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2 Synthetic analysis of natural variants yields insights into the evolution
3 and function of auxin signaling F-box proteins in *Arabidopsis thaliana*

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ABSTRACT

2 The evolution of complex body plans in land plants has been paralleled by gene
3 duplication and divergence within nuclear auxin-signaling networks. A deep mechanistic
4 understanding of auxin signaling proteins therefore may allow rational engineering of
5 novel plant architectures. Towards that end, we analyzed natural variation in the auxin
6 receptor F-box family of wild accessions of the reference plant *Arabidopsis thaliana* and
7 used this information to populate a structure/function map. We used a synthetic assay
8 to identify natural hypermorphic F-box variants, and then assayed auxin-associated
9 phenotypes in accessions expressing these variants. To directly measure the impact of
10 sequence variants on auxin sensitivity, we generated transgenic plants expressing the
11 most hypermorphic natural alleles. Together, our findings link evolved sequence
12 variation to altered molecular performance and phenotypic diversity at the organism-
13 level. This approach demonstrates the potential for combining synthetic biology
14 approaches with quantitative phenotypes to harness the wealth of available sequence
15 information and guide future engineering efforts of diverse signaling pathways.

16

INTRODUCTION

17 Auxin controls many aspects of plant development and environmental
18 adaptation. Natural and synthetic auxins have been used to control plant growth in
19 fields, greenhouses and laboratories for nearly a century. In recent years, the gene
20 families of biosynthetic and metabolic enzymes, transporters and perception machinery
21 that determine the spatial, temporal and developmental specificity of auxin signals have
22 been identified (Enders and Strader 2015). Recent work has just begun to determine

1 how functionally robust the auxin signaling machinery is to mutation (Yu *et al.* 2013,
2 2015), and to measure the propensity for mutations to produce novel plant phenotypes
3 that result in evolutionary innovation (Delker *et al.* 2010; Rosas *et al.* 2013). As auxin
4 effects are so wide-ranging, it is not surprising to find that significant variation exists in
5 auxin sensitivity and auxin-induced transcription across *A. thaliana* accessions (Delker
6 *et al.* 2010), perhaps contributing to morphological diversity. As such mapping
7 evolutionary trajectories in auxin signaling could facilitate the engineering of numerous
8 plant traits, such as root architecture, shoot branching or leaf venation—all traits
9 associated with crop yield (Mathan *et al.* 2016).

10 Auxin is perceived by a coreceptor complex consisting of an F-box protein
11 (TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOXES, TIR1/AFB;
12 hereafter referred to as AFBs), an auxin molecule and a member of a transcriptional
13 coreceptor/corepressor family (AUXIN/INDOLE-3-ACETIC ACID PROTEINS, Aux/IAAs)
14 (Lavy and Estelle 2016). The F-box domain of the AFB associates with a Skp/Cullin/F-
15 box (SCF) ubiquitin ligase complex that facilitates ubiquitination of the Aux/IAA proteins,
16 targeting them for degradation (Lavy and Estelle 2016). In low auxin conditions, Aux/IAA
17 proteins interact with and repress a family of transcription factors, the Auxin Response
18 Factors (ARFs) (Guilfoyle and Hagen 2007). Auxin response genes are turned on when
19 local auxin accumulation triggers degradation of Aux/IAAs thereby relieving the
20 repression on ARFs.

21 *A. thaliana* has six AFB genes, *TIR1* and *AFB1-AFB5* (Dharmasiri *et al.* 2005a).
22 The N-terminal F-box domain is modular and functionally conserved in *TIR1* and *AFB2*,
23 both of which form functional E3 ubiquitin ligase complexes with components in yeast

1 and animals (Nishimura *et al.* 2009; Zhang *et al.* 2015). The C-terminal domain of the
2 AFBs is a leucine-rich repeat (LRR). LRR domains offer a highly evolvable scaffold for
3 binding small molecules and proteins and perform diverse functions across all domains
4 of life (Bella *et al.* 2008). The AFB LRR domain allows auxin sensing by interacting with
5 both auxin and the Aux/IAA transcriptional repressor/co-receptor proteins (Dharmasiri *et*
6 *al.* 2005a; Tan *et al.* 2007; Calderón Villalobos *et al.* 2012). The identity of the subunits
7 and their affinity for one another governs the rate of Aux/IAA degradation which, in turn,
8 governs transcriptional dynamics, cell fate and morphological change (Dreher *et al.*
9 2006; Pierre-Jerome *et al.* 2014; Guseman *et al.* 2015; Galli *et al.* 2015).

10 Here, we paired an examination of the natural coding sequence variation in the
11 AFB family with quantification of functional variation. We used a synthetic auxin-induced
12 degradation assay in yeast to assess the function of natural variants in isolation from
13 the rest of the auxin response network. Variants with altered function were then
14 evaluated in their native context by quantifying auxin-associated root growth inhibition in
15 accessions containing these polymorphisms. Finally, we directly measured the
16 contribution to auxin sensitivity of several of the alleles with greatest effect by
17 generating transgenic plant lines expressing these variants under a constitutive
18 promoter. Through this work, we have generated a higher resolution structure/function
19 map of the AFB family and provided evidence that single polymorphisms within this
20 family can modify plant architecture.

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MATERIALS AND METHODS

2 **Materials, media composition and general growth conditions**

3 PCRs were performed with Phusion (cloning reactions; NEB, Ipswich, MA),
4 GoTaq (diagnostics; Promega, Madison, WI) or GemTaq (genotyping; MGQuest,
5 Lynnwood, WA) with primers from IDT (Coralville, Iowa). Media were standard
6 formulations as described in (Pierre-Jerome *et al.* 2017). Plants were grown on 0.5x LS
7 media (Caisson Laboratories, Smithfield, UT) containing 0.5% sucrose and 0.7%
8 phytoagar (plantmedia, Dublin, OH). Seeds were obtained from the Arabidopsis
9 Biological Resource Center (Columbus, OH).

10 **Analysis of sequence variation**

11 A reference dataset of the genome locations of the TIR1/AFB family and COI1
12 was assembled from the TAIR10 database on 28 July 2015. Transcript and coding
13 sequences were identified using the ENSEMBL biomart version of TAIR10. The 1001
14 genomes Salk dataset (28 June 2010) was obtained from <http://1001genomes.org/>.
15 Single nucleotide polymorphisms (SNPs) and one base pair deletions with a quality
16 (PHRED) score of 25 and above (i.e. “quality_variant_filtered” files) were used for the
17 following analysis using a custom R scripts unless otherwise specified. SNPs located in
18 genes of interest were isolated and mapped to their respective gene structures using
19 the VariantAnnotation package (Obenchain *et al.* 2014). Coding variants were identified
20 and assembled for each gene and each accession. A dN/dS matrix of all-by-all pairs of
21 accessions was calculated for each gene using the kaks function within the seqinr R-
22 package (Charif and Lobry 2007), which implements the method of Nei and Gojobori
23 (Nei and Gojobori 1986). Additionally, the genes were split into F-box and LRR

1 domains, with the F-box defined as the N-terminus of the protein to I50 of TIR1 and the
2 corresponding residues of the other genes according to the alignment generated by Tan
3 et al. (Tan *et al.* 2007). The N-terminal extension of AFB4 and 5 were excluded.
4 Domain-wise dN/dS matrices were calculated as above. Incalculable and infinite values
5 were excluded from these matrices prior to extraction of median values and outlier
6 pairs. Annotated code and supplemental data are in S6 Appendix.

