (Helix Medical, dimension 0.31mm diameter). Grafted seedlings were maintained for 48 hours (for phenotype grafts) and 72 hours (for GUS staining grafts) on AS containing plates to heal, and then they were transferred to KNO_3 containing medium for either 14 days (extended Fig. 4A) or 24 hours (experiments Fig.4C, Fig 5A, SI Appendix Fig.S4E).

Grafted seedlings 24 hours after transfer on KNO_3 containing medium were subjected to GUS reporter expression analyses. Typically, scions of *PIN7::GUS* or *ARR5::GUS* reporter lines

were grafted on roots of either wild type or various mutant lines. At least 4 successful grafts per combination were analyzed for *GUS* expression. The experiments were repeated 2 times.

In grafted seedlings grown for 14 days on KNO_3, adventitious roots were routinely removed and valid grafts determined on the basis of healthy primary root growth. Grafted seedlings were imaged using Epson Perfection V700 flatbed scanner. A total of 43 different grafts were performed, 33 were considered successful (evident by restoration of root growth). The reported phenotype was visible in 70% of the successful grafts.

Imaging and image analysis of PIN-GFP expression

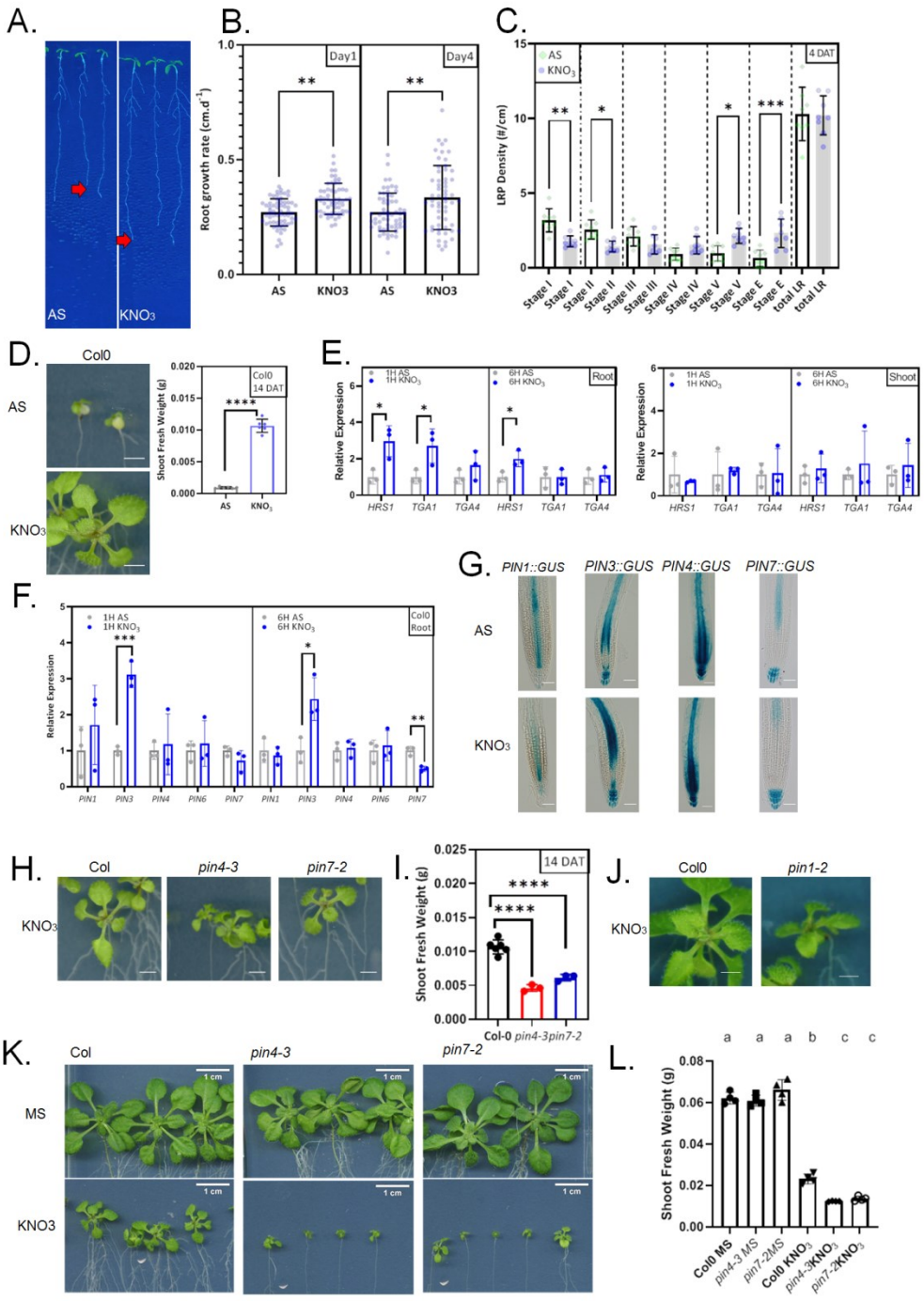
7-day-old seedlings were transferred on the AS or KNO_3 containing media for 3 hours, afterward mounted on slides in a droplet of water and imaged with Zeiss, LSM800 vertical confocal microscopes equipped with a 20×/0.8 Plan-Apochromat M27 objective. Fluorescence signals for GFP (excitation 488 nm, emission 507 nm) and Chlorophyl A signal for auto-fluorescence (excitation 640nm, emission 645-700 nm) to verify the authenticity of membrane bound-signal were detected. Maximum intensity Z-stack projections of confocal images of at least 4 cotyledons from 4 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 adaxial side per cotyledon was measured using the segmented line function to mark the cell membrane to obtain a quantification of mean grey value using Fiji (v152). Significant differences were calculated by Tukey's Test following one-way ANOVA. Experiments were conducted 2-3 times, representative images and quantification from one experiment are shown.

