Cell Reports

Supplemental Information

Meta-regulation of *Arabidopsis* Auxin Responses Depends on tRNA Maturation

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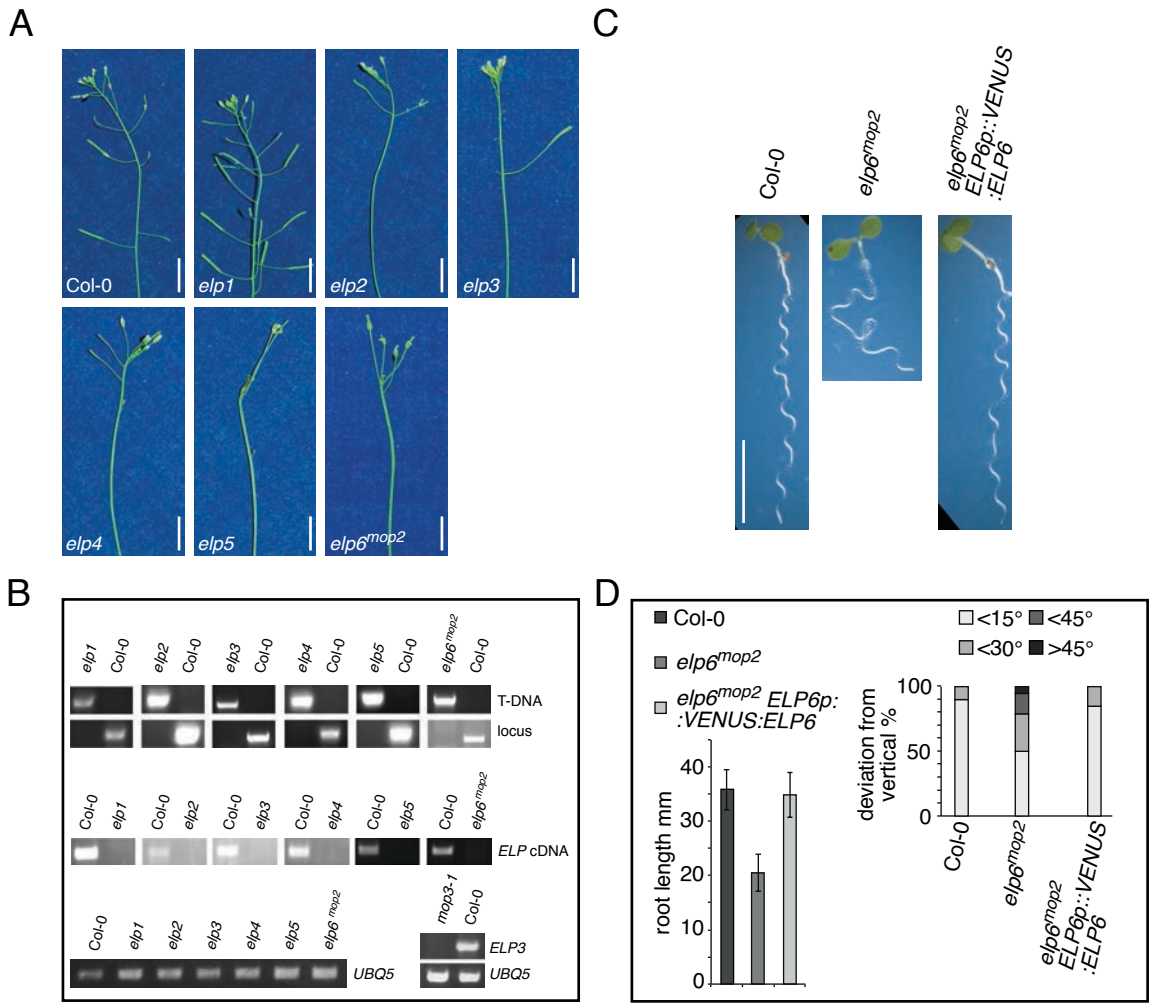