7 **Strain construction**

8 Plasmids were designed using j5 (Hillson *et al.* 2012) and constructed by
9 aquarium (www.aquarium.bio). TIR1 and AFB2 were separately inserted into pGP8G
10 (Havens *et al.* 2012) downstream of a GPD promoter and followed by 3X-FLAG-6X-HIS
11 tandem affinity purification tag, via Golden Gate cloning (Engler *et al.* 2009). Mutations
12 were introduced into the parent vectors via two-fragment Gibson assembly (Gibson *et*
13 *al.* 2009). The coding sequence of the gene of interest was confirmed by sequencing
14 (Genewiz, South Plainfield, NJ).

15 Plasmids were digested with *PmeI* before Lithium PEG (37) transformation into
16 W303-1A ADE2+ yeast (MAT α , leu2-3,112 trp1-1 can1-100 ura3-1 his3-11,15 ybp1-1).
17 Correct integration of transformed colonies was confirmed by diagnostic PCR across
18 the 3' boundary of homologous recombination, relative to the gene of interest. Similarly,
19 pGP4GY-IAA1 and -IAA17 (Havens *et al.* 2012) were transformed into W814-29B yeast
20 (MAT α ade2-1 trp1-1 can1-100 ura3-1 leu2-3,112 his3-11,15). Confirmed transformants
21 were struck to isolation on YPAD plates. *AFB* strains were individually mated with each
22 *Aux/IAA* strain using standard methods (Pierre-Jerome *et al.* 2016).

1 **Auxin-induced degradation assays in yeast**

2 Assays were essentially as described in (Pierre-Jerome *et al.* 2017) using a BD
3 special order cytometer with a 514 nm laser exciting fluorescence that is cutoff at 525
4 nm prior to PMT collection (Becton-Dickinson, Franklin Lakes, NJ). Events were
5 annotated, subset to singlet yeast, and normalized to initial levels of fluorescence using
6 the flowTime R package (<http://www.github.com/wrightrc/flowTime>). Full dataset is
7 available via FlowRepository (<http://tinyurl.com/j268y5e>). Additional detail in S6
8 Appendix.

9 **Root growth inhibition assays**

10 After sterile seeds were stratified at 4°C in the dark for 3 days (or 1 week for wild
11 accessions), they were transferred to long day conditions at 20°C for 4 days. Plants
12 were then transferred to plates containing either DMSO carrier or 2,4-
13 dichlorophenoxyacetic acid (2,4-D) with root tips aligned to a reference mark. Plants were
14 scanned after an additional 4 days of growth. Root growth was measured using ImageJ
15 (Rasband 1997) and an Intuos Pro drawing pad (Wacom, Portland, Oregon). Additional
16 detail in S6 Appendix.

17 **Construction and analysis of transgenic plants**

18 Genes of interest were inserted via Golden Gate cloning (Engler *et al.* 2009) into
19 pGreenII (Hellens *et al.* 2000) with a pUBQ10 promoter and 3X-FLAG-6X-HIS tandem
20 affinity purification tag. Plasmids were transformed into *Agrobacterium tumefactions*
21 GV3101 with pSOUP (Hellens *et al.* 2000) via electroporation, and transformants were
22 selected on plates with 50 µg/mL gentamycin and 25 µg/mL kanamycin. Plants were
23 transformed by floral dip (Zhang *et al.* 2006), and transformants were selected on plates

1 with 30 µg/mL hygromycin at four days post germination after an initial light exposure for
2 seven hours. Root growth inhibition phenotypes were quantified in T2 generation of
3 three independent transformants as described above. Each plant was genotyped for the
4 presence of the hygromycin resistance gene after the growth assay, using the forward
5 primer (GATGTTGGCGACCTCGTATT) and the reverse primer
6 (GTGCTTGACATTGGGGAGTT). Additional detail in the S6 Appendix.

7 Plasmids, strains and sequence files are available upon request or via Addgene.
8 All code used to perform analysis and visualization is provided in S6 Appendix. All data
9 including raw images are available upon request.

10 RESULTS

11 We identified polymorphisms across the entire AFB gene family in the 170 *A.*
12 *thaliana* accessions of the SALK subset of the 1001 Genomes Project (Schmitz *et al.*
13 2013). We found 1,631 polymorphisms within coding regions, and, of these, 273
14 polymorphisms were predicted to result in amino acid substitutions (Table 1 and S1
15 Fig). *AFB3* had the highest level of nonsynonymous mutation relative to synonymous
16 mutation, suggesting it may be undergoing neo-functionalization. *AFB4*, critical for
17 response to the synthetic auxin picloram (Prigge *et al.* 2016), had the largest number of
18 coding sequence polymorphisms (more than 15X the number found in *TIR1*) and the
19 largest number of nonsynonymous polymorphisms (18X the number in *TIR1*) including
20 the only two nonsense polymorphisms identified in this dataset. In contrast, *AFB1*,
21 which is largely incapable of forming a functional SCF complex (Yu *et al.* 2015), has
22 very similar ratio of nonsynonymous to synonymous changes as *TIR1*. Many of the

1 accessions contained nonsynonymous polymorphisms in multiple members of the AFB
2 family (S2 Table). These additional mutations tended to occur more frequently in sister
3 pairs (TIR1 and AFB1, AFB2 and AFB3, AFB4 and AFB5).

4 None of the identified accessions have nonsynonymous polymorphisms in both
5 *TIR1* and *AFB2* (S2 Table). This may mean that *AFB2* and *TIR1* serve partially
6 redundant yet distinct functions, a conclusion supported by genetic analysis (Dharmasiri
7 *et al.* 2005a; Parry *et al.* 2009). The majority of the mutations in *TIR1* and *AFB2*
8 occurred in positions of high diversity across the Col-0 AFB family, and most were
9 located in surface residues of the LRR domain (Fig 1A). The majority of these mutations
10 spanned the exterior helices and loops of the fourth through eighth LRRs (Fig 1C). This
11 region was recently identified as being responsible for SCF^{TIR1} dimerization (Dezfulian
12 *et al.* 2016) and is also proximal to the S-nitrosylation site (Terrile *et al.* 2012). A pair of
13 mutations exists on the surface spanning the final three LRRs and the C-terminal cap
14 (Fig 1D). This region may interact with the KR motif known to strongly affect auxin-
15 induced degradation rates (Dreher *et al.* 2006; Moss *et al.* 2015). A final pair of
16 mutations was found on the interior surface of the LRR domain horseshoe (Fig 1E).