For monitoring *PIN1::PIN1-GFP*, clearing method using Clearsee® solution was utilized to diminish chlorophyll autofluorescence and to enhance deep imaging and optical sectioning of the major vasculature extending between the petiole and the first vasculature branching junction in the cotyledon. Clearing process was performed according to the manufacturer protocols[19]. Maximum intensity Z-stack projection images of at least 4 different cotyledons of 4 different seedlings per treatment were obtained. Plasma membrane of cells within vasculature were marked using the segmented line function in Fiji (v152) and a mean grey value as a quantification of PIN1-GFP intensity obtained. Experiments were repeated 2-3

times; representative images and quantification from one repetition are shown. Significant differences were calculated by Tukey's Test following one-way ANOVA.

Measurements of endogenous cytokinins

Quantification of cytokinin metabolites were performed according to the method described by [20] including modifications described by [21]. Samples (20 mg FW) were homogenized and extracted in 1 ml of modified Bieleski buffer (60% MeOH, 10% HCOOH and 30% H_2O) together with a cocktail of stable isotope-labeled internal standards (0.25 pmol of CK bases, ribosides, *N*-glucosides, and 0.5 pmol of CK *O*-glucosides, nucleotides per sample added). The extracts were applied onto an Oasis MCX column (30 mg/1 ml, Waters) conditioned with 1 ml each of 100% MeOH and H_2O, equilibrated sequentially with 1ml of 50% (v/v) nitric acid, 1 ml of H_2O, and 1 ml of 1M HCOOH, and washed with 1 ml of 1M HCOOH and 1 ml 100% MeOH. Analytes were then eluted by two-step elution using 1 ml of 0.35M NH_4OH aqueous solution and 2 ml of 0.35M NH_4OH in 60% (v/v) MeOH solution. The eluates were then evaporated to dryness *in vacuo* and stored at -20°C. Cytokinin levels were determined by an ultra-high performance liquid chromatography-electrospray tandem mass spectrometry (UHPLC-MS/MS) using stable isotope-labelled internal standards as a reference (Rittenberg and Foster, 1940). Separation was performed on an Acquity UPLC® System (Waters, Milford, MA, USA) equipped with an Acquity UPLC BEH Shield RP18 column (150x2.1 mm, 1.7 µm; Waters), and the effluent was introduced into the electrospray ion source of a triple quadrupole mass spectrometer Xevo™ TQ-S MS (Waters). Five independent biological replicates were performed.

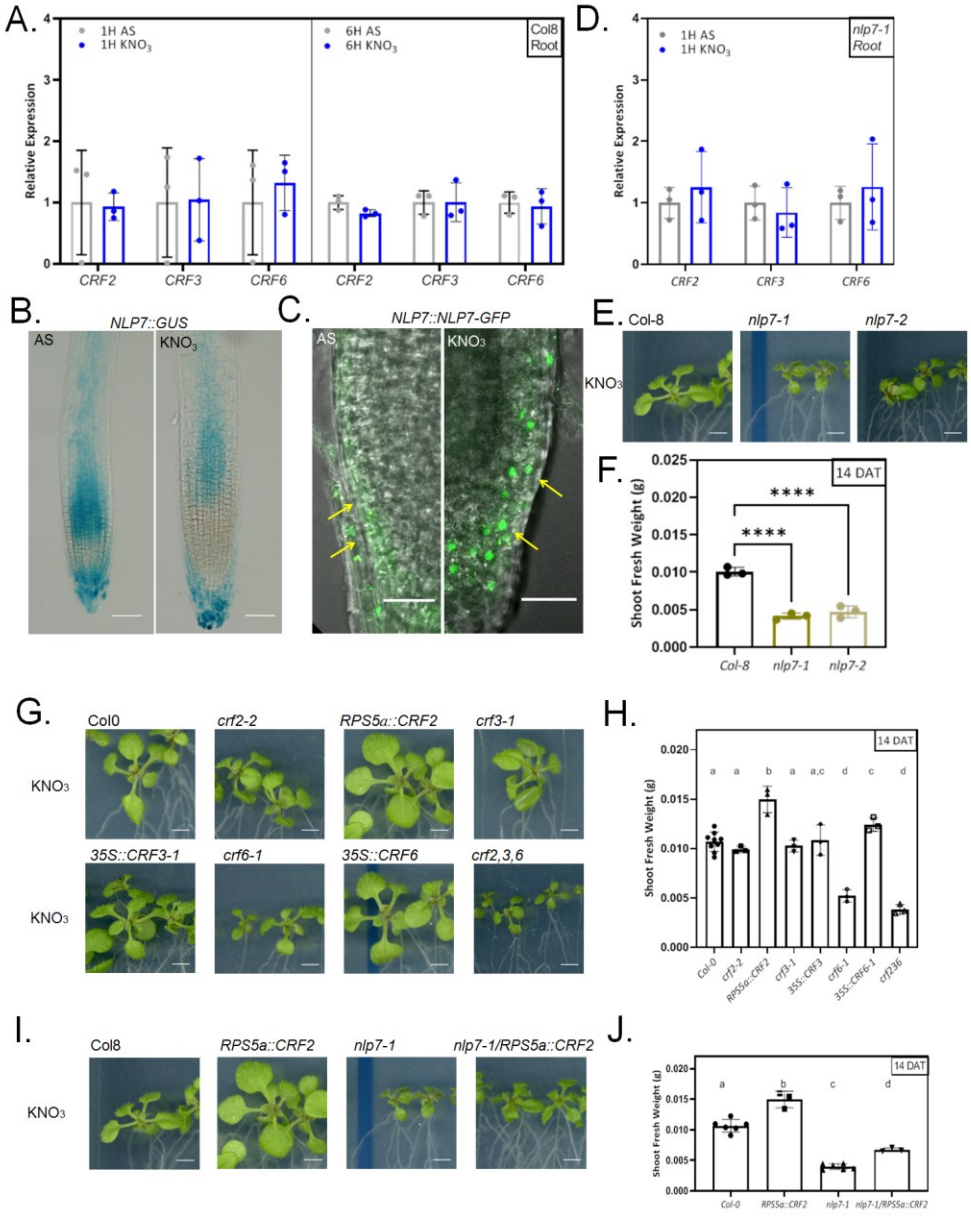


None

Fig. S1. Modulation of *PIN* expression is part of nitrate-mediated transcriptional reprogramming