Figure S1, related to Figure 1: Identification and characterization of *elp/mop* loss-of-function mutants. **A)** Comparison of Col-0 and *elp* inflorescences at 35 DAG. Note aberrations in lateral organ positioning, characteristic for *Arabidopsis elp* mutants. **B)** Top panels: Genotyping of *elp* alleles performed with primers summarized in Table 1. Middle panels: RT-PCR analysis performed with *ELP* locus-specific primers for all 6 mutant loci. Col-0 cDNA was used as control. All cDNA samples gave a positive result with *UBQ5*-specific primers (bottom left). Bottom: RT-PCR performed with *mop3-1* and Col-0 cDNA. *Mop3-1* does not express a full-length *ELP3* transcript. *UBQ5* served as a control. **C)** 8-day-old Col-0, *elp6*^{mop2} and *elp6*^{mop2} *ELP6p::VENUS:ELP6* grown on vertically oriented agar plates. **D)** Left: Root length of Col-0, *elp6*^{mop2} and *elp6*^{mop2} *ELP6p::VENUS:ELP6* at 6 DAG. 30 seedlings were analyzed for each dataset. Standard deviations are indicated. Right: Directional root growth of Col-0, *elp6*^{mop2} and *elp6*^{mop2} *ELP6p::VENUS:ELP6* at 8 DAG. At least 40 seedlings were analyzed for each dataset. Size bars: A,C = 10 mm.

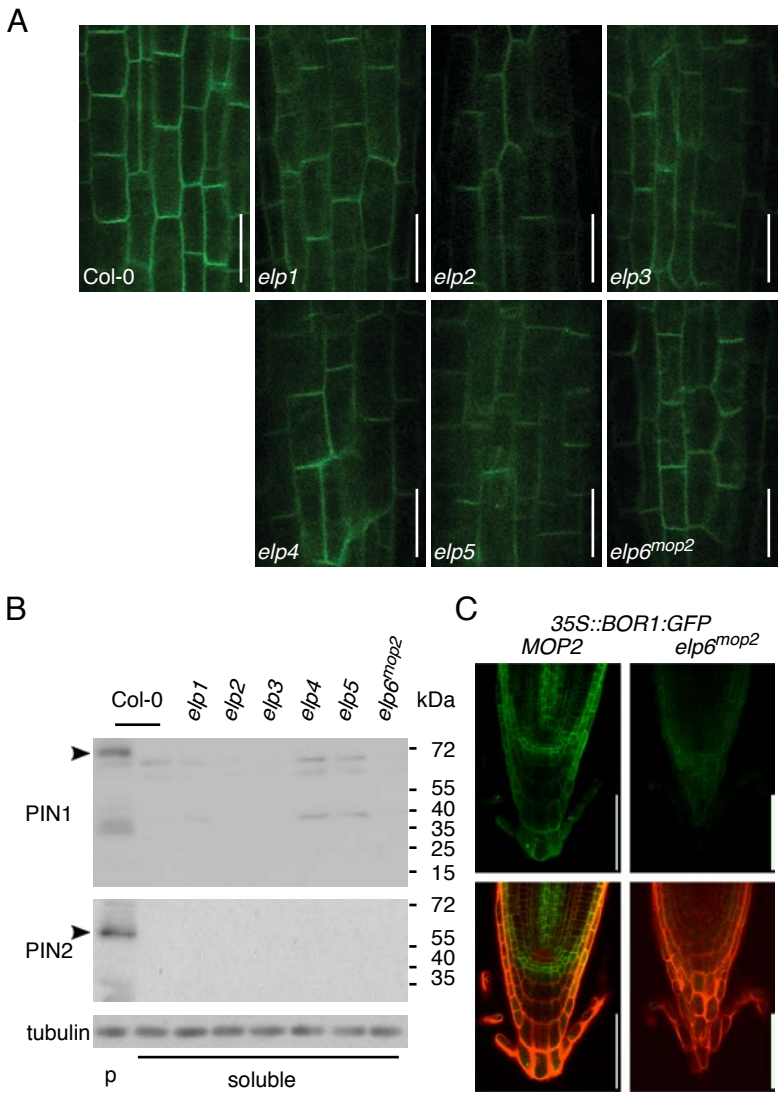

Figure S2, related to Figure 1: A) Distribution of *PIN1p::PIN1:GFP* signals (green) in wild type and *elp* mutant root meristem stele cells at 5 DAG. **B)** Western blot performed with soluble protein fractions derived from wild type and *elp* mutant protein extracts (6 DAG), probed with anti-PIN1 (top panel) and anti-PIN2 (middle panel). As a positive control, Col-0 membrane fraction protein extracts ("p") were used. Black arrowheads indicate position of PIN1- and PIN2-specific signals in the membrane fraction. Bottom panel displays signals obtained after probing with anti-tubulin. **C)** Expression of *35S::BOR1:GFP* (green) in *elp6*^{mop2}. *35S::BOR1:GFP* was crossed into *elp6*^{mop2} and resulting *MOP2* and *elp6*^{mop2} progeny, homozygous for the reporter protein was analyzed. Seedlings were stained with PI (red) to visualize cell boundaries. Size bars: A = 10 µm; C = 50 µm.

