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Supplemental Information

The AFB4 Auxin Receptor

Is a Negative Regulator

of Auxin Signaling in Seedlings

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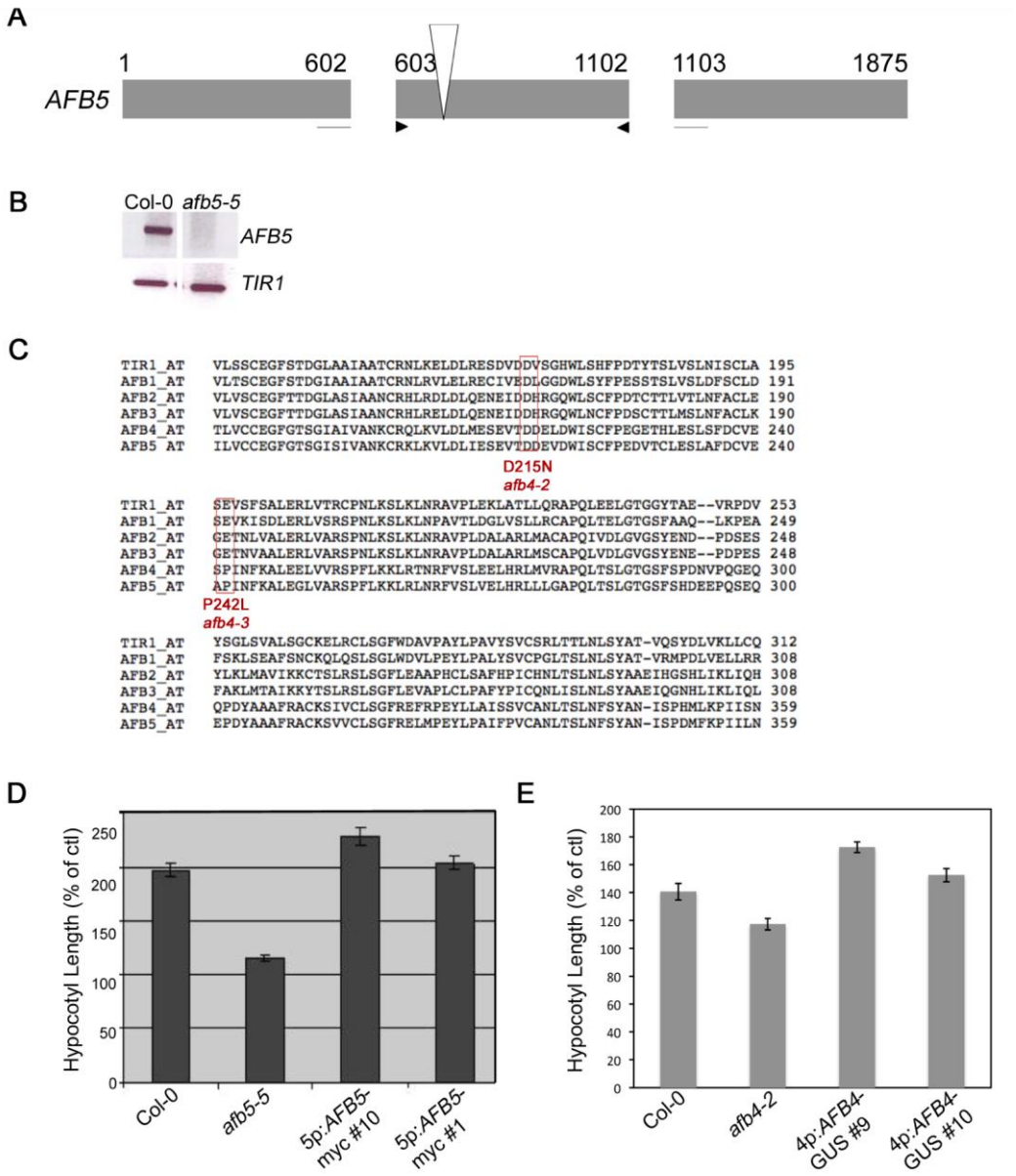


Figure S1. The Mutant Alleles for *AFB4* and *AFB5* (Related to Figure 2)

(A) Diagram of *AFB5* gene and T-DNA insertion site. Arrows indicate primer position for RT-PCR.