(**A**) Wild-type (Col0) seedlings grown for 7 days on AS and transferred for 4 days to either AS or KNO_3 containing media; red arrows indicate primary root tips. **(B, C)** Root growth rate (B) and lateral root primordia (LRP) density (C) quantified after transfer of 7-day-old seedlings for one (B) and four (B,C) days on AS or KNO_3. Significant differences determined by one-way ANOVA followed by Tukey's multiple comparison test, **P<0.01, n=60 seedlings (B) * P<0.05,**P<0.01, ***P<0.001 (C) n= 8 seedlings/treatment. **(D)** Representative images of shoots of wild type (Col0) seedlings 14 DAT to AS and KNO_3 and quantification of shoot fresh weight.

Significant differences determined by Student's t test, ****p<0.0001 (n= 3 biological replicates, 8 shoots per biological replicate) **(E,F)** RT-qPCR analysis of early nitrate responsive genes in Col0 roots and shoots (E) and *PIN* genes in Col0 roots (F) normalized to *UBQ10 (AT4G05320*) levels 1 and 6 HAT to AS or KNO_3 containing medium. All RT-qPCR reactions were carried out with biological and technical triplicates. Statistical difference determined by Student's t-test * P<0.05,**P<0.01 ***P< 0.001. **(G)** Expression analyses of *PIN::GUS* reporters in roots of 7-day-old seedlings 6 HAT to AS and KNO_3. (**H-J**) Representative images of shoots of Col0, *pin4-3, and pin7-2* seedlings (H), and Col0 and *pin1-2* (J) 14 DAT on AS and KNO_3 containing media (H,J) and quantification of shoot fresh weight (I). Significant differences determined by one-way ANOVA followed by Tukey's multiple comparison test, ****p<0.0001, (n=3 biological replicates, 8 shoots per biological replicate) (I). Scale bars 4 mm (D,H,J), 50 µm(G) . (**K**,**L**) Representative images (K) and fresh weight quantification (L) of shoots of Col0, *pin4-3, and pin7-2* seedlings grown either continuously for 21 days on MS media (upper panel) or after 7 days on AS transferred for 14 days on KNO_3 containing media (lower panel). Significant differences determined by one-way ANOVA followed by Tukey's multiple comparison test, different lower case letter indicate (*p<0.05), (n=4 biological replicates, 8 shoots per biological replicate), Scale bars 1 cm (K).



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232 **Fig. S2. Cytokinin response factors mediate shoot developmental adaptations to nitrate**

233 **(A,D)** RT-qPCR expression analysis of *CRF* genes normalized to *UBQ10 (AT4G05320*) 1 and 6 HAT on AS or KNO_3

234 containing media in Col8 (A) and *nlp7-1* (D) roots. All RT-qPCR reactions were carried out with biological and

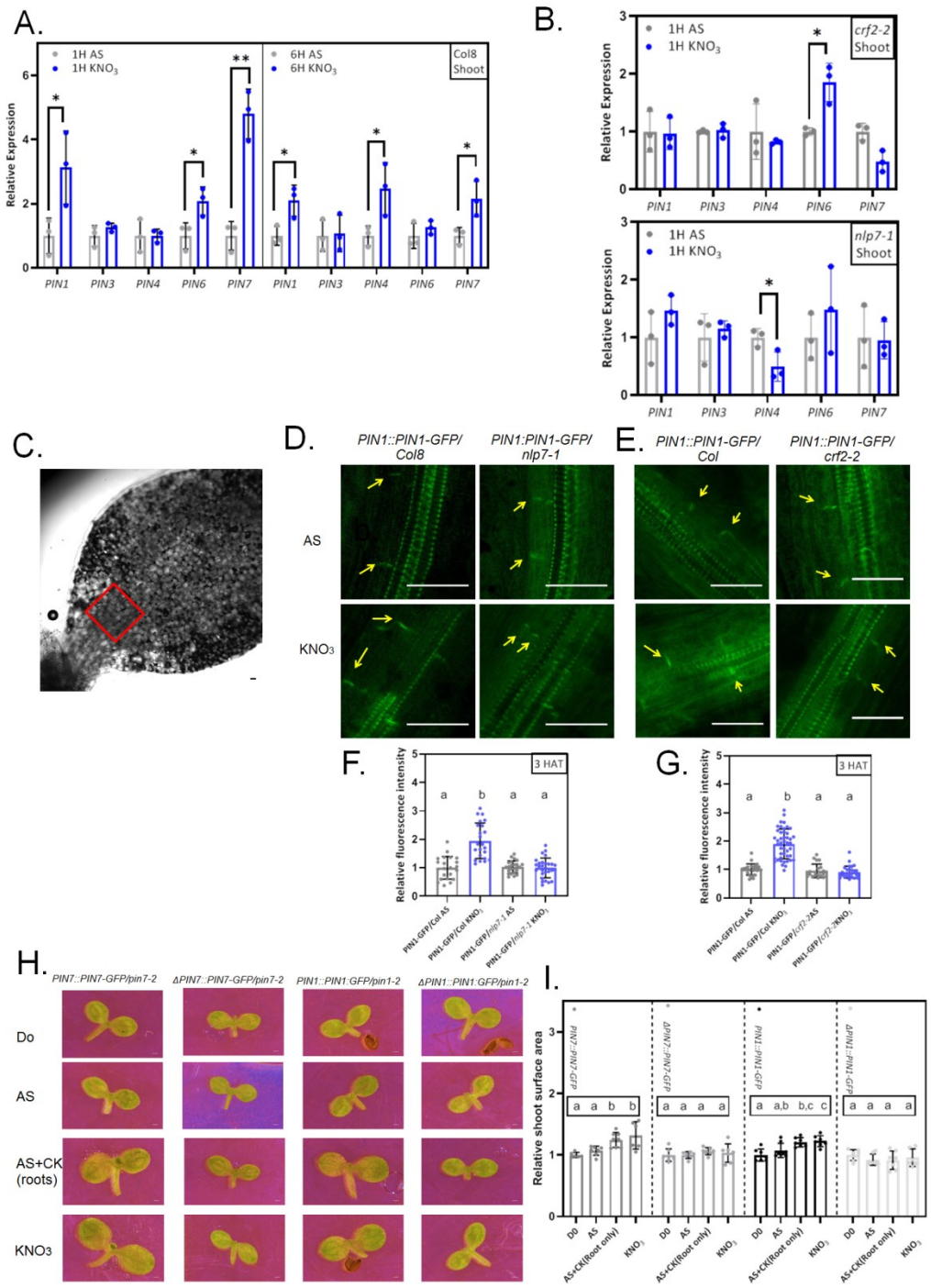
235 technical triplicates. Statistical difference determined with Student's t-test *P<0.05,**P<0.01. **(B,C)** Expression

