Supporting Information

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SI Materials and Methods

Plant Growth Assays. Seeds were plated onto media containing 1/2 × MS media, 1% sucrose, 1% agar, and stratified for 2–4 days before growth at 22 °C under continuous light at 100 μmol $m^{-2}s^{-1}$. For auxin growth assays, 4-day-old seedlings were transferred onto fresh MS media ± auxin for an additional 4 days after which the length of roots after transfer was measured. Growth is expressed as a percentage of growth of the equivalent genotype on media without auxin. Lateral root assays were performed in a similar manner except the number of emerged lateral roots and primary root length was measured after an additional 9 days. Emerged lateral roots were counted using a Nikon SMZ1500 stereomicroscope. Mutant alleles have been previously described or isolated from the SALK T-DNA insertion collection: *tir1–1* (1), *tir1–10* (Salk_090445), *afb1–3* (2), *afb1–5* (Salk_144884), *afb2–3* (2), *afb3–4* (Salk_068787).

Generation of Transgenic Lines. Promoter fusion lines were previously described (3). The TIR1/AFB1 translational GUS fusion lines were created by amplifying the cDNAs regions of each gene and placing them downstream of the appropriate promoter region in the pBluescript plasmid. The promoter-cDNA clones were PCR amplifying and cloned into the pENtr vector (Invitrogen) and subsequently by LR Clonase reaction into the destination vector pMDC163 (4). The primer sequences used are as follows:

TIR1 promotor (−14 bp to −1,979 bp), TIR1pSalF- (aaagtcgacgagtacgaaacccgagactagg), TIR1pEcoR- (aaagatatccctcgagatctcgatgatcg).

TIR1 cDNA (+1 to +1,783), TIR1EcoF- (aaagaattcatgcagaagcgaatagccttgt), TIR1BamR- (aaaggatcctaatccgttcgtagtaatga).

AFB1 Promotor (−6 bp to −1,805 bp), AFB1pSalF- (aaagtcgacgataagaagatgggcacgt), AFB1pEco- (aaagatatccagaaacagagtgagagag).

AFB1 cDNA (+1 to +1,758), AFB1EcoF- (aaagaattcatgggtctccgattcccacct), AFB1BamR- (aaaggatccctttatggctagatgtgaaact).

AFB2 promotor (−3 to −1,849), AFB2pSalF- (aaagtcgaccgaccgtttcaagtttca), AFB2pEcoR- (aaagatatcctaacttcaccagcaagattc).

AFB2 cDNA (+1 to +1,725), AFB2EcoF- (aaagaattcatgaattatttcccagatgaagt), AFB2BamR- (aaaggatccgagaatccacacaaatggcgg).

AFB3 Promotor (−3 bp to −1,655), AFB3pSalF- (aaagtcgacgtgggtctattattgagt), AFB3pClaR- (aaaatcgattcctctattgattgtcgaac).

AFB3 cDNA (+1 to +1,734), AFB3BamF- (aaagaattcatgaattatttcccagacgagg), AFB3NotR- (aaagcggccgcctaaagaatcctaacatatgg).

We also created a new TIR1 transcriptional GUS fusion line in pMDC163 using the primers described above.

The generation of *pmiR393a-GFP* and *pmiR393b-GFP* transgenic lines were previously described (5). Confocal images of GFP expression were obtained using a Leica TCS SP confocal microscope. To visualize the cell walls, roots were stained for 1 min in 1 μg/mL propidium iodide (in water), washed briefly, and mounted in water.

We amplified the promoter regions of *miR393a* (−70 bp to −2,578 bp) and *miR393b* (−87 bp to −2,532 bp) genes into pMDC163 as described above. Primer sequences are as follows: miR393apromF- aactgcaaagttaagtgggc, miR393aR- ttgtcaatggatcgtgttcc, miR393bpromF- tggtgatgatgacacaaagg, and miR393bpromR- agatgaccatgatcctcttc.

The *35S:miR393a* line has been previously described (5). To create the *35S:miR393b* clone we amplified the region surrounding the *miR393b* mature gene (−273 bp to +218 bp) and cloned it into the pENtr vector and subsequently into the pMDC32 destination vector (4).

We transformed each destination binary vector into electrocompetent *Agrobacterium* strain GV3101 and then transformed *Arabidopsis* by floral dip. Selection of transgenic seedlings was performed by growth on hygromycin-containing plates (30 μg/mL).