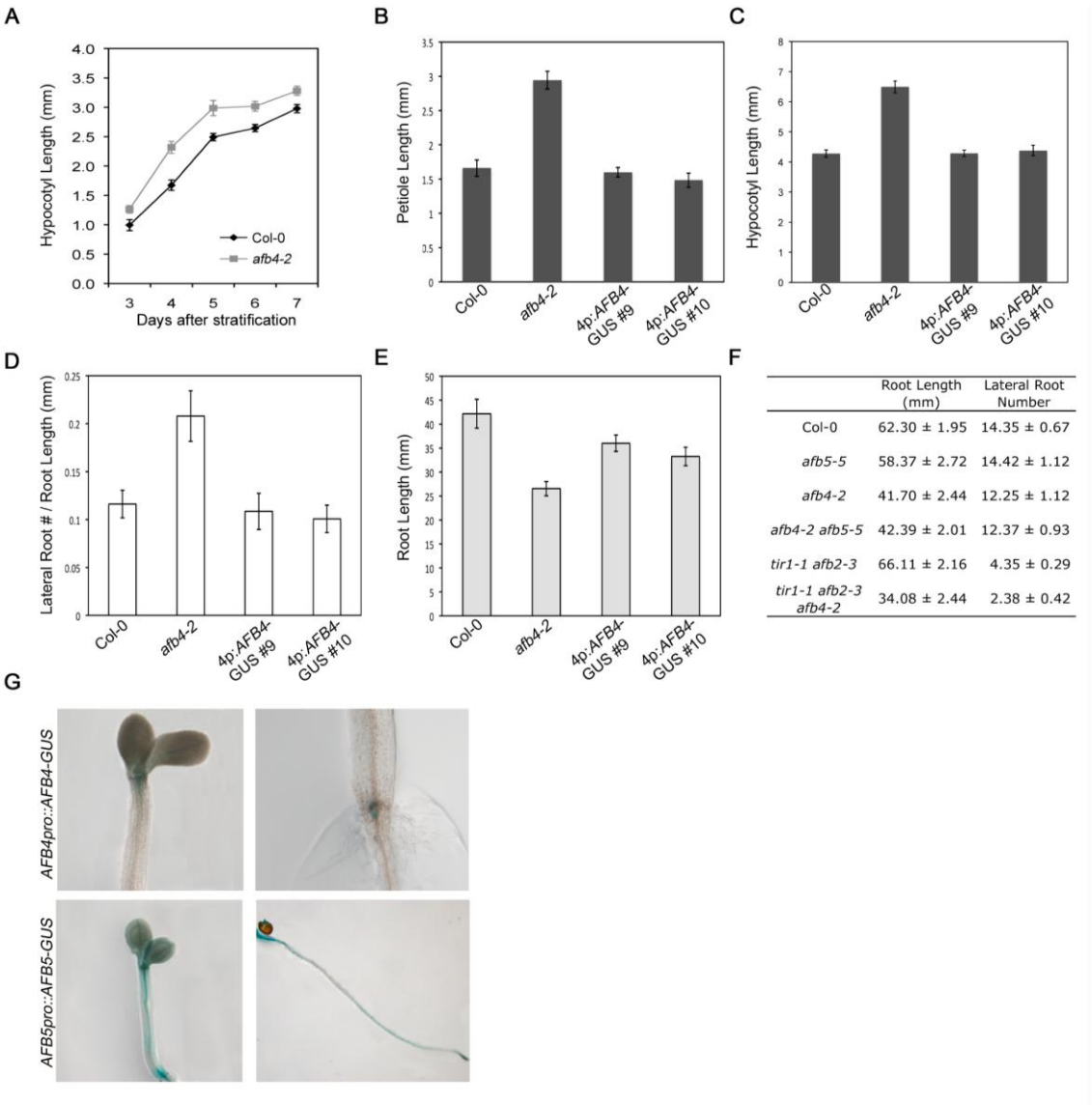
(B) RT-PCR demonstrating loss of AFB5 transcript in *afb5-5*. Reactions with TIR1 primers were used as a control for afb5-5 cDNA integrity.