236 analyses of *NLP7::GUS* reporter in roots of 7-day-old seedlings 6 HAT to AS or KNO_3 containing media (B) and of

237 *NLP7::NLP7-GFP* 30 minutes after transfer to AS or KNO_3 (C). Yellow arrows indicate the nuclear localization of

238 NLP7 on KNO_3. **(E,F)** Representative images of shoots from 7-day-old wild type (Col8) and *nlp7* seedlings 14

DAT on AS or KNO_3 containing media (E) and quantification of shoot fresh weight (F). Significant differences determined by one-way ANOVA followed by Tukey's multiple comparison test, ****p<0.0001 (n= 3 biological replicates, 8 shoots per biological replicate). **(G-J)** Representative images of shoots from 7-day-old wild type (Col0) and transgenic lines 14 DAT on AS and KNO_3 (G,I) and quantification of shoot fresh weight (H,J). Significant differences determined by one-way ANOVA followed by Tukey's multiple comparison test, different lower case letter indicate (P<0.05) (n= 3 biological replicates**,** 8 seedlings per biological replicate). Scale bars 50 µm (B), 24 µm (C), 4 mm (E,G,I).



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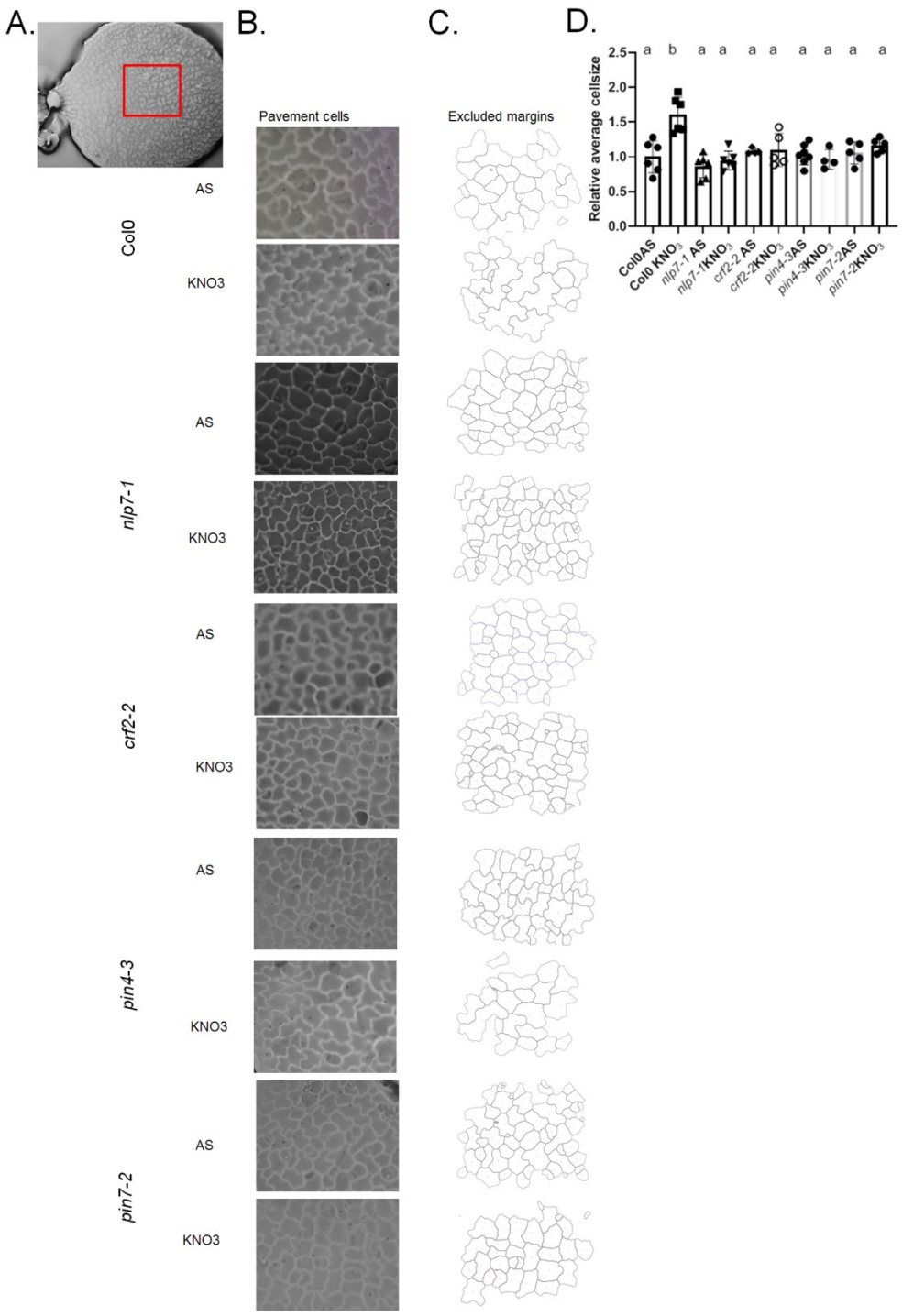
251 **Fig. S3. NLP7 fine-tunes expression of *PINs* and shoot developmental adaptation to nitrate through CRFs**