17 **Synthetic yeast assays reveal functional variation in *TIR1* and *AFB2***

18 An auxin-induced degradation assay has been established in yeast using
19 heterologous expression of either *TIR1* or *AFB2* (Havens *et al.* 2012). We used this
20 synthetic assay to quantify the function of AFB natural variants in the absence of the
21 potentially confounding effects of feedback from the auxin pathway itself or from
22 modulation by other integrating pathways. Natural variants were engineered into the
23 Col-0 reference sequence with co-occurring polymorphisms cloned individually and in

1 combination. Each AFB was then constitutively co-expressed in yeast with fluorescently
2 labeled Aux/IAA targets. Auxin-induced degradation was measured for two targets,
3 IAA1 and IAA17, as these substrates show distinct patterns of behavior when assayed
4 with Col-0 TIR1 and AFB2. TIR1^{Col} induces degradation of IAA1 and IAA17 at similar
5 rates, while AFB2^{Col} causes IAA17 to degrade much faster than what is observed for
6 IAA1 (Havens *et al.* 2012). We focused on polymorphisms in the LRR domain that were
7 predicted to be functionally divergent (having any pairwise d_N/d_S value greater than
8 one), but analysis of the few additional polymorphisms is shown in Figures S4 and S5.

9 Some natural variants increased function compared to the Col-0 reference, while
10 others decreased or nearly abrogated function (referred to hereafter as hypermorphs,
11 hypomorphs and amorphs, respectively) (Fig 2). Of the *TIR1* polymorphisms, T154S
12 was hypermorphic and E239K-S546L was strongly hypomorph (Fig 2A). E239K alone
13 was nearly amorphic, and adding S546L only slightly restored activity. T491R was the
14 only clear hypermorph identified among the *AFB2* polymorphisms (Fig 2B). D176E was
15 slightly hypermorphic, whereas A254V was a moderate hypomorph. In combination,
16 these two mutations were largely additive, giving a response quite similar to AFB2^{Col}.
17 AFB2^{Q169L} was also a moderate hypomorph. Two *AFB2* alleles, R396C and R204K,
18 were strong hypomorphs, and T179M was amorphic in our assays. Interestingly, the two
19 most highly represented variants, TIR1^{T154S} (present in 5 accessions) and AFB2^{R204K} (6
20 accessions), show strong functional divergence from their respective wild-type proteins.

1 **Accessions containing a hypermorphic *TIR1* allele are hypersensitive to** 2 **auxin**

3 We next assessed whether the functional variation observed in the synthetic
4 assays was manifested in phenotypic differences in the respective accessions. To do
5 this, we measured inhibition of primary root growth in the presence of exogenous auxin,
6 a bioassay that has been used extensively to identify and characterize mutants in the
7 *AFB* gene family (Gray *et al.* 1999; Dharmasiri *et al.* 2005a; b; Parry *et al.* 2009). We fit
8 a log-logistic dose response model to the data to allow a more precise comparison. One
9 parameter, the effective dose of auxin required to elicit fifty percent of the maximum root
10 growth inhibition (ED50), was the most effective metric for differentiating among the
11 genotypes we assayed. Two *tir1* mutants in the Col-0 background (a point mutation *tir1-*
12 *1* and a null insertion *tir1-10*) were also included in our analysis. Both mutants had
13 significantly higher ED50s as expected (Fig 3A and C). A loss of function *afb2* allele did
14 not significantly affect the root growth response in our assays, although *tir1-1 afb2-3*
15 double mutants had a much larger ED50 than the *tir1-1* single mutant.

16 In general, the root growth response of the accessions we analyzed was only
17 subtly different from that of Col-0 (S6 Appendix, pg. 38-40), with one notable exception.
18 Four out of five accessions carrying *TIR1*^{T154S} were hypersensitive to auxin, following
19 the pattern predicted by the hypermorphic behavior of that variant in yeast (Fig 3B and
20 C). This led us to hypothesize that the *TIR1*^{T154S} acts as a natural gain-of-function allele
21 with the capacity to impact organ-level auxin responses.

1 **A common *TIR1* allele confers auxin hypersensitivity to Col-0**

2 We next generated transgenic Col-0 lines expressing *TIR1*^{Col} or *TIR1*^{T154S} under
3 a constitutive promoter to quantify the phenotypic effect of *TIR1*^{T154S}. As was observed
4 in yeast and in the wild accessions, *TIR1*^{T154S} increased auxin sensitivity relative to
5 *TIR1*^{Col} (Fig 3D) in root inhibition bioassays (e.g., 20 nM 2,4-D, $p < 10^{-6}$, full statistical
6 analysis shown in S6 Appendix). The trend of increased sensitivity conferred by
7 *TIR1*^{T154S} could be observed even in the absence of exogenous auxin, suggesting a
8 differential response to endogenous auxin levels.

9 We similarly quantified the effect of *AFB2*^{T491R}, the only hypermorphic *AFB*
10 variant we identified. These plants had somewhat shorter roots than plants expressing
11 *AFB2*^{Col} under low auxin and mock treatments. While *AFB2*^{T491R} had a significant effect
12 on root growth inhibition compared with *AFB2*^{Col} ($p = 0.009$), the interaction between
13 transgene and treatment was not significant. This is consistent with the finding that the
14 hypersensitive response of *AFB2*^{T491R} was strongest in the absence of exogenous auxin
15 and became undetectable at high auxin levels—the opposite trend as what was
16 observed with *TIR1*^{T154S}. One possibility is that these results reflect a degree of
17 specialization in *TIR1* and *AFB2* responses at distinct auxin dosages. In support of that
18 hypothesis, loss of *AFB2* function had a much weaker effect on root growth compared
19 with loss of *TIR1*, but the combination was strongly auxin resistant (Fig 3A and
20 C)(Dharmasiri *et al.* 2005a).

1 **Dimerization domain variation affects dominance relations between TIR1** 2 **alleles**

3 One of the unexpected findings in our analysis of auxin response across
4 genotypes was a subtle but highly reproducible difference between the two induced
5 alleles of *tir1* in the Col-0 background (Fig 3A, C). The point mutation *tir1-1* showed a
6 consistently stronger loss of auxin sensitivity than the T-DNA insertion *tir1-10*, raising
7 the possibility that *tir1-1* might be acting as a dominant negative or antimorph rather
8 than as a simple loss-of function. In support of that interpretation, *tir1-1* mutants are
9 semi-dominant (Ruegger *et al.* 1998), and the *tir1-1* allele (*G147D*) and several other
10 mutations in nearby residues negatively affect SCF^{TIR1} dimerization and activity
11 (Dezfulian *et al.* 2016).

12 We turned to the yeast synthetic system to further investigate this question. By
13 transforming a single copy of each allele into haploid yeast strains of each mating type,
14 we created all pairs of alleles via mating. We also created *tir1*^{K159*} a mimic of the *tir1-10*
15 T-DNA insertion allele. As expected, *tir1*^{K159*} was an amorph, behaving similarly to an
16 empty expression cassette (S5 Fig). *TIR1* dosage had little effect on auxin response in
17 these assays, as *TIR1/tir1-10* heterozygotes responded similarly to *TIR1* homozygotes
18 (Fig 4A). In contrast, expression of *tir1-1* completely abrogated *TIR1* activity (Fig 4B),
19 providing strong evidence that *tir1-1* is indeed a dominant negative allele.