Glucoronidase Assay. X-Gluc assays were performed by incubating seedlings in X-Gluc buffer (50 mM sodium phosphate buffer pH 7, 0.1%Triton X-100, 1 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (GBT) overnight at 37 °C. Seedlings were cleared by ethanol series (70%, 50%, 30%, and 10%) and mounted in 50% glycerol or choral hydrate for microscopy. Bright-field images were taken using Nikon SMZ1500 and Nikon E800 microscopes.

RNA Techniques. N_2-frozen tissue was ground up using mortar and pestle and RNA was extracted either using QIAGEN RNeasy plant kit or using Tri-reagent (Sigma). For semiquantitative RT-PCR poly(dT) was performed on 1–5 μg RNA using SuperScript II (Invitrogen). PCR was subsequently performed by standard techniques with Ex-Taq (Takara). Northern blots were undertaken using standard techniques. DNA probes were amplified using the following primers: GUSprobeF- atgttacgtcctgtagaaacc, GUSprobeR- cgaaacgcagcacgatacgc, TIR13utrF- ggatataagacaaagaaac, TIR13utrR- aacaaagctctcttagatc. Standard techniques for random priming were used to label DNA probes with $^{[32]}$P.

Quantitative PCR. Plants were grown on 1/2 MS, 1% bactoagar at 23 °C, 150 μmol·m^{-2}·s^{-1} under continuous light. Six-day-old plants were transferred on medium supplemented with −/+1 μM of IAA for 6 h. RNA was extracted from roots using Qiagen RNeasy plant mini kit with on-column DNase treatment (RNase free DNase set, Qiagen). Poly(dT) cDNA was prepared from 3 μg total RNA using the transcriptor first strand cDNA synthesis kit (Roche). Quantitative PCR was performed using SYBR Green Sensimix (Quantace) on a Stratagene Mx3005P apparatus. PCR was carried out in 96-well optical reaction plates heated for 5 min to 95 °C, followed by 40 cycles of denaturation for 10 s at 95 °C and annealing-extension for 30 s at 60 °C. Target quantifications were performed with the following primer pairs that lie 5′ of the *miR393* recognition site in each gene: TIR1F-cctaaactgcagcgcctct, TIR1R-ggttgaagcaagcacctca, AFB1F-actgatggtatcgctgctattg, AFB1R-agttgaactctctggaaaatagctaag, AFB2F-cgtgcctcgaaggagaaac, AFB2R-tttggagacctagcaacaagc, AFB3F-tgataaactttacctctaccgaacag, and AFB3R-cctaacatatggtggtgcatctt. Expression levels were normalized to UBA (At1g04850) using the following primers: UBAF- agtggagaggctgcagaaga and UBAR-ctcgggtagcacgagcttta. All qRT-PCR experiments were performed in triplicates and the values presented represent means ± SE.

In Vitro Protein Expression and Pulldown Reactions. The cDNAs of *TIR1*, *AFB1*, *AFB2*, and *AFB3* were amplified and cloned into pTNT (Promega) using an EcoRI and BamHI restriction sites. *Aux/IAA* cDNAs were amplified and cloned into pGEX-4T3

(Amersham Pharmacia) using EcoRI and XhoI restriction sites. Full length TIR1, AFB1, AFB2, and AFB3 proteins were obtained by in vitro translation using TNT coupled wheat germ extract system (Promega) in the presence of [35S]translabeled methionine. GST-tagged Aux/IAA proteins were recombinantly expressed and purified from *Escherichia coli* using standard procedures.

For pulldown assays, 20 μL of labeled TIR1, AFB1, AFB2, and AFB3 proteins were incubated for 2 h at 4 °C with >10 μg of GSH-sepharose immobilized GST-Aux/IAA proteins in 200 μL of lysis buffer (50 mM Tris p 8.0, 200 mM NaCl, 10% glycerol, 0.1% Tween-20, protease inhibitors) in the presence of 100 μM IAA. After washing the samples with 10 bed volumes of lysis buffer, beads were resuspended in one bed volume of sample buffer, denaturated, and separated on SDS/PAGE. Products were detected by autoradiography.