252 **(A,B)** RT-qPCR analysis of *PIN* genes normalized to *UBQ10 (AT4G05320*) levels 1 HAT to AS or KNO_3 containing
253 medium in Col8 shoots (A) *nlp7-1* and in *crf2-2* shoots (B). All RT-qPCR reactions were carried out with
254 biological and technical triplicates. Statistical difference determined with a Student's t-test * P<0.05. (**C-J**)
255 Representative image of a cotyledon, red square indicate imaging area(C). Monitoring (D,E) and quantification

(F,J) of PIN1-GFP membrane signal in vasculature (yellow arrows) of cotyledons 3HAT to AS or KNO_3 supplemented media in *nlp7-1* (D,F) and *crf2-2* (E,G). Different lower-case letters indicate one-way ANOVA followed by a Tukey's multiple comparison test ($P< 0.05$). n= at least 2 cells of vasculature in at least 4 different seedlings per treatment measured. **(H,I)** Imaging (G) and surface area quantification (H) of cotyledons from 7-day-old *PIN7::PIN7-GFP,pin7-2, ΔPIN7::PIN7-GFP,pin7-2, PIN1::PIN1-GFP,pin1-2* and *ΔPIN1::PIN1-GFP,pin1*-2 seedlings grown on AS (D0), and 24 HAT to either AS, AS supplemented with 100nM cytokinin (CK, 6-Benzylaminopurine) or KNO_3 containing media. Relative size quantified as cotyledon mean surface area on AS or AS plus CK or KNO_3 24 HAT compared to D0 (n=6-8 seedlings per treatment). Experiment repeated twice; results from one representative experiment are presented. Different lower-case letters indicate the significant One-way ANOVA followed by Tukey's multiple comparison test (at least $P< 0.05$) **(H)**. Scale bars 12 µm (D,E), 100 µm (H).



<u>Fig. S4.</u> Pavement cell size growth 24HAT to AS and KNO_3 containing media

(**A-C**) Pavement cells at the adaxial side in the middle part of cotyledons (A, red square) were visualized [15](B), automated map of pavement cell extracted using CellSeT program [16](C) and average cell size was quantified (**D**). Seedlings of wild-type, *nlp7*, *crf2-2*, *pin4-3* and *pin7-2* mutants were grown for 7 days on media supplemented with AS and transferred to either AS or nitrate containing medium for 24 hours. The relative cell size when compared to Col0 on AS calculated (n=4-7 seedlings, 2 biological repeats). Different lower-case

276 letters indicate the significant One-way ANOVA followed by Tukey's multiple comparison test (at least *P<
277 0.05)