(C) Alignment of TIR1/AFB proteins demonstrating *afb4* tilling line point mutation sites and their conservation among the family members.

(D) Hypocotyl elongation assay on 5μM picloram demonstrating complementation of *afb5-5* with AFB5-myc expressed from the *AFB5* promoter.

(E) Hypocotyl elongation assay on picloram demonstrating complementation of *afb4-2* following a cross with a line carrying the *AFB4:AFB4-GUS* construct. Seedlings were grown for 4 days and transferred to 5μM picloram for 2 days.

All error bars represent standard error.



	Root Length (mm)	Lateral Root Number
Col-0	62.30 ± 1.95	14.35 ± 0.67
afb5-5	58.37 ± 2.72	14.42 ± 1.12
afb4-2	41.70 ± 2.44	12.25 ± 1.12
afb4-2 afb5-5	42.39 ± 2.01	12.37 ± 0.93
tir1-1 afb2-3	66.11 ± 2.16	4.35 ± 0.29
tir1-1 afb2-3 afb4-2	34.08 ± 2.44	2.38 ± 0.42

Figure S2. The *afb4-2* Mutation Accounts for Several Auxin-Related Defects in the Developing Seedling (Related to Figure 3)

(A) Hypocotyl growth assay with wild-type and *afb4-2* seedlings beginning 3 days after stratification under short day (SD) photoperiods at 22°C. Hypocotyl length was measured for the next 4 days.

(B-E) Complementation of *afb4-2* phenotypes with the line described in Figure S1E.

(B and C) Petiole (B) and hypocotyl length (C) of 6 day old seedlings.

(D and E) Lateral root number (D) and primary root length (E) of 10 day old seedlings grown under long day photoperiods.

(F) Primary root length and lateral root number measurements used to calculate the lateral root #/mm root length shown in figure 3C. Mean length and lateral root number is represented with standard error.

(G) *AFB4:AFB4-GUS* and *AFB5:AFB5-GUS* activity in 4 day old SD grown wild-type seedlings.

All error bars represent standard error.

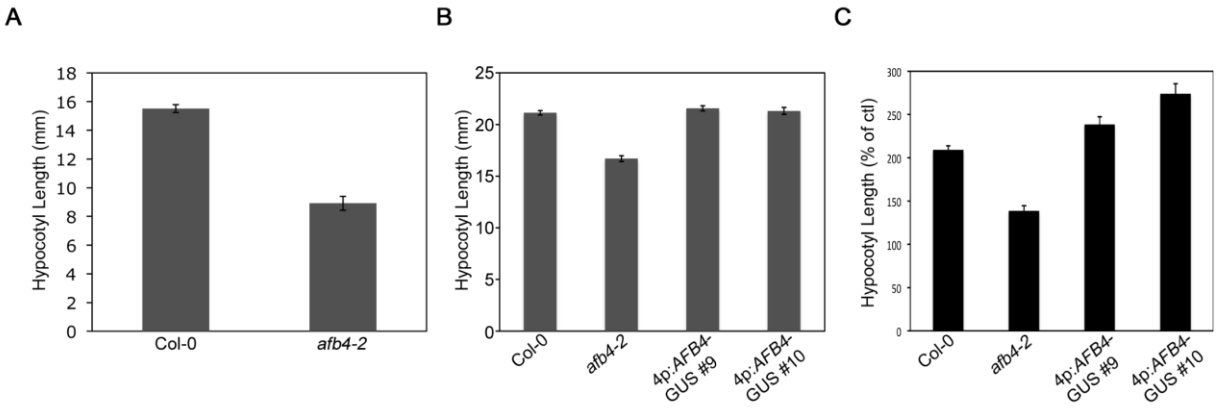


Figure S3. Dark-Grown Hypocotyl Growth of *afb4-2* (Related to Figure 4)

(A) Hypocotyl length of wild-type and *afb4-2* seedlings following 7 days in the dark. Seedlings were stratified for 2 days and subjected to 1hr of light before being shifted to constant dark conditions.