20 **DISCUSSION**

21 The analysis of intraspecific variation in auxin sensitivity presented here critically
22 extends previous work on the evolution of this pathway by focusing on protein level

1 functional variation. Synthetic assays allowed for direct quantification of differences in
2 the ability of TIR1 and AFB2 variants to facilitate ubiquitin-mediated degradation of their
3 substrates. The creation of a structure/function map of natural variation revealed several
4 areas of the F-box-LRR protein scaffold that can accommodate mutations, while
5 modulating auxin sensitivity. This analysis further underscored the importance of the
6 AFB dimerization domain (Dezfulian *et al.* 2016) to regulate SCF activity.

7 The *AFB* family provides a test case for genome evolution after gene duplication,
8 as there is evidence of both significant novelty and redundancy between family
9 members (Dharmasiri *et al.* 2005a; Walsh *et al.* 2006; Parry *et al.* 2009; Hu *et al.* 2012).
10 Analysis of coding sequence polymorphisms in this study revealed significant
11 differences across the gene family. These apparent differences in evolutionary
12 trajectories raise the possibility for lineage-specific functional specialization. In support
13 of this idea, several of the polymorphisms analyzed here were found in multiple
14 accessions across a wide geographic area. These accessions, as well as those
15 accessions with phenotypes not predicted by our synthetic functional analysis, should
16 facilitate future examination of evolutionary robustness and plasticity in nuclear auxin
17 signaling and downstream gene networks.

18 Functional diversification is occurring within the *Arabidopsis TIR1* lineage.
19 Differences observed in *TIR1* variants analyzed in isolation in synthetic assays were
20 frequently predictive of plant phenotype, pointing to a potential role for divergence in
21 receptor function in allowing for optimization of auxin responses in new environments.
22 The integrated biochemical and phenotypic analysis of natural variants refined the map
23 of functionally relevant residues in TIR1 and AFB2, as well as generated hypotheses

1 about differential evolutionary paths for different *AFB* family members. This information,
2 along with the general evolvability of the LRR scaffold (Bella *et al.* 2008), make the
3 AFBs prime candidates for engineering novel traits in crops.

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FIGURE CAPTIONS

2 **Fig 1. Clusters of natural variation in *TIR1* and *AFB2*.** (A) Identified mutations tend to occur in residues
3 of high diversity within the *Arabidopsis* AFB family. A top down view of the LRR domain of the TIR1
4 structure (PDB:2P1Q) is shown with the F-box domain in the bottom right and the LRR domain spiraling
5 counterclockwise. The backbone of the TIR1 structure (Tan *et al.* 2007) was colored according to protein
6 sequence diversity with conserved residues in blue and diverging residues in red. Diversity was
7 calculated as Shannon Entropy using an alignment of the protein sequences of the *Arabidopsis* AFB
8 family (TIR1, AFB1-5). All mutations are shown as sticks. AFB2 variants are in light blue and TIR1
9 variants are in purple. Previously identified TIR1 mutants are in dark blue (Ruegger *et al.* 1998; Yu *et al.*
10 2013). The IAA7 degron is shown as a light green ribbon with side-chains as sticks. The N-terminal
11 residue of the IAA7 degron is in lighter green and the C-terminal residue is darker green. Circles around
12 polymorphisms match the detailed views shown in panels C, D and E. (B) Mutations face the Cullin
13 subunit of the predicted SCF^{TIR1} structure. ASK1 (light grey) was aligned with SKP1 from the human
14 SKP2-SKP1-Cul1-RBX1 structure (PDB: 1LDK, shown in dark grey), docking with TIR1 (gold). Putative
15 E2 location is labeled. (C) The dimerization domain on the N-terminal side of the LRR horseshoe contains
16 the majority of natural variation in *TIR1* and *AFB2*. The *tir1-1* allele (*tir1*^{G147D}) is in light purple. (D) Two
17 variants were located on the C-terminal side of the LRR close to the N-terminus of the degron. (E) Two
18 additional variants were located inside the LRR horseshoe, near the inositol-hexakisphosphate cofactor.

19 **Fig 2. Synthetic assays reveal significant functional variation in naturally occurring AFB**
20 **polymorphisms.** Nonsynonymous mutations in the LRR domains of *TIR1* (A) and *AFB2* (B) with dN/dS
21 values >1 were synthesized and co-expressed in yeast with fluorescently labeled IAA1 or IAA17.
22 Degradation was assessed using flow cytometry on cultures exposed to different concentrations of the
23 auxin indole-3-acetic acid (IAA) for one hour. Error bars represent 95% confidence intervals around the
24 median fluorescence calculated from three independent experiments. In many cases, intervals are small
25 enough that they appear as a single line. The reference Col-0 variant is shown in grey.

26 **Fig 3. Auxin sensitivity varies only subtly in wild accessions.** (A) The impact of auxin on root growth
27 (normalized to mock treated controls) was measured in 8-day-old seedlings. Each measurement is shown

1 as a transparent grey point. Solid lines represent fits from a log-logistic dose response model with a
2 lighter ribbon indicating 95% confidence intervals. The Col-0 curve is reproduced in light grey in each
3 panel to facilitate comparisons. (A) Assays on the reference accession Col-0, and mutants in the Col-0
4 background, are shown (B) Auxin sensitivity of accessions containing the hypermorphic *TIR1*^{T154S} allele.
5 (C) Estimated ED50 values for selected accessions and controls. Parameters were compared to Col-0
6 values and Student's t-tests were used to estimate the likelihood that the ratio of parameters equals 1. P-
7 values were corrected for multiple testing using the Benjamini-Hochberg method. (D) Some natural
8 polymorphisms were sufficient to alter auxin sensitivity in plants. Mean root growth (large points) and 95%
9 confidence intervals (error bars) of transgenic plants from three independent lines of Col-0 expressing
10 either reference alleles or one of the putative hypermorphs *TIR1*^{T154S} and *AFB2*^{T491R}. All transgenes were
11 expressed under the pUBQ10 promoter. The number of plants measured for each condition is shown
12 above the X-axis with individual measurements indicated by small points.

13 **Fig 4. *tir1-1* is a dominant negative allele.** Yeast expressing YFP-IAA17 and pairwise combinations of
14 (A) *TIR1* and *tir1-10* (*tir1*^{K159*}) or (B) *TIR1* and *tir1-1* (*tir1*^{G147D}) alleles were treated with various
15 concentrations of auxin for one hour before YFP-IAA17 fluorescence was measured by flow cytometry.
16 Mean fluorescence +/- SE was calculated from four experiments. Some error bars are within the points.

17 SUPPORTING INFORMATION CAPTIONS

18 **S1 Fig. Polymorphisms in the *AFB* genes of the 170 analyzed accessions.** Using a sliding 5-codon
19 window, synonymous (blue dotted) and nonsynonymous (red solid) polymorphisms were counted across
20 each *AFB* gene for all 170 accessions. A vertical black dotted line separates the F-box and LRR domain
21 of each gene and also identifies the target site of miR393. Nonsynonymous mutations functionally
22 characterized in this study are indicated.