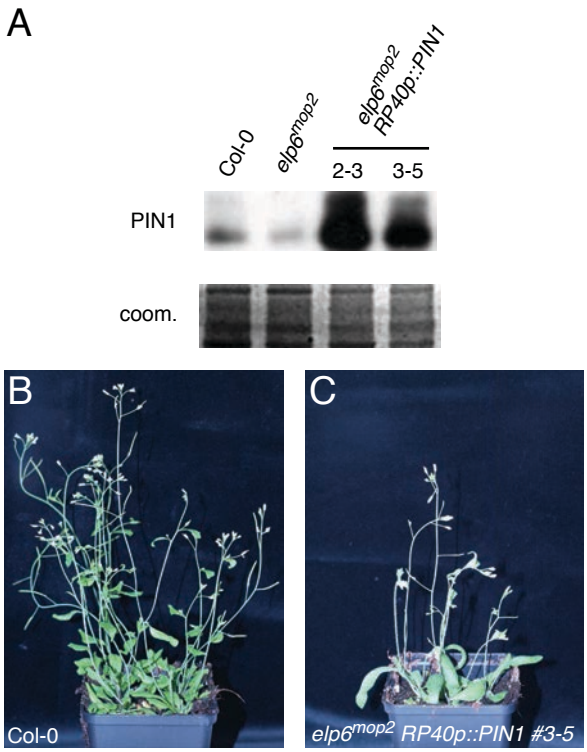

Figure S3, related to Figure 2: A) Determination of *PIN1* expression in *RP40p::PIN1* overexpression lines. Membrane proteins were extracted from 5-day-old seedlings, separated by SDS-PAGE and probed with anti-PIN1 antibody. Coomassie-staining (coom.) demonstrated comparable protein loading for all samples. **B,C)** Comparison of Col-0 (B) and *elp6*^{mop2} *RP40p::PIN1* (C) plants at the stage of flowering.