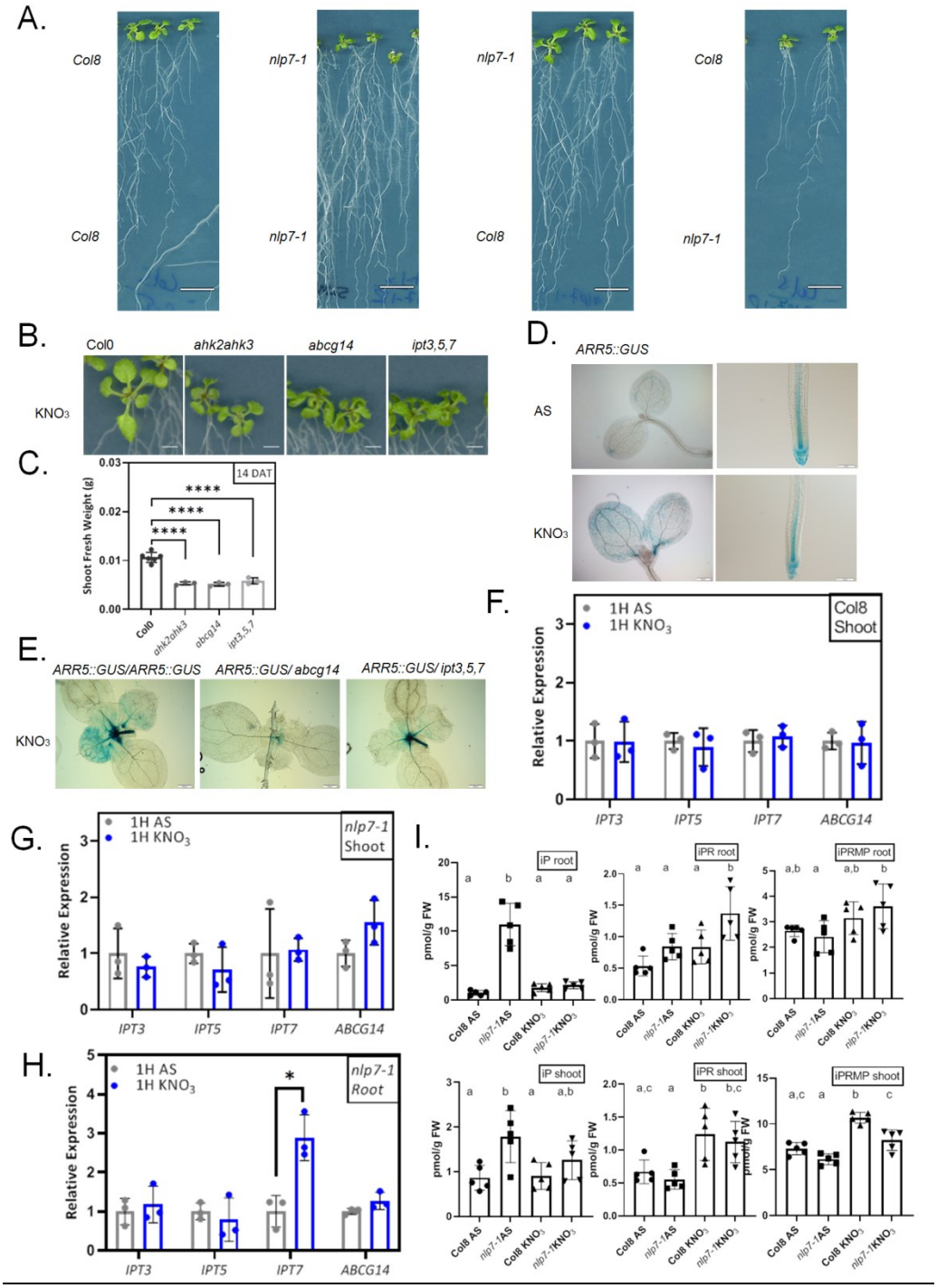


Fig. S5. NLP7-mediated signaling in roots controls cytokinin levels in shoots

(A) Representative images of grafted seedlings and shoots (close-ups) of Col8R(root)/Col8S(shoot), *nlp7*-1R/*nlp7-1S*, Col8R/*nlp7-1S* and *nlp7*-1R/Col8S 14DAT to KNO_3 containing medium. **(B,C)** Representative images of shoots of 7-day-old wild type Col0, *ahk2ahk3, abcg14,* and *ipt3,5,7* seedlings 14 DAT to KNO_3 (B) and shoot fresh weight quantification (C). Significant differences determined by one-way ANOVA followed by Tukey's

multiple comparison test, ****p<0.0001 (n= 3 biological replicates, 8 shoots per biological replicate). **(D)** Expression analyses of *ARR5::GUS* in shoots of 7-day-old seedlings 6 HAT to AS orKNO_3 containing media. **(E)** Representative images of *ARR5::GUS* expression in the shoots grafted on roots of *ARR5::GUS*, *abcg14* or *ipt3,5,7*, respectively. Grafted seedlings after 72 hours of healing on AS medium, then transferred to KNO_3 for 24 hours. **(F-H)** RT-qPCR analysis of *IPTs* and *ABCG14* expression normalized to *UBQ10 (AT4G05320*) in Col8 shoots (F) and *nlp7-1* shoots (G) and roots (H) 1 HAT to AS or KNO_3 containing media. All RT-qPCR reactions were carried out with biological and technical triplicates. Statistical difference was calculated with a t-test *P<0.05. **(I).** Quantification of *isopentenyladenine* (*iP)*, *isopentenyladenine riboside* (*iPR) isopentenyladenine riboside 5'-monophosphate*(*iPRMP*) in roots and shoots of Col8 and *nlp7-1* seedlings 6 HAT on AS and KNO_3 containing media. Different lower-case letters indicate one-way ANOVA followed by Tukey's multiple comparison test (P< 0.05) (n= 5 biological replica/genotype/treatment). Scale bars 4mm (B), 200 µm shoots, 50 µm roots (D), 200µm (E).

Tissue	Line	Treatment	Total iP-types		iP7G	iP9G
AS	Col8	Root	34.39 ± 2.04		26.45 ± 1.51	3.72 ± 0.15
	nlp7-1		**40.39 ± 4.55**	*	**22.81 ± 2.57**	* 3.33 ± 0.40
KNO3	Col8		34.77 ± 3.77		25.30 ± 2.55	3.69 ± 0.40
	nlp7-1		37.85 ± 1.26		26.83 ± 0.48	3.88 ± 0.26
AS	Col8	Shoot	85.30 ± 2.29		65.83 ± 3.09	10.63 ± 0.58
	nlp7-1		84.62 ± 3.02		64.94 ± 3.56	11.19 ± 0.83
KNO3	Col8		92.81 ± 5.15		68.52 ± 4.70	11.47 ± 0.54
	nlp7-1		87.91 ± 5.06		66.60 ± 3.96	**10.71 ± 0.23** *