23 **S2 Table. Accessions containing nonsynonymous variants in *TIR1* or *AFB2*.**

24 **S3 Fig. Characterization of additional *TIR1* polymorphisms.** Nonsynonymous mutation in the F-box
25 domain of *TIR1* and with dN/dS value <1 were synthesized and co-expressed in yeast with fluorescently
26 labeled IAA1 or IAA17. Degradation was assessed using flow cytometry on cultures exposed to different

1 concentrations of the auxin, indole-3-acetic acid (IAA) for one hour. Error bars represent 95% confidence
2 intervals around the median fluorescence calculated from three independent experiments. In many cases,
3 intervals are small enough that they appear as a single line.

4 **S4 Fig. Characterization of additional AFB2 polymorphisms.** Nonsynonymous mutations in the F-box
5 domain of AFB2 and or with dN/dS values <1 were synthesized and co-expressed in yeast with
6 fluorescently labeled IAA1 or IAA17. Degradation was assessed using flow cytometry on cultures
7 exposed to different concentrations of the auxin, indole-3-acetic acid (IAA) for one hour. Error bars
8 represent 95% confidence intervals around the median fluorescence calculated from three independent
9 experiments. In many cases, intervals are small enough that they appear as a single line.

10 **S5 Fig. *tir1-10* is an amorph in synthetic auxin-induced degradation assays.** A yeast expression
11 cassette recapitulating the *tir1-10* allele ($TIR1^{K159*}$) was co-expressed with YFP-IAA17 as a homozygous
12 diploid and along with full-length $TIR1^{Col}$ and an empty expression cassette (null). Each yeast strain was
13 treated with various concentrations of auxin for one hour during log-phase growth. YFP-IAA17
14 fluorescence was measured by flow cytometry. Mean fluorescence +/- SE calculated from four
15 experiments are represented by points and error bars respectively. Some error bars are within the points.

16 **S6 Appendix. Supplemental information.** Complete analytical methods, detailed protocols and
17 additional figures for each section of the main text.

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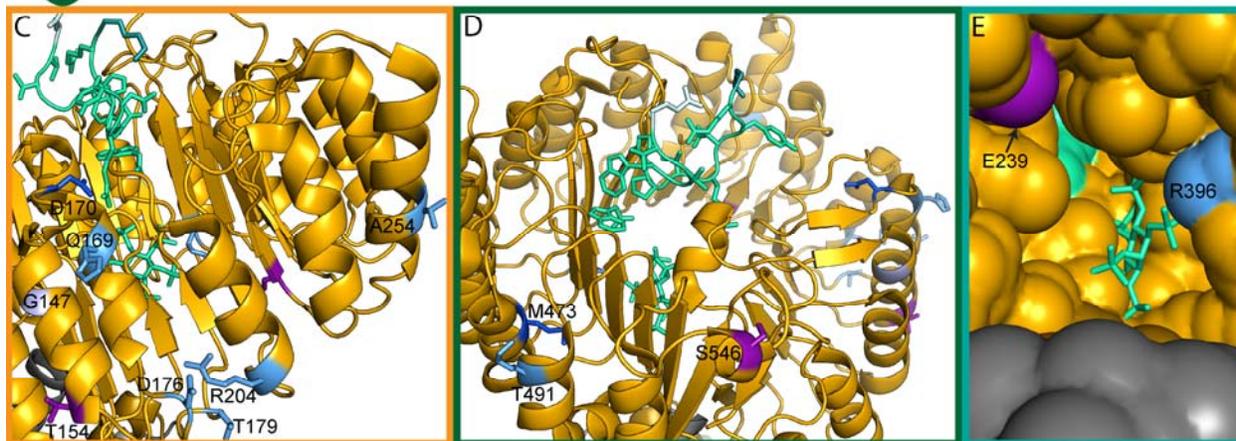
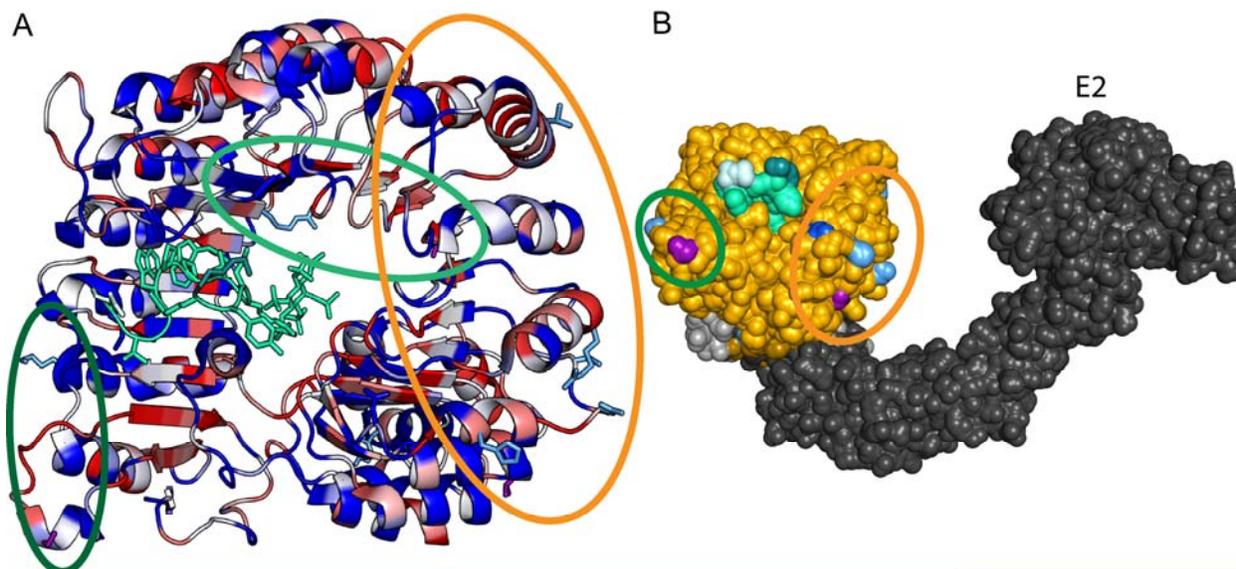
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1 **Table 1: Sequence variation in the AFB gene family.**

	Pro	Splice	Intron	5' UTR	3' UTR	Coding	Nonsyn	Nonsense	Syn
<i>TIR1</i>	120	0	96	120	19	41	8	0	33
<i>AFB1</i>	40	0	23	38	123	76	12	0	64
<i>AFB2</i>	23	0	88	200	11	275	22	0	253
<i>AFB3</i>	26	0	16	123	173	122	53	0	69
<i>AFB4</i>	57	0	180	51	22	646	147	2	497
<i>AFB5</i>	15	0	107	14	412	471	31	0	440
Total	281	0	510	546	760	1631	273	2	1356