Phylogenetic Analysis. Protein sequences were extracted from whole-genome sequence databases: *Arabidopsis thaliana* (TAIR v8, http://www.arabidopsis.org), *Carica papaya* (http://www.ncbi.nlm.nih.gov/nuccore/ABIM00000000), *Populus trichocarpa* (v.1.1, http://genome.jgi-psf.org/Poptr1_1), *Ricinus communis* (TIGRcastorWGSr0.1, http://castorbean.jcvi.org/), *Vitis vinifera* (Vitis_vinifera_peptide_v1, http://www.genoscope.cns.fr/spip/Vitis-vinifera-e.html), *Oryza sativa* (TIGRv5.0, http://rice.plantbiology.msu.edu/), and *Physcomitrella patens* (Phypa1_1.FilteredModels, http://genome.jgi-psf.org/Phypa1_1). *Brassica rapa* and *Solanum lycopersicum* genes were identified in GenBank accession numbers AC189644, AC232438, CU695329, EX050213, AC189535, AC232530, AK320427, CU928132, and AC215365. The AFB6 homolog from the palm, *Phoenix dactylifera*, was identified in Scaffold_1804030 (http://www.qatarweill.cornell.edu/research/datepalmGenome). Loblolly pine sequences were initially identified in the EST databases, and full-length ORF cDNAs were sequenced for *PintaTIR1* (GQ503647), *PintaAFB4A* (GQ503648, GQ503649), and *PintaAFB6* (GQ503650) although inverse PCR followed by RT-PCR was used to identify the 5′ end of the *PintaAFB4A* gene. The *PintaAFB4B* sequence was derived from transcript assemblies (http://plantta.jcvi.org/) TA24266_3352, AA556825, BM493781, and TA22701_3352. The translated sequences were aligned using *T-COFFEE* (6) and adjusted using *MACCLADE* (7). For Bayesian inference, analysis was run using *MRBAYES 3.1.2* (8) with the parameters aamodelpr = mixed, nst = 6, and rates = invgamma.

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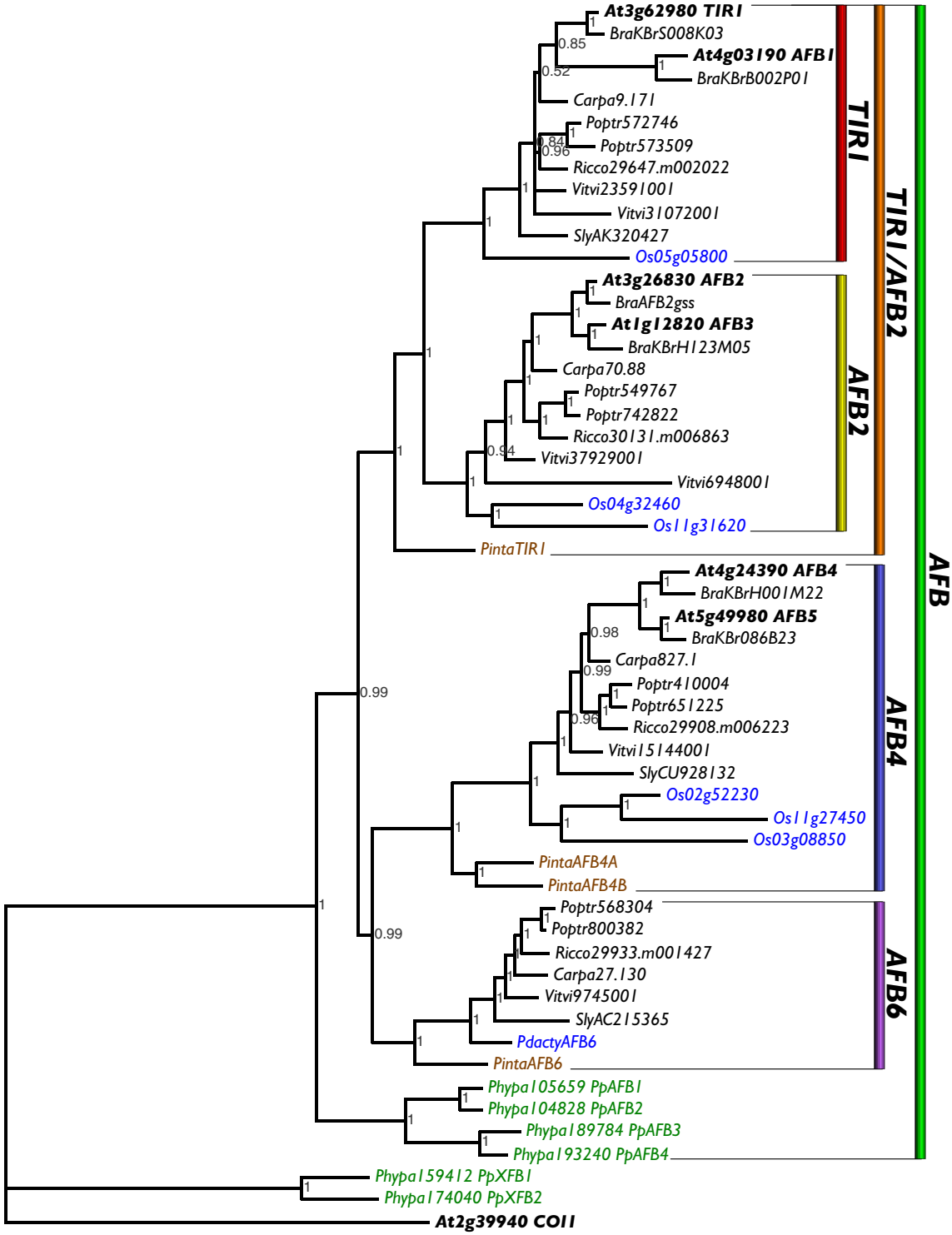


