Complex regulation of the TIR1/AFB family of auxin receptors

G. Parry[a], L. I. Calderon-Villalobos[b], M. Prigge[b], B. Peret[c], S. Dharmasiri[a,1], H. Itoh[d], E. Lechner[a,2], W. M. Gray[d], M. Bennett[c], and M. Estelle[a,b,3]

[a]Department of Biology, Indiana University, Bloomington, IN 47401; [b]Section of Cell and Developmental Biology, University of California at San Diego, La Jolla, CA 92093; [c]Centre for Plant Integrative Biology, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, United Kingdom; and [d]Department of Plant Biology, University of Minnesota, 250 Biological Sciences Center, St. Paul, MN 55108

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Auxin regulates most aspects of plant growth and development. The hormone is perceived by the TIR1/AFB family of F-box proteins acting in concert with the Aux/IAA transcriptional repressors. *Arabidopsis* plants that lack members of the TIR1/AFB family are auxin resistant and display a variety of growth defects. However, little is known about the functional differences between individual members of the family. Phylogenetic studies reveal that the TIR1/AFB proteins are conserved across land plant lineages and fall into four clades. Three of these subgroups emerged before separation of angiosperms and gymnosperms whereas the last emerged before the monocot-eudicot split. This evolutionary history suggests that the members of each clade have distinct functions. To explore this possibility in *Arabidopsis*, we have analyzed a range of mutant genotypes, generated promoter swap transgenic lines, and performed in vitro binding assays between individual TIR1/AFB and Aux/IAA proteins. Our results indicate that the TIR1/AFB proteins have distinct biochemical activities and that TIR1 and AFB2 are the dominant auxin receptors in the seedling root. Further, we demonstrate that *TIR1*, *AFB2*, and *AFB3*, but not *AFB1* exhibit significant posttranscriptional regulation. The microRNA *miR393* is expressed in a pattern complementary to that of the auxin receptors and appears to regulate *TIR1/AFB* expression. However our data suggest that this regulation is complex. Our results suggest that differences between members of the auxin receptor family may contribute to the complexity of auxin response.

plant hormone | plant development

The plant hormone auxin influences virtually every developmental program in plants (1, 2). Auxin acts by regulating transcription through the action of at least three protein families called the TIR1/AFB F-box proteins (3, 4), the Aux/IAA transcriptional repressors (5, 6), and the ARF transcription factors (7, 8). The TIR1 F-box protein acts as an auxin receptor and directly links auxin perception to degradation of the Aux/IAA proteins (9, 10). Structural studies recently revealed that auxin acts as a "molecular glue" to increase the strength of the interaction between TIR1 and the Aux/IAA protein, thus promoting their ubiquitination and degradation (11–14).

In *Arabidopsis* there are 29 *Aux/IAA* genes (5, 6). The only known function of Aux/IAA proteins is to repress transcription of the auxin-regulated genes. According to the current model, Aux/IAAs recruit the corepressor TPL to promoters through interactions with both TPL and ARF proteins (15–18). The presence of auxin, and the subsequent degradation of the Aux/IAA proteins, releases this repression and permits ARF-dependent transcription (19).

TIR1 is a member of a small gene family that contains 5 additional AFB proteins (3). Previous studies have shown that TIR1, AFB1, AFB2, and AFB3 all function as auxin receptors and that the respective genes are broadly transcribed throughout the plant (3). Genetic experiments indicate that reducing the number of functional TIR1/AFB proteins in the plant results in increasing resistance to exogenous auxin. Further, the analysis of triple and quadruple *tir1/afb* mutants demonstrated that these genes have overlapping functions and collectively are essential for *Arabidopsis* growth and development (3). However many questions remain concerning the specific function of each gene. In this study, we show that individual members of the family make an unequal contribution to the auxin response and that they exhibit important differences in expression, biochemical activity, and function.