- 2 Polymorphisms with high quality support from resequenced accessions of the SALK 1001 genomes
3 dataset were isolated and assigned to gene body locations according to TAIR10 annotations. Pro –
4 promoter, Splice – splice site, UTR – untranslated region, Nonsyn – nonsynonymous, Syn – synonymous.
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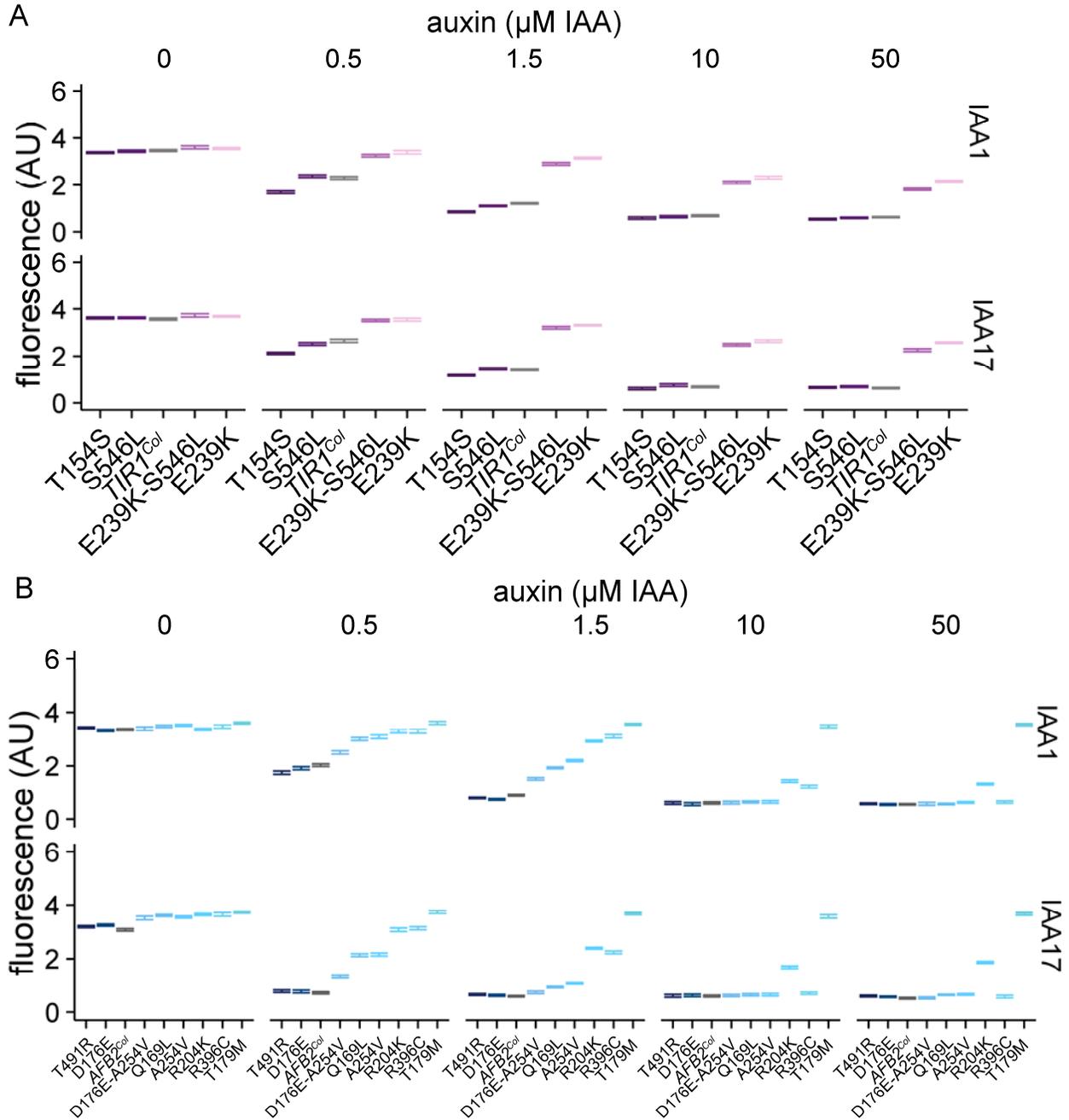
1 Figure 1



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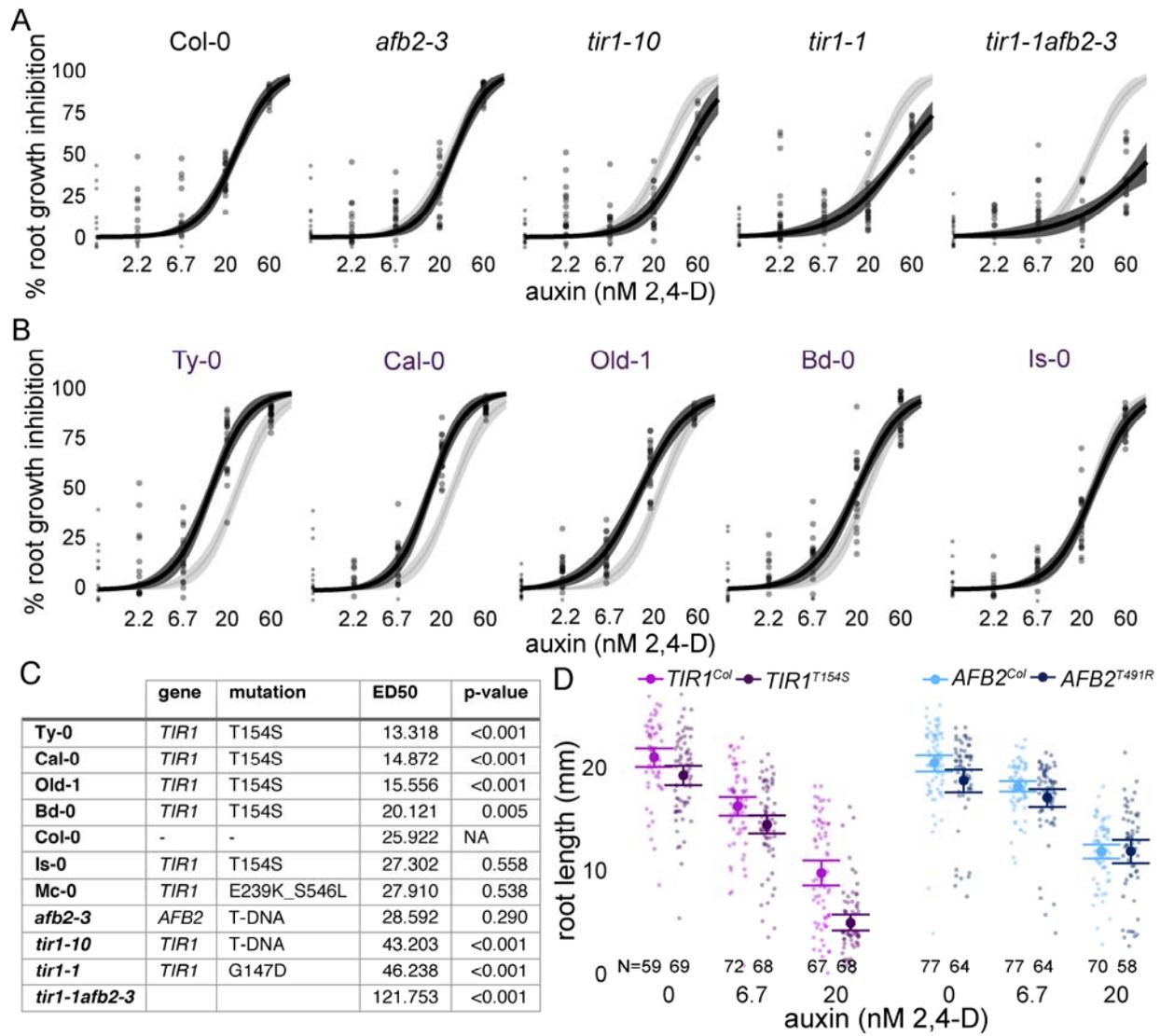
1 Figure 2