Tissue	Line	Treatment	Total tZ-types		tZRMP	tZOG	tZROG	tZ7G	tZ9G	
AS	Col8	Root	28.24 ± 4.57		<LOD	9.19 ± 2.23	0.08 ± 0.02	7.63 ± 0.58	10.46 ± 2.45	
	nlp7-1		**19.91 ± 2.29**	*	<LOD	6.88 ± 0.84	0.11 ± 0.02	**4.90 ± 0.72** ***	**7.34 ± 1.08**	*
KNO3	Col8		26.31 ± 3.39		<LOD	8.74 ± 1.58	0.14 ± 0.04	7.07 ± 0.77	8.99 ± 1.56	
	nlp7-1		26.49 ± 1.18		<LOD	9.56 ± 0.98	0.18 ± 0.03	6.57 ± 0.10	8.56 ± 0.42	
AS	Col8	Shoot	45.04 ± 5.61		4.12 ± 0.94	10.35 ± 1.57	0.74 ± 0.19	17.43 ± 0.88	12.58 ± 1.80	
	nlp7-1		45.59 ± 4.60		3.34 ± 1.16	12.03 ± 1.45	0.68 ± 0.12	**15.54 ± 0.26** **	13.00 ± 2.03	
KNO3	Col8		52.80 ± 3.11		6.35 ± 1.82	12.37 ± 0.97	0.87 ± 0.16	18.06 ± 0.69	12.48 ± 0.57	
	nlp7-1		**48.12 ± 1.49**	*	4.37 ± 0.89	12.95 ± 1.03	0.71 ± 0.13	**15.85 ± 0.95** **	12.27 ± 0.31	

Tissue	Line	Treatment	Total cZ-types		cZ	cZR	cZRMP	cZROG	cZ7G	cZ9G
AS	Col8	Root	128.54 ± 6.87		0.71 ± 0.05	2.05 ± 0.21	18.92 ± 4.71	2.25 ± 0.17	98.23 ± 1.43	6.38 ± 1.72
	nlp7-1		113.94 ± 15.56		0.68 ± 0.04	**2.67 ± 0.37** *	14.60 ± 4.38	**2.74 ± 0.37** *	88.32 ± 13.46	4.92 ± 0.69
KNO3	Col8		129.51 ± 8.48		0.86 ± 0.15	2.43 ± 0.38	19.07 ± 2.61	2.58 ± 0.56	98.44 ± 8.12	6.13 ± 1.09
	nlp7-1		130.95 ± 6.19		0.93 ± 0.07	**3.42 ± 0.63** *	16.08 ± 1.59	2.78 ± 0.42	100.86 ± 8.31	6.88 ± 0.94
AS	Col8	Shoot	161.77 ± 6.56		0.36 ± 0.04	2.45 ± 0.34	15.74 ± 3.52	7.17 ± 1.22	130.43 ± 7.36	5.62 ± 1.13
	nlp7-1		164.38 ± 3.38		0.39 ± 0.03	2.25 ± 0.15	16.66 ± 2.85	5.69 ± 0.68	133.01 ± 2.75	6.38 ± 1.10
KNO3	Col8		174.37 ± 11.38		0.36 ± 0.03	2.91 ± 0.23	23.10 ± 4.35	7.33 ± 0.54	134.11 ± 9.36	6.56 ± 0.15
	nlp7-1		165.38 ± 9.36		0.37 ± 0.02	**2.37 ± 0.24** *	18.32 ± 4.21	**5.74 ± 0.46** **	131.59 ± 7.61	6.98 ± 0.38

<u>Supplementary Table 1.</u> Cytokinin content in wild type (Col8) and *nlp7-1* shoots and roots

Quantification of cytokinins in roots and shoots of Col8 and *nlp7-1* seedlings 6 HAT to AS or KNO_3 containing media, mean value and standard deviation from 5 biological replicas, significant difference calculated unpaired t test, *P<0.05.

Abbreviations:

iP7G, isopentenyladenine 7-glucoside, iP9G; isopentenyladenine 9-glucoside
tZRMP; tZ riboside 5'-monophosphate, tZOG; transzeatin O-glucoside, tZROG; trans-zeatin-O-glucoside riboside, tZ7G; trans-zeatin N7-glucoside, tZ9G; trans-zeatin N9-glucoside
cZ ;cis-zeatin, cZR; cis-zeatin riboside, cZRMP ; cis-zeatin riboside-5' –monophosphate, cZROG; cis-zeatin-O-glucoside riboside, cZ9G; cis-zeatin N9-glucoside, cZ7G; cis-zeatin N7-glucoside.

Primer Name	Sequence	Purpose
PIN1 F	GGCATGGCTATGTTCAGTCTTGGG	qRTPCR
PIN1 R	ACGGCAGGTCCAACGACAAATC	qRTPCR
PIN3 F	AAGGCGGAAGATCTGACCAAGG	qRTPCR
PIN3 R	TGCTGGATGAGCTACAGCTTTG	qRTPCR
PIN4 F	ACAACGTGGCAACGGAACAATC	qRTPCR
PIN4 R	GCCGATATCATCACCACCACTC	qRTPCR
PIN6 F	TTCATGGCTGGTGCTTCCCTTC	qRTPCR
PIN6 R	GCCTGTACGATAGCAGCATGTAAC	qRTPCR
PIN7 F	ATTGCGTGTGGCCATTGTTCAAGC	qRTPCR
PIN7 R	GCAAACACAAACGGCACGATCC	qRTPCR
NLP7 F	TCTCCGGTGTTCCTTCTTTCCG	qRTPCR
NLP7 R	CAGCTGCTGATGGAGAAGAGTAAG	qRTPCR
UBQ 10 R	CACACTCCACTTGGTCTTGC	qRTPCR
UBQ10 F	TGGTCTTTCCGGTGAGAGTCTTCA	qRTPCR
CRF2 F	GAAGAGCCATCCATGACAACGG	qRTPCR
CRF2 R	AGAACAAGGGCGCCGAGAAATC	qRTPCR
CRF3 F	ACAACGTCGTCACCGGAGTTTC	qRTPCR
CRF3 R	ACGCCGGAGATTGAGTTTCATCG	qRTPCR
CRF6 F	TCAAAGGACCTAAAGCGCTCACG	qRTPCR
CRF6 R	TGGAGATCGATAACCGGCGTTG	qRTPCR
TGA1 F	GACCCTCCGATCTTCTCAAGGTTC	qRTPCR
TGA1 R	TCAACGCGTCTTCTGCTTGCTG	qRTPCR
TGA4 F	AAAGTCGTTTGCGCAAGAAAGC	qRTPCR
TGA4 R	AGCATTGGTATCTACTCCGTTCCC	qRTPCR
HRS1 F	TGGTGTGCCTCAAGTAACTGCTG	qRTPCR
HRS1 R	TCCAATTCGCTTGAAGAAGGTGAC	qRTPCR
IPT3 F	TGGTCTATTCAGAGAGTGGATGCG	qRTPCR
IPT3 R	AGCATCCATCTTGGACCTTCGC	qRTPCR
IPT5 F	TGTCGTGCGGAATAGTGTACGG	qRTPCR
IPT5 R	AACCGCGTTGACCAACGATCTC	qRTPCR
IPT7 F	TGTTGACGCCACTGAGGTGTTC	qRTPCR
IPT7 R	TCTCCCAAGCCTCGTCTTGTTC	qRTPCR
ABCG14 F	TCTACTATGGCGCTGCCTCTTC	qRTPCR
ABCG14 R	TCTGAGTATCAGGTGGGATTCCG	qRTPCR