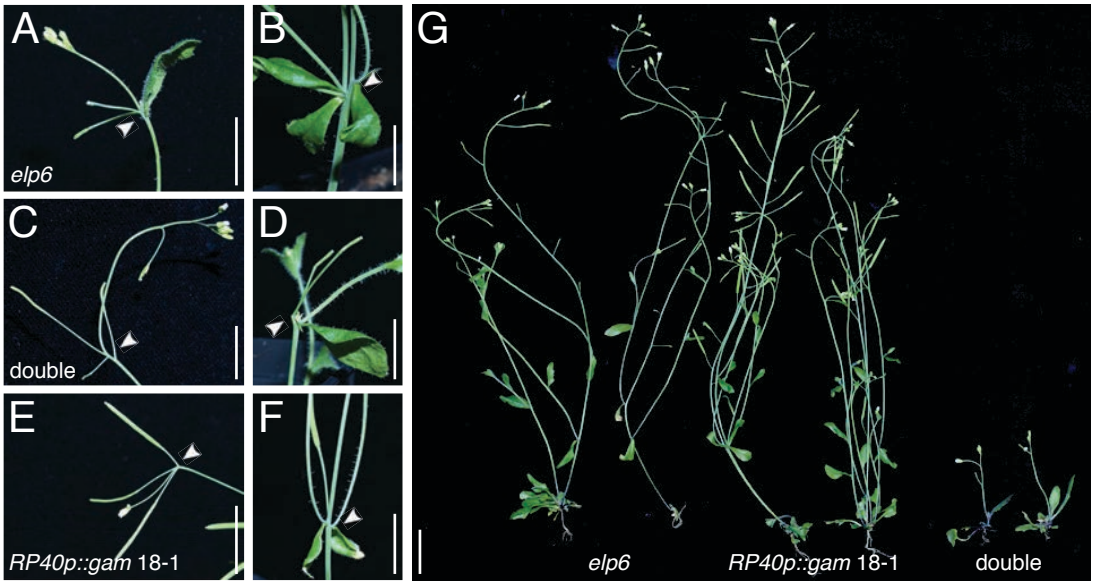

Figure S4, related to Figure 3: Comparison of *elp6*^{mop2}, *RP40p::gam* 18-1 and *elp6*^{mop2} *RP40p::gam* 18-1 ("double") at the stage of flowering. **A,B)** Details of *elp6*^{mop2} inflorescence (A) and inflorescence axes (B) at 30 DAG. **C,D)** Details of *elp6*^{mop2} *RP40p::gam* 18-1 ("double") inflorescence (C) and inflorescence axes (D) at 38 DAG. **E,F)** Details of *RP40p::gam* 18-1 inflorescence (E) and inflorescence axes (F) at 30 DAG. White arrowheads indicate aberrations in organ positioning (A-E). **G)** Comparison of *elp6*^{mop2}, *RP40p::gam* 18-1 and *elp6*^{mop2} *RP40p::gam* 18-1 ("double") plantlets at 30 DAG. Note the delay in growth of *elp6*^{mop2} *RP40p::gam* 18-1. Size bars: A-F = 10 mm; G = 20 mm.

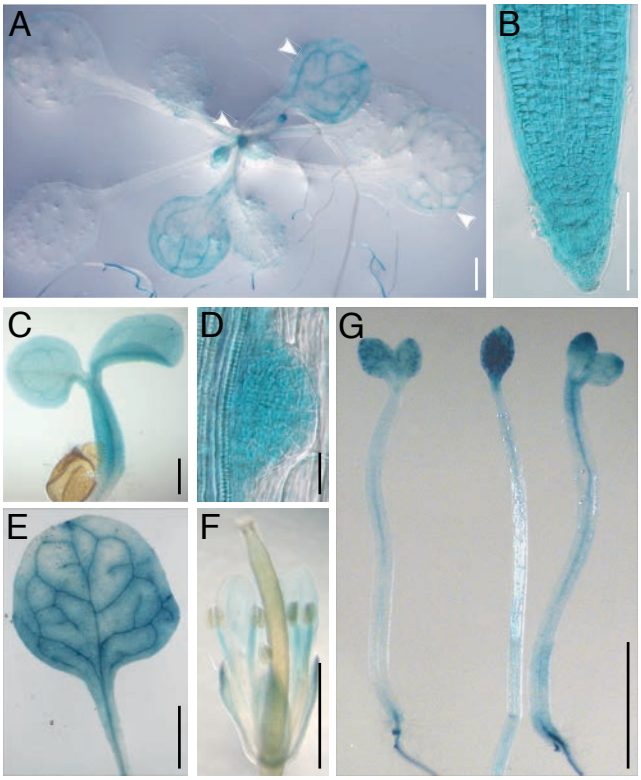

Figure S5, related to Figure 5: Expression of *AtRNLp::GUS* in Col-0. **A,B)** *AtRNLp::GUS* activity in a 12-day-old plantlet (A) and a primary root meristem (B). Arrowhead indicates pronounced GUS activity in vasculature and developing organs. **C)** Aerial portion of a seedling at 4 DAG. **D)** Later stage lateral root primordium. **E)** True Leaf. **F)** Flower. **G)** Etiolated hypocotyls: Size bars: A,E,G = 2 mm; B = 50 µm; C,F = 1 mm; D = 25 µm.