(B) Complementation of the dark grown hypocotyl phenotype of *afb4-2* using the line described in Figure S1E.

(C) Complementation of the 29°C induced hypocotyl elongation resistance of *afb4-2* using the line described in Figure S1E. Seedlings were grown for 4 days at 22°C and shifted to 29°C for an additional 2 days. Elongation is expressed as a percentage of the 22°C control.

All error bars represent standard error.

Supplemental Experimental Procedures

Growth Assays

For auxin inhibited root growth assays, 5-day-old seedlings were transferred onto fresh MS media ± auxin for 3 additional days after which root length was measured. Hypocotyl assays were performed similarly except the seedlings were transferred at day 4 for a 2-day treatment unless otherwise stated. Emerged lateral roots were counted on 10-day-old long day grown seedlings using a Nikon SMZ1500 dissecting scope. Petiole length was measured on 7-day-old SD grown seedlings. All measurements were performed using a Nikon SMZ1500 dissecting scope and ImageJ software (http://rsbweb.nih.gov/ij/index.html).

Generation of Transgenic Lines

The TIR1-myc line was generated as previously described [1]. The AFB4 and AFB5 c-myc lines were generated using a 2-kb 5' upstream region of the *AFB5* gene with the *AFB4* and *AFB5* cDNA. The *AFB5* promoter was used for expressing AFB4 due to the low activity of the *AFB4* promoter. These constructs were transformed into the *afb5-5* mutant background. Transformants were confirmed for their ability to restore picloram sensitivity in the hypocotyl (Fig S1D,E). The GUS fusions were generated with the 2-kb promoter region of each gene ahead of the *GUS* cDNA for the translational fusions and the *AFB4* and *AFB5* cDNA fused to *GUS* in the transcriptional fusions.

Protein Expression and Pull-down Experiments

For pull-down assays, GST-IAA3 and GST-IAA7 were recombinantly expressed in *E.coli* and purified with glutathione agarose beads (SIGMA) using standard methods. Seedlings expressing c-myc-tagged AFB4, AFB5 and TIR1 were grown for 8 days in liquid MS medium. TIR1-myc expression was induced by treatment with 30μM Dex for 24hrs. The ASK1-antibody was generated as previously described [1]. For the various auxin comparisons (Fig 2E) seedlings were incubated for 2hrs in 50μM of the compounds or an equivalent volume of DMSO prior to harvest. For all other pull-down experiments samples were incubated with auxin for 45min following harvest. Tissue was harvested by grinding to a powder in liquid nitrogen and vortexed vigorously in extraction buffer (50mM Tris pH7.5, 150mM NaCl, 10% glycerol, 0.1% NP-40, complete protease inhibitor (Roche), 50μM MG-132). Cellular debris was removed by centrifugation and total protein concentration was determined by Bradford assay. Each pull-down reaction included 1mg total protein extract and equal volumes of GST-IAA protein bound to agarose beads for each sample in a 500μl total volume. The pull-down reactions were incubated at 4°C for 45min with rocking and transferred to a Micro Bio-Spin Chromatography Column (Bio-Rad). Samples were washed 3 times in 1 ml extraction buffer without protease inhibitors or MG-132 in the presence or absence of auxin. Samples were eluted using reduced glutathione (Sigma) and separated on SDS-PAGE and stained with Ponceau (0.1% (w/v) Ponceau S in 5%(v/v) acetic acid) for loading control. AFB/TIR1-myc proteins were detected by immunoblotting with anti-c-myc-Peroxidase antibody (Roche). Proteins were visualized using the ECL Plus Western Blotting Detection System (Amersham).