Results

Evolutionary History of the TIR1/AFB Protein Family. To learn more about the evolutionary history of the TIR1/AFB proteins, we performed a phylogenetic analysis using the available sequences from land plants. Our results show that the proteins are conserved across land plants and have radiated during vascular plant evolution (Fig. S1). The seed plant TIR1/AFB proteins are more closely related to each other than to those from the nonvascular plant *Physcomitrella patens*, indicating that the genome of an ancestor to vascular plants likely encoded a single TIR1/AFB protein. Two duplication events established three distinct lineages—"TIR1/AFB2," "AFB4," and "AFB6"—before the angiosperm and gymnosperm lineages diverged roughly 300 million years ago (Mya). The duplication event distinguishing the TIR1 and AFB2 clades occurred early within the angiosperm lineage before the split between monocot plants and eudicot plants, possibly coinciding with a proposed whole-genome duplication event at the base of the angiosperm lineage (20). AFB6 homologs were lost independently early in the Brassicaceae and Poaceae families. Finally, the α-duplication event at the base of the Brassicaceae family ≈34 Mya (21) resulted in the three pairs of paralogs present in the *Arabidopsis thaliana* genome.

The TIR1 and AFB Proteins Do Not Contribute Equally to Auxin Response in the Root. In a previous study we characterized a series of single and higher order mutants deficient in the *TIR1*, *AFB1*, *AFB2*, and *AFB3* genes (3). However, many of the mutant combinations were mixed *Ws* and *Col-0* backgrounds, complicating the analysis. We have now obtained *Col-0* alleles for each gene. The *tir1–10*, *afb1–3*, *afb1–5*, *afb2–3*, and *afb3–4* alleles carry T-DNA insertions, whereas the *afb2–5* allele was recovered in a screen for enhancers of *tir1–1* (Fig. S2) (22). The effects of

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[1]Present address: Texas State University, Department of Biology, 601 University Drive, San Marcos, TX 78666.

[2]Present address: Institut de Biologie Moleculaire des Plantes du CNRS, 12 Rue du General Zimmer, 67084 Strasbourg Cedex, France.

[3]To whom correspondence should be addressed. E-mail: mestelle@ucsd.edu.

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each new mutation on RNA levels were determined by RT-PCR (Fig. S2). The results show that the *tir1–10*, *afb1–3*, *afb1–5*, and *afb3–4* alleles do not produce full-length transcript. The *tir1–10* and *afb1* alleles are likely to be nulls because the T-DNA insertion is within the coding region near the 5′ end of the gene. In contrast the *afb2–3* allele has a T-DNA insertion 37 bp upstream of the transcriptional start site that results in reduced levels of *AFB2* transcript (Fig. S2*C*). The *afb2–5* allele has a nucleotide substitution that results in substitution of glycine 70 with an arginine. Thus the *afb2* alleles may retain some function. Similarly, because the T-DNA insertion in *afb3–4* is near the end of the gene, it is possible that this gene retains some function. We generated a full series of double, triple, and quadruple mutants using these alleles and determined the effects of 2,4-D on root elongation in each genotype. Of the single mutants, only *tir1–1* is clearly resistant to 2,4-D (Fig. S3 *A* and *B*). However the *tir1–1 afb2* double mutant is more resistant to auxin than *tir1–1* alone, clearly showing that AFB2 contributes to auxin response in the root. Similar results were obtained when *tir1–1* was combined with the *Ws* allele *afb2–1* (3). In contrast, the response of the *tir1afb1*, *tir1afb3*, and *tir1afb1afb3* mutants is similar to that of *tir1* seedlings, indicating that the loss of AFB1 and/or AFB3 does not affect the response to auxin when the seedling is already lacking TIR1 (Fig. S3 *A* and *B*). Further, the response of *afb1afb2*, *afb1afb3*, and *afb2afb3* roots is similar to wild type, indicating that TIR1 is sufficient for normal response to 2,4-D, even in the absence of any two of the three AFB proteins (Fig. S3*A*). We used qRT-PCR to examine the relative expression of each *TIR1/AFB* gene in the root tissue of each single mutant background and did not observe any evidence for compensatory regulation (Fig. S4*A*).

The relative contribution of AFB1 and AFB3 to auxin response in the root is revealed by the analysis of higher order mutants. The *tir1afb2afb3* line but not *tir1afb1afb2* triple mutant is more resistant to 2,4-D than the *tir1afb2* double mutant, implying that AFB3 contributes to auxin response in the root but AFB1 does not (Fig. S3*B*). However, AFB1 does appear to have a role later in plant development because *tir1afb1afb2* plants are smaller in stature than *tir1afb2* plants (Fig. S3*H*). The *tir1afb2afb3* has a more severe phenotype than *tir1afb1afb2*. A significant proportion of these plants are embryo or seedling lethal (Fig. S5). This phenomenon was previously observed in the mixed ecotype triple mutant lines (3). Thus our data indicate that *AFB3* plays a more significant role than *AFB1* in auxin response.