Fig. S1. *TIR1/AFB* genes radiated during vascular plant evolution. The phylogeny of *TIR1/AFB* genes was inferred using Bayesian methods and an alignment of full-length TIR1/AFB protein sequences. The colors of protein names indicate the phylogenetic affinities of the plant whose genome encodes the protein: black, eudicot; blue, monocot; brown, gymnosperm; and green, moss (*Inset*). Numbers at nodes indicate posterior probabilities. Distinct clades of *TIR1/AFB* genes are designated to the *Right*. The tree was rooted using the *Arabidopsis* COI1 protein and moss members of a family F-box proteins (XFB) absent in seed plants. Abbreviations: At, *Arabidopsis thaliana*; Bra, *Brassica rapa*; Carpa, *Carica papaya*; Poptr, *Populus trichocarpa*; Ricco, *Ricinus communis*; Vitvi, *Vitis vinifera*; Sly, *Solanum lycopersicum*; Os, *Oryza sativa*; Pdacty, *Phoenix dactylifera*; Pinta, *Pinus taeda*; and Phypa, *Physcomitrella patens*.

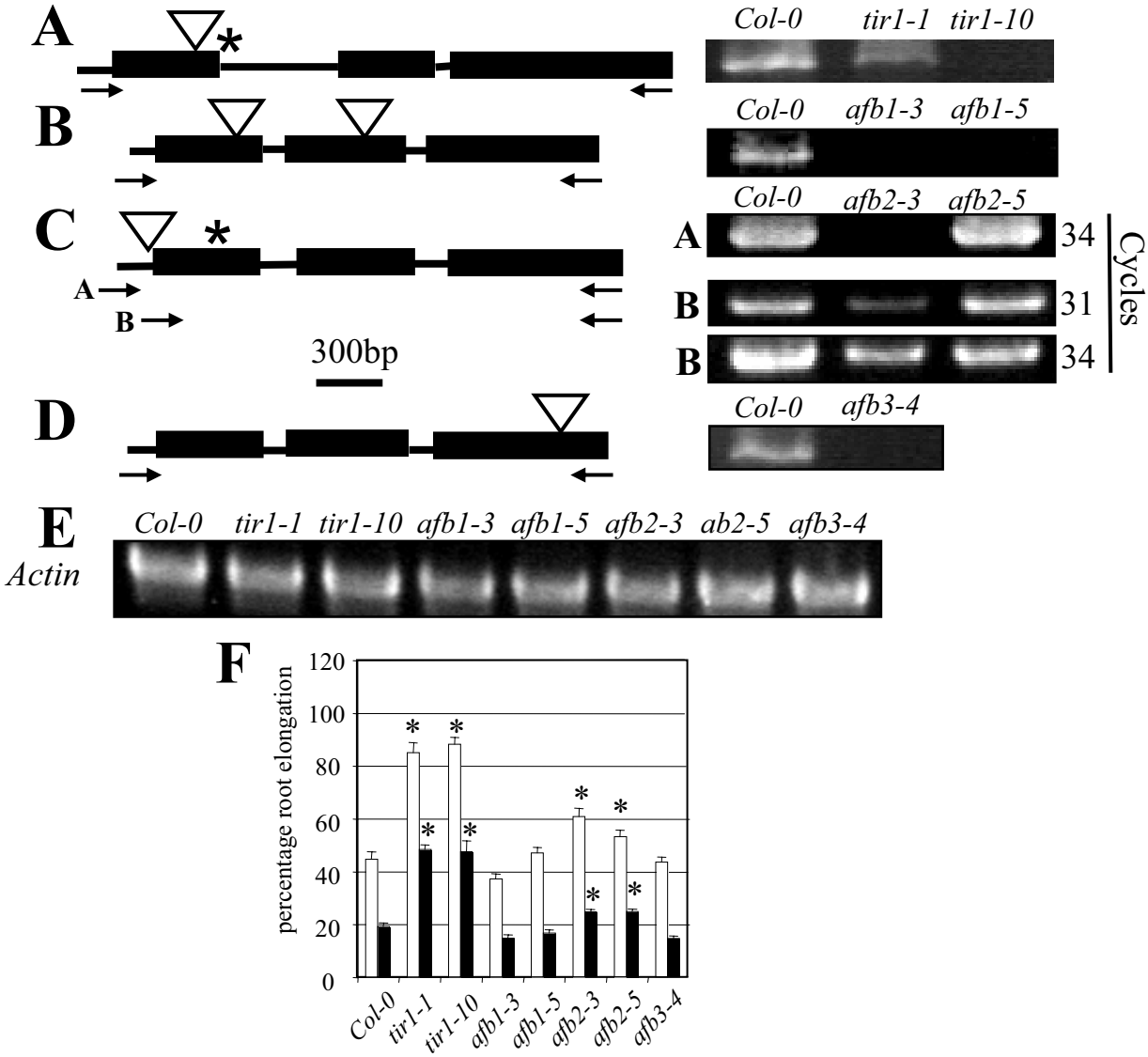


Fig. S2. New *TIR1/AFB* alleles. (*A–D*) Gene structure showing exons (filled bars) and untranslated regions (black lines) in *TIR1* (*A*), *AFB1* (*B*), *AFB2* (*C*), or *AFB3* (*D*). White triangles denote positions of T-DNA insertions in *tir1–10* (*A*), *afb1–3* (*B*, *Left*), *afb1–5* (*B*, *Right*), *afb2–3* (*C*), and *afb3–4* (*D*) alleles. Asterisks denote the position of the point mutations *tir1–1* (*A*) and *afb2–5* (*C*). RT-PCR reactions were performed using RNA isolated from floral and leaf tissue of wild-type and mutant plants. Arrows denote positions of primer pairs used in each PCR. In *C*, primer set *B* was used in PCR for 31 and 34 cycles to show differences in expression level between alleles. (*E*) Amplification by RT-PCR of the *ACTIN2* gene in wild-type and mutant alleles. (*F*) Root elongation assay as described in Fig. 1*A*. Samples marked by an asterisk are significantly different from wild-type samples that grew equivalent auxinconcentrations (Student's *T*-test, $P < 0.005$).