GUS Staining Assays

Seedlings were fixed in 90% acetone for 20 min followed by incubation in GUS staining solution (50mM sodium phosphate buffer pH 7, 0.1% Triton X-100, 10mM EDTA and 0.5mg/ml 5-bromo-4-chloro-3-indolyl-β-glucuronic acid in N,N-dimethylformamide) overnight at 37°C. Seedlings were cleared in 70% (v/v) ethanol and imaged using a Nikon SMZ1500 dissecting scope.

RNA Extraction and Quantitative PCR

Hypocotyl, cotyledon and root tissue frozen in liquid N_2 and ground using a mortar and pestle was used for RNA extractions using the INVITROGEN PureLink RNA mini kit. RNA yield was quantified using the Thermo Scientific NanoDrop 2000. For quantitative RT-PCR, 1μg RNA, pre-treated with DNase using the DNA-free Kit (Ambion) according to manufacturer's instructions, was used for generating cDNA with SuperScript III First-Strand Synthesis (Invitrogen) and 20-mer oligo(dT) primers. Quantitative RT-PCR was performed using SyBR green and the primers listed in the table below. Primer pairs were evaluated for specificity and efficiency using three serial dilutions of cDNA using the CFX96^{TM} Real-Time PCR Detection System (Bio-Rad). Data was normalized to the reference primer PP2AA3 [2] according to the ΔΔCt method. All other primers were designed using QuantPrime [3]. Two biological replicates were performed, each replicate containing roughly 700 individual seedlings that were dissected into cotyledon, hypocotyl and root samples.

Annotation	AGI	5'->3' Primer Sequences (F/R)
TIR1	AT3G62980	ATCGCTGCCACTTGCAGGAATC TGGCCACTAACGTCGTCAACATC
AFB1	AT4G03190	GCTACTGTCCGAATGCCTGATCTTG GCCTTGTTCCGTCAGAGGTATGTTG
AFB2	AT3G26810	GCCGCTAATTGCAGGCATCTTC AGTCGTGCAAGTGTCTGGGAAAC
AFB3	AT1G12820	AGGTTGAAGCGGATGGTTGTAACAG GCAAGTCCAGCTCACGAAGATGC
AFB4	AT4G24390	TGCTCAAGCCCATCATAAGCAAC TCGAGTCAAGAGCCCAGAAGACTC
AFB5	AT5G49980	TGCCAACAAGTGCAGAAAGCTG TCCACTTCATCATCCGTGACCTC
ACS8	AT4G37770	TGGCCTTCCTTCCTTCAAGAATGC TGAGAGTCTCGTTAGCCGGAGTAG
IAA5	AT1G15580	TCCGCTCTGCAAATTCTGTTCG ACGATCCAAGGAACATTTCCCAAG
IAA19	AT3G15540	GGTGACAACTGCGAATACGTTACCA CCCGGTAGCATCCGATCTTTTCA
SAUR15	AT4G38850	TTGAGGAGTTTCTTGGGTGCTAAG GCCATGAATCCTCTTGGTGTCG
GH3.1	AT2G14960	CGATCGTCGCCAGCTTCTTTAC CCCGGCACATACAAATTCATTACG
GH3.3	AT2G23170	ACCGGAGATTCAACGTATTGCC CAGAGCTTGTGAGGAACTCTGTG
HAT2	AT5G47370	AACGTCGAGGAAGAAGCTCAGG AGCTTCTGTTTGGGATTGAGAGTG

Quantification of IAA

Tissues were pooled, weighed, and frozen in liquid nitrogen for quantification of free IAA content. The frozen samples contained between 10 and 15 mg of tissue (fresh weight) and were collected in five replicates for hypocotyl and three replicates for cotyledon + hypocotyl samples. Sample extraction and purification was performed according to [5] with 500 pg $^{13}C_6$-IAA internal standard added to each sample before extraction. After derivatization, the samples were analyzed by gas chromatography-selected reaction monitoring-mass spectrometry as described previously [5].

Supplemental References

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