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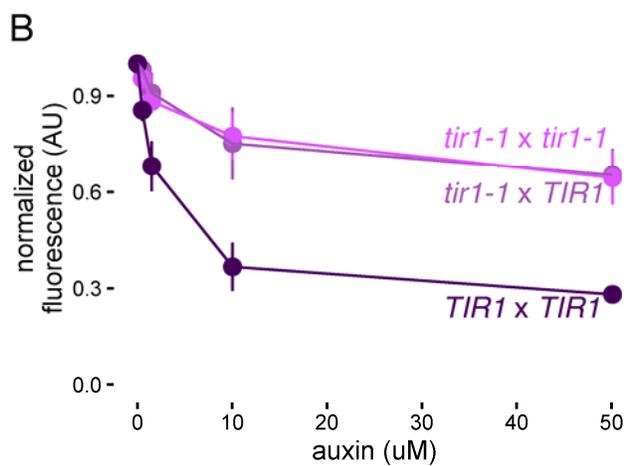
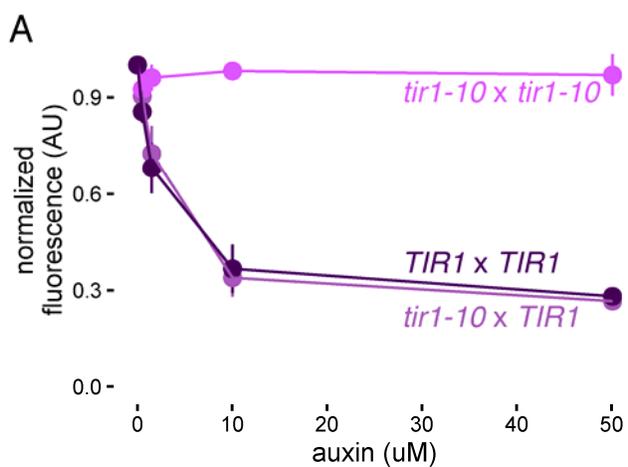
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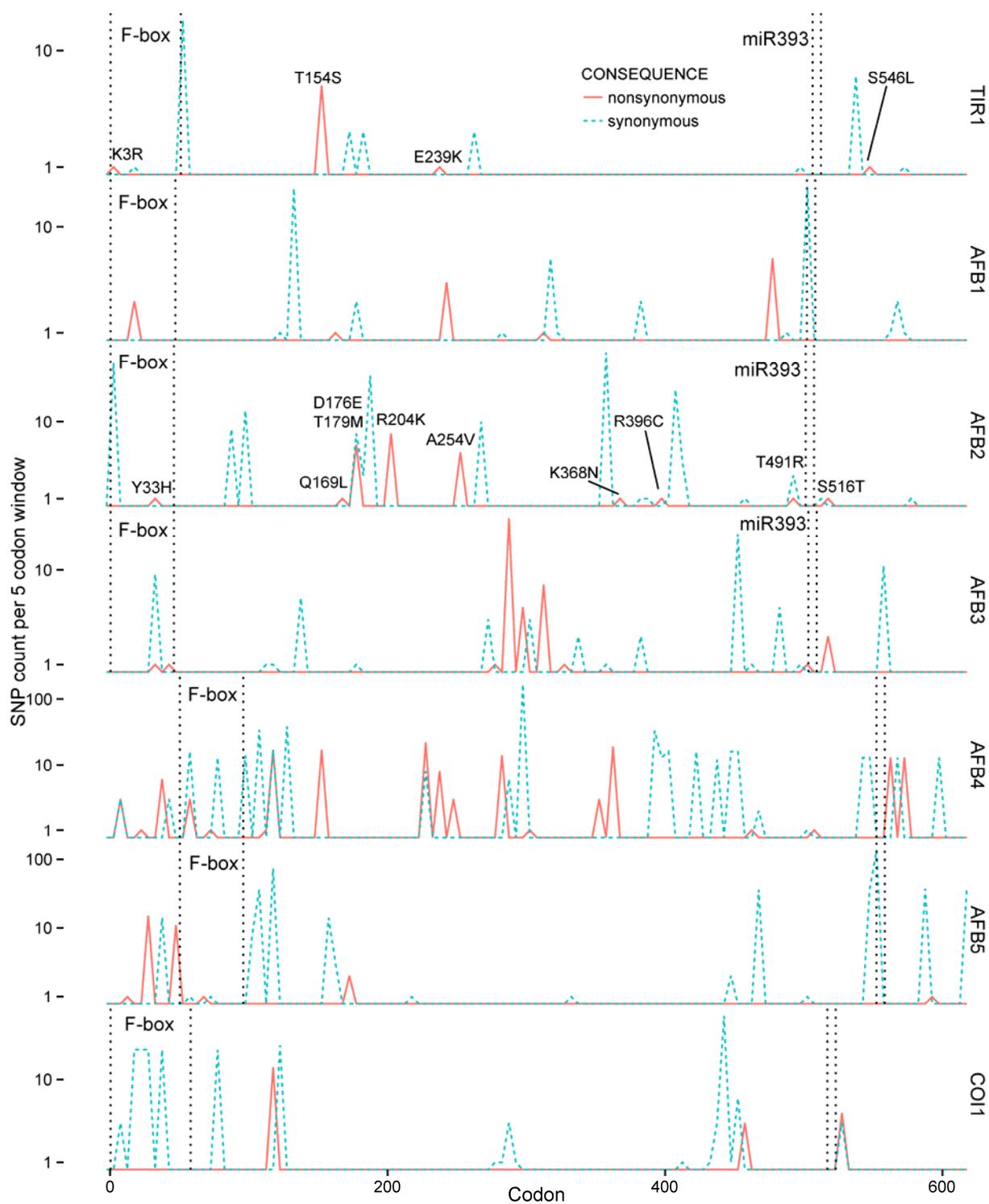
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1 S1 Figure



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1 **S2 Table: Accessions containing nonsynonymous variants in *TIR1* or *AFB2*.**

accession	Germplasm	characterized mutation	<i>TIR1</i>	<i>AFB1</i>	<i>AFB2</i>	<i>AFB3</i>	<i>AFB4</i>	<i>AFB5</i>
Bd-0	CS76445	TIR1_T154S	1	0	0	1	1	0
Cal-0	CS76460	TIR1_T154S	1	1	0	1	0	0
Is-0	CS76517	TIR1_T154S	1	0	0	0	0	0
Old-1	CS76567	TIR1_T154S	1	0	0	0	0	0
Ty-0	CS76619	TIR1_T154S	1	1	0	1	2	0
Mc-0	CS76548	TIR1_E239K_S546L	2	1	0	0	2	0
Ts-1	CS76615	AFB2_Y33H_T491R	0	0	2	0	0	0
Knox-18	CS76530	AFB2_Q169L	0	0	1	1	2	1
Co-1	CS76468	AFB2_D176E_A254V	0	0	2	0	8	0
Da(1)-12	CS76470	AFB2_D176E_A254V	0	0	2	0	1	0
Dra-0	CS76476	AFB2_D176E_A254V	0	0	2	0	1	0
Bor-1	CS76453	AFB2_T179M	0	0	1	0	0	0
Gel-1	CS76492	AFB2_R204K	0	0	1	1	2	0
Gre-0	CS76497	AFB2_R204K	0	0	1	1	0	1
Pna-17	CS76575	AFB2_R204K	0	0	1	1	0	0
RRs-10	CS76592	AFB2_R204K	0	0	1	1	0	1
Tol-0	CS76614	AFB2_R204K	0	0	1	1	0	1
Tul-0	CS76618	AFB2_R204K	0	0	1	1	0	1
Br-0	CS76455	AFB2_R396C	0	1	1	2	1	0