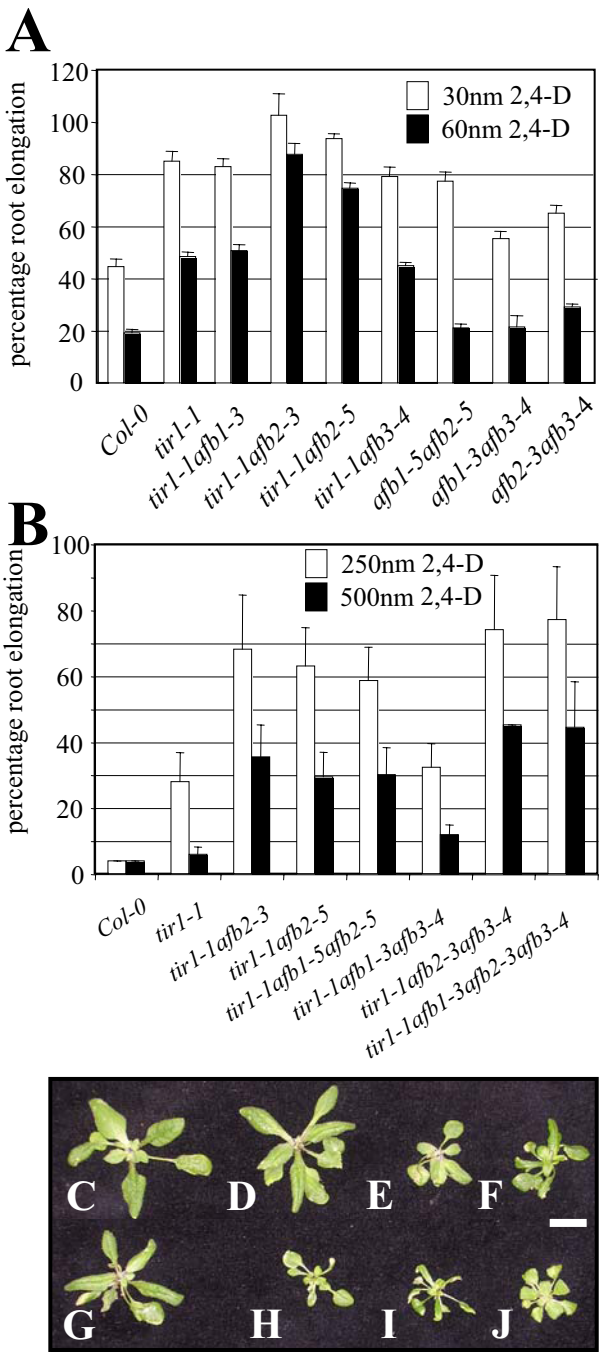


Fig. S3. Role of the TIR1/AFB genes in auxin response. (*A*) Four-day-old seedlings grown on MS media were transferred to media containing 2,4-D. Root elongation was measured after an additional 4 days and expressed as a proportion of growth in the absence of auxin. (Scale bars, SE.) (*B*) Experiment conducted as in 1 *A* except that seedlings