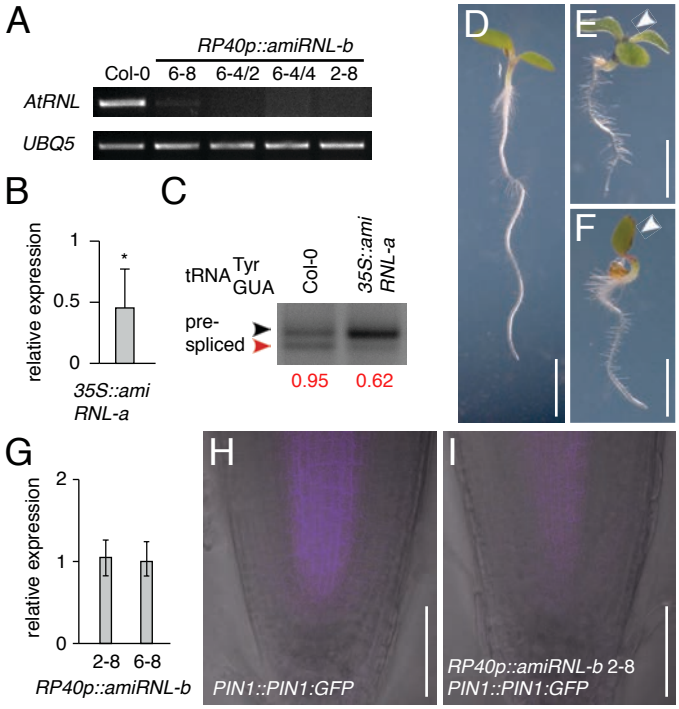

Figure S6, related to Figures 5 and 6: A) RT-PCR displaying *AtRNL* levels in 4 *RP40p::amiRNL-b* silencer lines. *UBQ5* was used for normalization. **B)** *AtRNL* transcript levels in a *35S::amiRNL-a* silencer line selected from 5 lines characterized by similar growth defects (Col0 = 1). Data from 3 biological repeats, each with 3 technical repeats are displayed. Standard deviations are shown (* = two-tailed t-test, $p < 0.05$). **C)** Quantification of $\mathrm{tRNA}^{\mathrm{Tyr}}_{\mathrm{GUA}}$ levels in Col-0 and *35S::amiRNL-a* by quantitative PCR. Upper band corresponds to non-spliced $\mathrm{tRNA}^{\mathrm{Tyr}}_{\mathrm{GUA}}$ precursor (black arrowhead); lower band corresponds to processed $\mathrm{tRNA}^{\mathrm{Tyr}}_{\mathrm{GUA}}$ (red arrowhead). Numbers below, display ratio between spliced and precursor band intensities. **D-F)** Comparison of Col-0 (D) and *35S::amiRNL-a* (E,F) seedlings at 4 DAG. *35S::amiRNL-a* develops shorter, slightly agravitropic roots and frequently shows an aberrant number of cotyledons (19/49; white arrowhead), which is not observed in wild type controls (0/45). **G)** Comparison of *PIN1* transcript levels in Col-0 and *RP40p::amiRNL-b* silencer lines 2-8 an 6-8 at 6 DAG (Col-0 = 1). Data from 3 biological repeats each with 3 technical repeats are displayed. Standard deviations are indicated. **H,I)** Expression of *PIN1p::PIN1:GFP* (purple) in Col-0 (H) and *RP40p::amiRNL-b* 2-8 (I) primary root meristems. Size bars: D-F = 5 mm; H,I = 50 μm.

Supplemental table: primers for expression analysis (Ex) and genotyping (Gt).

locus/primer	Application	5'-Sequence-3'
PIN2	Ex	GCTACTAAAGCGATGCAGAATC
PIN2	Ex	GAAATCGATCCACTCATTATCGTT
ELP1	Gt	TCTGATGATATAAGGAAAAAG
ELP1	Gt	GTATAGGCGCTCATTCCACT
ELP1	Ex	GGGGTTGGCTACTTTCCTGATT
ELP1	Ex	CTGCAAGCAATAATCGTCTCT
ELP2	Ex, Gt	CAGGATGCAACAGAGTCGTC
ELP2	Ex, Gt	TGTGTAACGGTAATGAGAAGTC
ELP3	Ex, Gt	GGCGGATCCATGGCGACGGCGGTAGTGA
ELP3	Ex, Gt	CATGCTTACTCTGTTAGTCCA
ELP4	Ex, Gt	CTGCACCAAACGTTCGTAGTAGTA
ELP4	Ex, Gt	TTGATAAAAACGTAGCACAG
ELP5	Ex, Gt	GTGGCGAAGAAGGCGAACTC
ELP5	Ex, Gt	AGCGCATTTGTGACCTTCTC
ELP6	Ex, Gt	CCGGATCCATGGATCGTTCTTTGAATCTCCTCGAT
ELP6	Ex, Gt	CCGGATCCTCAGCTTCTGCAACCAGGATAGAAATA
TUB9	Ex	ACTCGTTGGGAGGAGGAACT
TUB9	Ex	ACACCAGACATAGTAGCAGAAATCAAG
UBQ5	Ex	ACCCCTTGAGGTTGAATCATC
UBQ5	Ex	GTCCTTCTTTCTGGTAAACGT
LBa1	Gt	TGGTTCACGTAGTGGGCCATCG
LBb1	Gt	GCGTGGACCGCTTGCTGCAACT
RBa1	Gt	GCGGCTGAGTGGCTCTTCCAACGTT
RBb1	Gt	GTCGTTTCCCGCCTTCAGTTTAA
GKTd	Gt	ATATTGACCATCATACTCATTGC
PIN1	Ex	TACTCCGAGACCTTCCAACTACG
PIN1	Ex	TCCACCGCCACCACTTCC
tRNA-His	Ex	GTGGCTGTAGTTTAGTGGT
tRNA-His	Ex	TGTGGCTGGGATTCGAG
tRNA-Ser	Ex	GGACGTGCCGGAGTGGTTATCGGGCATGACT
tRNA-Ser	Ex	GAACGGCAGGATTCGAACCTGCGCGGGCAAA
tRNA-Gln	Ex	TTCTATGGTGTAGTGGTtAGCACTCTGGACTTT
tRNA-Gln	Ex	AGGTCCTACCGGGAGTCGAACCCAGGT
tRNA-Glu	Ex	GATGTCGTCCAGCGGTtAGGATATCT
tRNA-Glu	Ex	CTCCGTTACCGGGAATCGAACCC
AT5g60390	Ex	TGAGCACGCTCTTCTTGCTTTCA
AT5g60390	Ex	GGTGGTGGCATCCATCTTGTTACA
AtRNL	Ex	TTTATGTACGCCGGTCA
AtRNL	Ex	CGGTTACCAGTTCCATAG
γ-toxin	Ex	CAACCAAATGAAAGAAGAAGCTC
γ-toxin	Ex	CTCTTCTACCAAGACCTAATTC