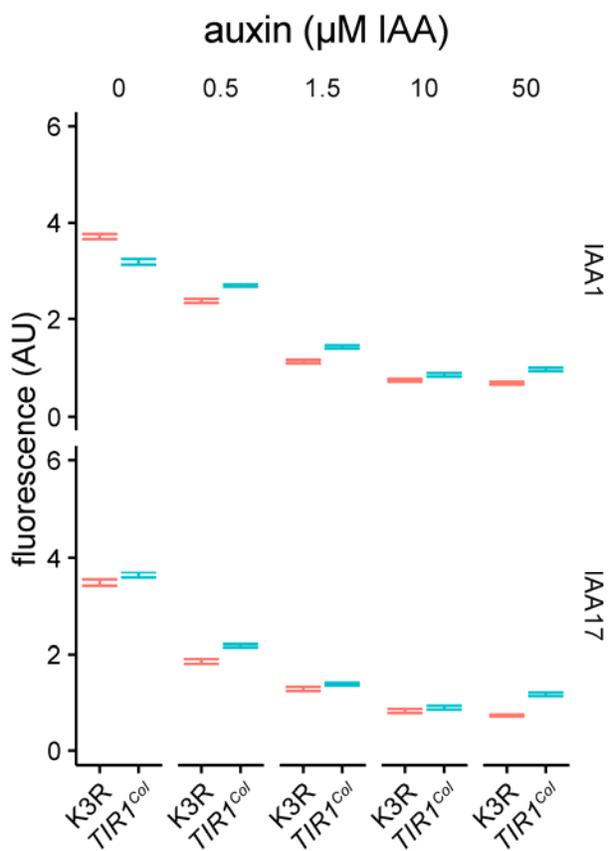
2 Germplasm, variants analyzed in this study and total nonsynonymous changes for each AFB gene are

3 indicated for each accession.

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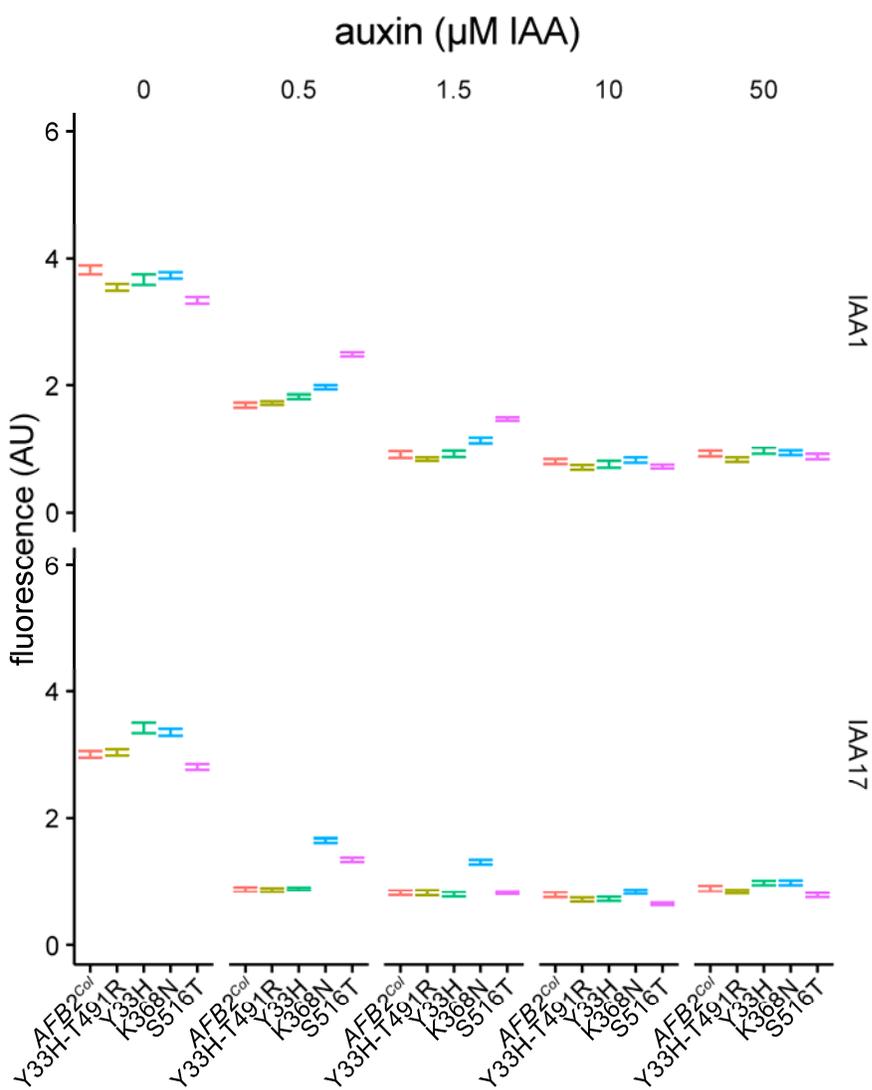
1 S3 Figure



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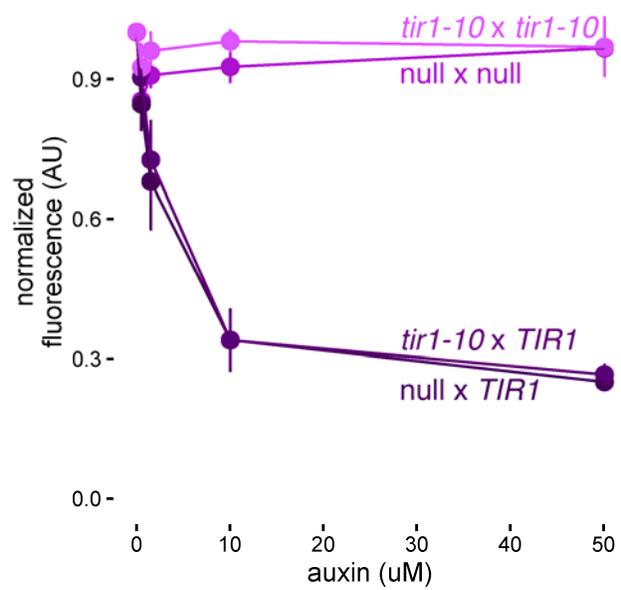
1 S4 Figure



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1 S5 Figure



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