were transferred to media containing 2,4-D. (*C–J*) Rosettes of 30-day-old plants. *Col-0* (*C*), *tir1–1* (*D*), *tir1–1afb2–3* (*E*), *tir1–1afb2–5* (*F*), *tir1–1afb1–3afb3–4* (*G*), *tir1–1afb1–5afb2–5* (*H*), *tir1–1afb2–3afb3–4* (*I*), and *tir1–1afb1–3afb2–3afb3–4* (*J*). (Scale bar, 1 cm.)

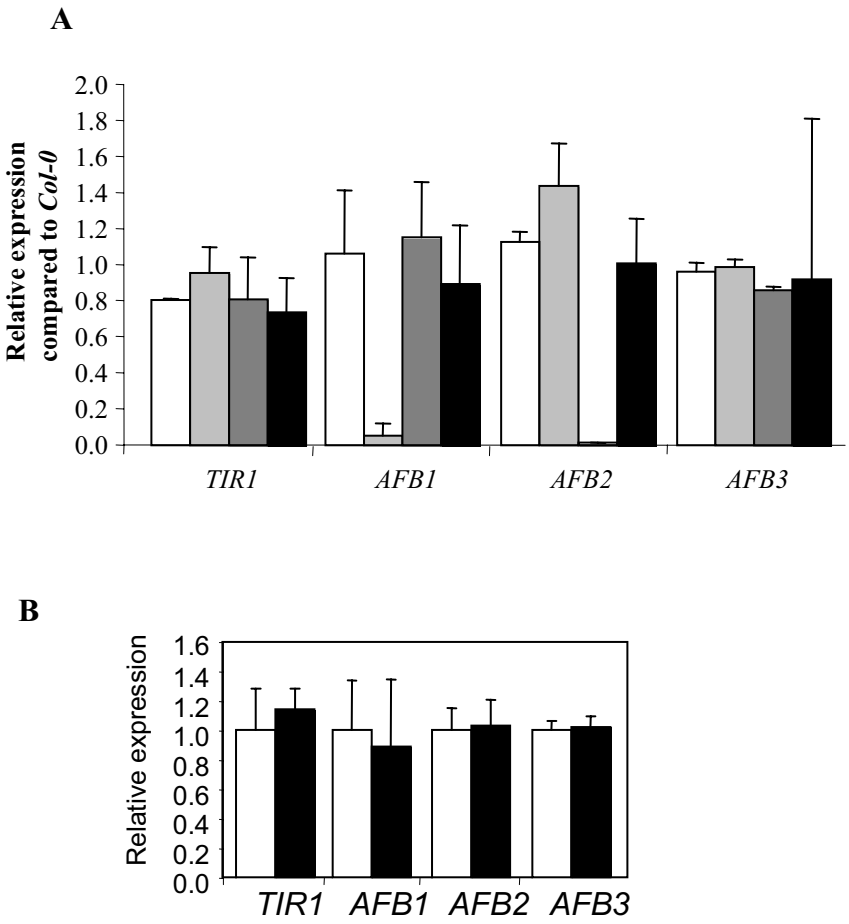
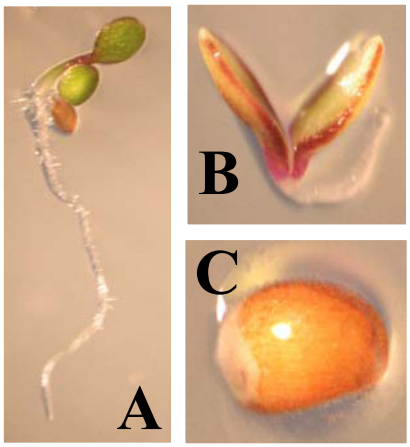