Supplemental Experimental Procedures

Plant growth and lines

Plants were grown on 0.5x Murashige-Skoog or on plant nutrient agar medium (5 mM KNO_3, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 250 mM KPO_4, 70 μM H_3BO_3, 14 μM $MnCl_2$, 500nM $CuSO_4$, 1 μM $ZnSO_4$, 200 nM Na_2MoO_4, 10 μM NaCl, 10 nM $CoCl_2$, 50 μM $FeSO_4$; pH adjusted to 5.7; supplemented with 1% (w/v) agar and 1% (w/v) sucrose (Haughn and Somerville, 1986); in a 16 hrs. light/8 hrs. dark regime at 22°C, if not indicated otherwise. *DR5rev::GFP*, *PIN1p::PIN1:GFP*, *35S::YFP:ELP3*, *35S::YFP:GCN5*, *35S::BOR1:GFP* reporter lines as well as *mop2-1* and *mop3-1* have been described elsewhere (Benkova et al., 2003; Earley et al., 2007; Malenica et al., 2007; Takano et al., 2005). For analysis of *AtRNL*, T-DNA insertion lines SALK_059581, SAIL_693_G06 and GK-258C06 were genotyped and tested for effects on transcription by using oligonucleotides 5'- AGCTCGCTTAGCTCAGATGA-3', 5'- GGCTCTGATCTGAGCATGAC-3', 5' CGGCTGTCTCACAGATAGGT-3', 5'-CCTAACTCGGTAACAGCAGTC-3', 5'- GCAAACCCAACAATGAGATC-3', 5'-GATATCTCAATGAATGCAG-3', 5'- GGGCTTACTGTGGCCCAATAG-3' and 5'-AACGACATCAACACGTGGC-3'. No differences in gene expression were observed in the SALK and SAIL lines, whereas we failed to identify a T-DNA insertion in the GABI line. We therefore decided to initiate an amiRNA silencing approach.

For determination of growth, seedlings and adult plants were analyzed at indicated time points. Root length, lateral root growth and root gravitropism were assessed manually on vertically grown seedlings. For determination of cotyledon vasculature seedlings were cleared in 70% (v/v) EtOH and viewed on a binocular. Phenotypes of flowering plants were determined on soil-grown seedlings incubated in a growth chamber at a 16 hrs. light/8 hrs. dark regime at 22°C. For analysis of *RP40p::PIN1* expression in *elp6*^{mop2} and wild type, we compared isogenic lines

obtained by crossing of and identification of double homozygote lines in resulting progeny generations For root growth assays, we used line *RP40p::PIN1* #2-3. Similarly, for analysis of *RP40p::gam* in *elp6*^{mop2} we obtained isogenic lines by crossing *RP40p::gam* and *elp6*^{mop2} parental lines. Effects of hormones and growth regulators were assessed by adding appropriate amounts of the compounds, dissolved as 1000x stocks in either EtOH or DMSO. Solvent only was added to the control medium.