Supplementary Table 2. Primers used in RT-qPCR

References

1. Benková, E. *et al.* Local, Efflux-Dependent Auxin Gradients as a Common Module for Plant Organ Formation. *Cell* **115**, 591–602 (2003).
2. *Arabidopsis protocols*. (Humana Press, 2014).
3. Šimášková, M. *et al.* Cytokinin response factors regulate PIN-FORMED auxin transporters. *Nature Communications* **6**, 8717 (2015).
4. Inzé, A. *et al.* A subcellular localization compendium of hydrogen peroxide-induced proteins: Hydrogen peroxide-induced protein compendium. *Plant, Cell & Environment* **35**, 308–320 (2012).
5. Castaings, L. *et al.* The nodule inception-like protein 7 modulates nitrate sensing and metabolism in Arabidopsis. *The Plant Journal* **57**, 426–435 (2009).
6. Miyawaki, K. *et al.* Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. *Proceedings of the National Academy of Sciences* **103**, 16598–16603 (2006).
7. Le Hir, R. *et al.* ABCG9, ABCG11 and ABCG14 ABC transporters are required for vascular development in Arabidopsis. *Plant J* **76**, 811–824 (2013).
8. Higuchi, M. *et al.* In planta functions of the Arabidopsis cytokinin receptor family. *Proceedings of the National Academy of Sciences* **101**, 8821–8826 (2004).
9. Friml, J., Wiśniewska, J., Benková, E., Mendgen, K. & Palme, K. Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. *Nature* **415**, 806–809 (2002).
10. Friml, J. *et al.* AtPIN4 Mediates Sink-Driven Auxin Gradients and Root Patterning in Arabidopsis. *Cell* **108**, 661–673 (2002).

11. Jeon, J. *et al.* A Subset of Cytokinin Two-component Signaling System Plays a Role in Cold Temperature Stress Response in Arabidopsis. *Journal of Biological Chemistry* **285**, 23371–23386 (2010).
12. D'Agostino, I. B., Deruère, J. & Kieber, J. J. Characterization of the Response of the Arabidopsis Response Regulator Gene Family to Cytokinin. *Plant Physiol.* **124**, 1706–1717 (2000).
13. Malamy, J. E. & Benfey, P. N. Organization and cell differentiation in lateral roots of Arabidopsis thaliana. *Development* **124**, 33–44 (1997).
14. Dubrovsky, J. G., Gambetta, G. A., Hernández-Barrera, A., Shishkova, S. & González, I. Lateral Root Initiation in Arabidopsis: Developmental Window, Spatial Patterning, Density and Predictability. *Annals of Botany* **97**, 903–915 (2006).
15. Horiguchi, G., Fujikura, U., Ferjani, A., Ishikawa, N. & Tsukaya, H. Large-scale histological analysis of leaf mutants using two simple leaf observation methods: identification of novel genetic pathways governing the size and shape of leaves. *The Plant Journal* **48**, 638–644 (2006).
16. Pound, M. P., French, A. P., Wells, D. M., Bennett, M. J. & Pridmore, T. P. CellSeT: Novel Software to Extract and Analyze Structured Networks of Plant Cells from Confocal Images. *The Plant Cell* **24**, 1353–1361 (2012).
17. Arvidsson, S., Kwasniewski, M., Riaño-Pachón, D. M. & Mueller-Roeber, B. QuantPrime – a flexible tool for reliable high-throughput primer design for quantitative PCR. *BMC Bioinformatics* **9**, 465 (2008).
18. Turnbull, C. G. N., Booker, J. P. & Leyser, H. M. O. Micrografting techniques for testing long-distance signalling in *Arabidopsis*. *The Plant Journal* **32**, 255–262 (2002).

19. Kurihara, D., Mizuta, Y., Sato, Y. & Higashiyama, T. ClearSee: a rapid optical clearing reagent for whole-plant fluorescence imaging. *Development* **142**, 4168–4179 (2015).
20. Svačinová, J. *et al.* A new approach for cytokinin isolation from Arabidopsis tissues using miniaturized purification: pipette tip solid-phase extraction. *Plant Methods* **8**, 17 (2012).
21. Antoniadi, I. *et al.* Cell-Type-Specific Cytokinin Distribution within the Arabidopsis Primary Root Apex. *Plant Cell* **27**, 1955–1967 (2015).