Fig. S4. Expression of *AFB* genes. (*A*) RNA was extracted from roots of *tir1–1* (white), *afb1–3* (light gray), *afb2–3* (dark gray), or *afb3–4* (black) seedlings. Primers corresponding to *TIR1*, *AFB1*, *AFB2*, or *AFB3* were used to perform qPCR and values are expressed relative to the expression in *Col-0* roots. (Scale bars, SD.) (*B*) RNA was extracted from roots of *Col-0* seedlings grown in the absence (white bars) or presence (black bars) of 1 μM IAA. Primers corresponding to *TIR1*, *AFB1*, *AFB2*, or *AFB3* were used to perform qPCR. Values are relative to the expression in the absence of IAA. (Scale bars, SD.)



D Genotype	A	B	C	n
tir1-1afb1-5afb2-5	100%	-	-	>200
tir1-1afb1-3afb3-4	100%	-	-	>200
tir1-1afb2-3afb3-4	37%	32%	31%	169
tir1-1afb1-3afb2-3afb3-4	10%	48%	42%	58

Fig. S5. Seedling phenotypes of higher order *tir1/afb* mutant seedlings. (*A–C*) Appearance of mutant seedlings. Representative seedlings are shown that form a root (*A*), are rootless (*B*), or do not germinate (*C*). (*D*) Table showing the proportions of each seedling type for each genotype.

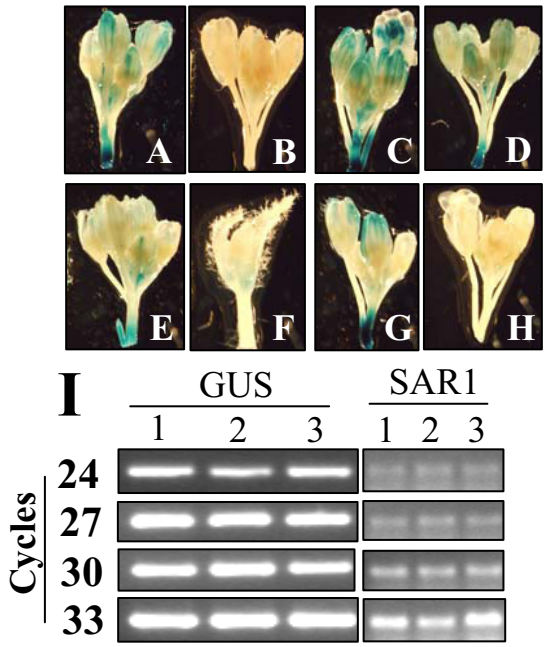


Fig. S6. *TIR1/AFB* gene expression involves posttranscriptional regulation. (*A-H*) GUS expression of flower buds in plants containing *pTIR1:GUS* (*A*) *pTIR1:cTIR1:GUS* (*B*), *pAFB1:GUS* (*C*), *pAFB1:cAFB1:GUS* (*D*), *pAFB2:GUS* (*E*), *pAFB2:cAFB2:GUS* (*F*), *pAFB3:GUS* (*G*), and *pAFB3:cAFB3:GUS* (*H*) transgenes. (*I*) RT-PCR performed on RNA extracted from flowers of plants containing *pTIR1:GUS* (*1*), *pTIR1:cTIR1:GUS line 2–2* (*2*), or *pTIR1:cTIR1:GUS line 24–4* (*B*). Primers were used to amplify from the coding regions of the transgenic GUS gene and the endogenous SAR1 gene (*40*). The PCR reactions were set up identically and run for *24*, *27*, *30*, or *33* cycles.