Generation of constructs

For generation of *RP40p::PIN1* the *PIN1* cDNA was cloned into RP40p-pApA, a derivative of pPZP221 (Butt et al., 2014). For generation of *RP40p::gam* we amplified γ-toxin lacking its signal peptide by using 5'-GGCCCGGGATGAAGATATATCATATATTTAGTG-3' and 5'-GGCCCGGGTTAGTCGACTACACATTTTCCATTCTGTAGATTA-3' according to Lu and colleagues (Lu et al., 2005). The confirmed clone was then introduced into RP40p-pApA.

For *ELP6::VENUS:ELP6* the *ELP6* cDNA was amplified using oligonucleotides 5'-CCGGATCCATGGATCGTTCTTTGAATCTCCTCGAT-3' and 5'-CCGGATCCTCAGCTTCTGCAACCAGGATAGAAATA-3', and cloned into pApA–pPZP221 (Leitner et al., 2012), 3' of an *ELP6* promoter fragment amplified with oligonucleotides 5'-CCGAATTCAAATATATAAACAAGTTTTTGGAA-3' and 5'-CCGAATTCGGAGAAATTTGGACGGAGAAGAAGA-3'. A VENUS tag (Nagai et al., 2002) was then introduced in frame immediately upstream of the *ELP6* Start-ATG.

For generation of silencer constructs, we used oligonucleotides 5'-GATATAGACTCAAATGGAACCTCTCTCTCTTTTGTATTCC-3', 5'-GAGAGGTTCCATTTGAGTCTATATCAAAGAGAATCAATGA-3', 5'-

GAGAAGTTCCATTTGTGTCTATTTCACAGGTCGTGATATG-3' and 5'-GAAATAGACACAAATGGAACTTCTCTACATATATATTCCT-3' to generate amiRNL-a directed against nucleotides 2969-2987 of the *AtRNL* cDNA. For amiRNL-b directed against 1203-1218 of the *AtRNL* cDNA we employed oligonucleotides 5'-GATACTGCTCACGATACGACCAATCTCTCTTTTGTATTCC-3', 5'-GATTGGTCGTATCGTGAGCAGTATCAAAGAGAATCAATGA-3', 5'-GATTAGTCGTATCGTCAGCAGTTTCACAGGTCGTGATATG-3' and 5'-GAAACTGCTGACGATACGACTAATCTACATATATATTCCT-3'. Resulting constructs were expressed under control of either the 35S-promoter (amiRNL-a), or the *RP40*-promoter (amiRNL-b). For generation of *AtRNLp::GUS* we used oligonucleotides 5'-GCTGTATATCTCCTCAATTGCTGATT-3' and 5'-CTTCGGTTTTACTTGCCACTTCTGC-3' to amplify a 2.2 kb *AtRNL* promoter fragment for expression of the GUS reporter gene in pPZP-GUS (Diener et al., 2000).

Expression analysis

For analysis of tRNA modifications in *elp* mutants, total RNA was isolated from 100 g Col-0, *elp3* and *elp6*^{mop2} seedlings, grown in liquid medium (23°C, 16 light, 6-days-old). Plant material was ground with mortar and pestle and ice-cold 0.9 % NaCl was added (2 ml/g), vortexed immediately and the same amount of phenol was added afterwards. Samples were homogenized on an orbital shaker for 30 min., followed by centrifugation at 10.000 x g for 30 min. The aqueous phase was re-extracted and precipitated with 0.7 vol. of isopropanol o/n at -20°C. After washing and drying, RNA pellets were dissolved in 2 ml of 2 M LiCl/0.05 M NH_4OAc and vortexed vigorously for 10 min, followed by o/n precipitation of mRNAs and ribosomal RNAs on ice. After centrifugation at 4°C for 30 min at 10.000 x g, supernatant fractions were precipitated with 2.5 vol of ice-cold EtOH o/n at -20°C. Small RNAs

were pelleted by centrifugation at 4°C for 30 min at 10.000 x g and washed in 70 % EtOH. Pellets were air-dried and resuspended in 100 µl RNase-free water. RNA integrity was analyzed on 2 % agarose gels and concentration was determined spectrophotometrically.

For analysis of $tRNA^{Tyr}$ splicing, cDNA from small RNA pools pretreated with RNAse-free DNAse-I (Roche, Vienna, Austria) was generated by using primer 5'-TCCGACCGGATTCGAACCAGTGA-3' to enrich for $tRNA^{Tyr}$ transcripts. cDNA samples were then used for PCR with primers 5'-CGGCTGTCTCACAGATAGGT-3' and 5'-CCTAACTCGGTAACAGCAGTC-3, specifically amplifying spliced and unprocessed $tRNA^{Tyr}$ followed by separation by gel electrophoresis. Relative signal intensities were determined by Image-J software.

For semi-quantitative RT-PCR we generated cDNA from mRNA samples according to Sieberer and colleagues (Sieberer et al., 2003). For qPCR we generated cDNA from total RNA samples by using the QuickPick™ total RNA isolation kit from BioNobile (Pargas, Finland). DNAseI (Roche, Vienna, Austria) treatment was performed after sample elution followed by reverse transcription with M-MuLV H plus Reverse Transcriptase (peqLab, Erlangen, Germany) in presence of either oligo-dT for generation of mRNA-derived cDNAs, or random hexameric primers for total RNA conversion. PCR was performed on a CFX96 Real Time System (Biorad, Hercules, CA, USA). Data were analyzed with CFX Manager Software (Bio Rad, version 2.1), Cq values were determined by single threshold methods, and combined data derived from biological replicates are presented (Livak and Schmittgen, 2001). For normalization, we used locus At5g60390 (*EF-1a*) for mRNA expression analysis (Czechowski et al., 2005). For determination of $\mathrm{tRNA}^{\mathrm{Gln}}_{\mathrm{UUG}}$ and $\mathrm{tRNA}^{\mathrm{Glu}}_{\mathrm{UUC}}$ abundance we normalized against levels of $\mathrm{tRNA}^{\mathrm{His}}_{\mathrm{GUG}}$ and $\mathrm{tRNA}^{\mathrm{Ser}}_{\mathrm{AGA}}$, which due to the absence of wobble uridines should not represent preferred

substrates for γ-toxin. Relative tRNA abundance in *RP40p::gam* lines was normalized to tRNA abundance obtained in wild type controls. Oligonucleotides used for expression analyses are summarized in the Supplemental Table.

Membrane protein extraction has been performed according to Leitner et al., (2012). In brief, root material was homogenized and resuspended in extraction buffer, as described previously (Abas and Luschnig, 2010). Samples were cleared by centrifugation (2,100 × g for 2 min). The supernatant was saved and the pellet was re-extracted. Samples were cleared by centrifugation, and combined supernatants were centrifuged (14,000 × g for 2 hours) to yield total membrane pellets. For Western blot analysis, membrane pellets or proteins precipitated from the supernatant were resuspended in sample buffer [0.5% (wt/vol) CHAPS, 3% (wt/vol) SDS, 30% (vol/vol) glycerol, 60 mM dithioerythritol, 50 mM Tris (pH 6.8), 1 mM PMSF, and 0.5x Roche Complete Mini Protease Inhibitor Mixture Tablets; (Abas and Luschnig, 2010)]. Proteins were separated by SDS/urea PAGE, transferred to nitrocellulose membranes, and probed with affinity-purified rabbit anti-PIN1 and anti-PIN2 (Abas and Luschnig, 2010), followed by HRP-conjugated goat-anti-rabbit IgG (1:20,000; Pierce). Mouse-anti-Tubulin (Sigma) was used for normalization, and detected after probing with HRP-conjugated rabbit-anti-mouse IgG (1: 10,000; Jackson).

For visualization of FP-tagged reporter lines, we used Leica SP2 and SP5 Confocal Laser Scanning Microscopes (CLSM). Seedlings were viewed either alive after brief staining in propidium iodide (100 µg/ml) to visualize cell boundaries, or after fixation in 3.7 % (v/v) formaldehyde in Microtubule Stabilization Buffer (MTSB; 50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$) for 15 minutes. After 3 washes in MTSB seedlings were mounted in MTSB containing 4,6,Diamidine-2-phenylindole

dihydrochloride (DAPI, 0.5 ng/ml) and viewed under the CLSM. For imaging, we used the following excitation conditions: 488 nm (GFP), 514 nm (VENUS, YFP), 405 nm (DAPI), 561 nm (propidium iodide).

HPLC analysis of tRNAs

Purified tRNA was digested with Nuclease P1 for 16 h at 37°C and then treated with bacterial alkaline phosphatase for 2 h at 37°C. The hydrolysate was analyzed by high-pressure liquid chromatography with a Develosil C-30 reversed-phase column as described (Gehrke et al., 1982; Gehrke and Kuo, 1990).

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