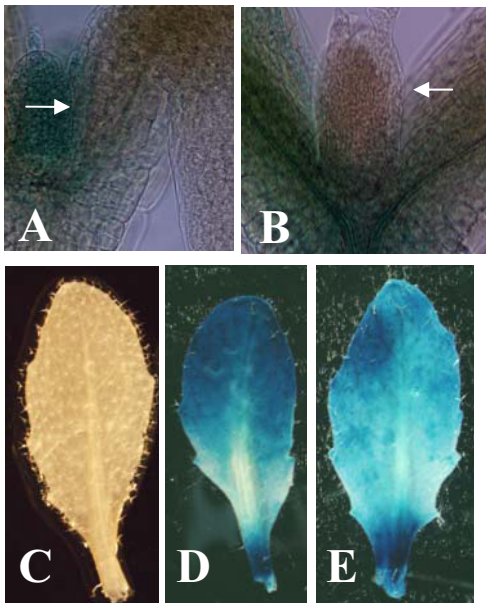


Fig. S7. Expression of *miR393* in leaves. (*A* and *C*) Expression of *pTIR1*:*cTIR1*:*GUS* in young (*A*) and older (*C*) leaves. (*B* and *D*) Expression of *pmiR393a*:*GUS* in young (*B*) or older (*D*) leaves. (*E*) Expression of *pmiR393b*:*GUS* in older leaf.

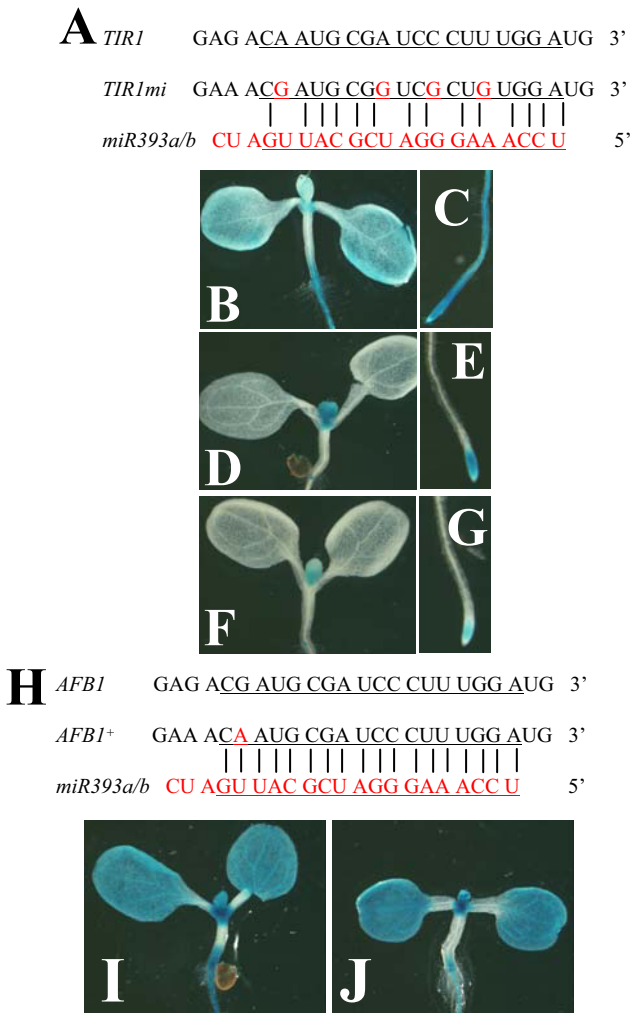


Fig. S8. MicroRNA-resistant forms of *TIR1*:*GUS* do not exhibit altered expression pattern. (*A*) *miR393* recognition sequences in *TIR1* and *TIR1mi*. Mutated nucleotides in *TIR1mi* are shown in red and mismatches with *miR393* are designated by ·. (*B–G*) Expression of *pTIR1*:*GUS* (*B* and *C*), *pTIR1*:*cTIR1*:*GUS* (*D* and *E*), and *pTIR1*:*cTIR1mi*:*GUS* (*F* and *G*) in 7-day-old seedlings showing that the mutated nucleotides have no effect on the expression pattern of TIR1:GUS. (*H*) *miR393* recognition sequences in AFB1 and $AFB1^{TIR1}$. The mutated nucleotide in $AFB1^{TIR1}$ is shown in red. (*I* and *J*) Expression of *pAFB1*:*cAFB1*:*GUS* (*I*) and *pAFB1*:$cAFB1^{TIR1}$:*GUS* (*J*) in 7-day-old seedlings.