The quadruple *tir1–1afb1–3afb2–3afb3–4* mutant displays the same range of phenotypes observed in *tir1–1afb2–3afb3–4* (Fig. S5). However in this case a higher proportion of the seedlings demonstrated the severe phenotype (Fig. S5). This phenotype is similar to that observed in the previously characterized *tir1–1afb1–1afb2–1afb3–1* quadruple mutant (3) and demonstrates that each *tir1/afb* plays a role in seedling development.

***TIR1*, *AFB2*, and *AFB3* Exhibit Posttranscriptional Regulation.** Previously we showed that *TIR1*, *AFB1*, *AFB2*, and *AFB3* are broadly transcribed throughout development (3). To further explore expression of these genes, we created a series of translational GUS fusion proteins. The GUS enzyme was fused to the C-terminal region of each protein and introduced into plants under the control of the same promoters as were used to make the transcriptional fusion lines (3). To determine whether the TIR1-GUS fusion was functional, we introduced this transgene into *tir1–1* plants and showed that the fusion protein restores normal auxin response to mutant seedlings (Fig. 3*H*). At least 10 independent transgenic lines were identified for each of *pTIR1:cTIR1-GUS*, *pAFB1:cAFB1-GUS*, *pAFB2:cAFB2-GUS*, and *pAFB3:cAFB3:GUS*. Homozygous lines with representative levels of expression were selected for further experimentation.

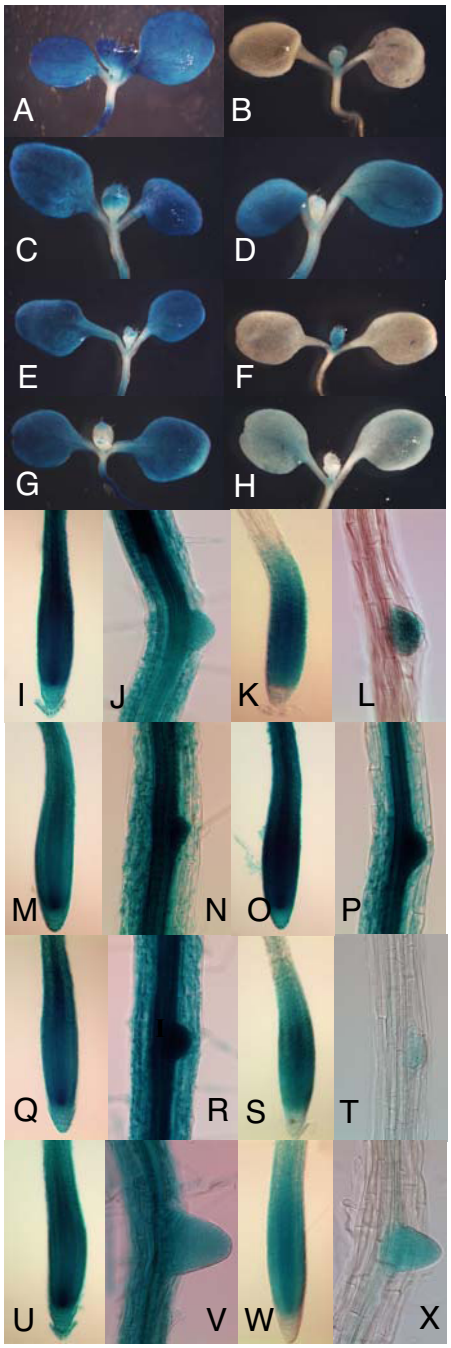

Fig. 1. TIR1/AFB gene expression involves posttranscriptional regulation. GUS expression in the cotyledons, young leaves (*A–H*), primary root tip, and young lateral roots (I–X) of 8-day-old transgenic seedlings. X-Gluc staining is representative of expression levels in seedlings containing *pTIR1:GUS* (*A*, *I*, and *J*), *pTIR1:cTIR1:GUS* (*B*, *K*, and *L*), *pAFB1:GUS* (*C*, *M*, and *N*), *pAFB1:cAFB1:GUS* (*D*, *O*, and *P*), *pAFB2:GUS* (*E*, *Q*, and *R*), *pAFB2:cAFB2:GUS* (*F*, *S*, and *T*), *pAFB3:GUS* (*G*, *U*, and *V*), and *pAFB3:cAFB3:GUS* (*H*, *W*, and *X*). Samples *A–H*, *J–L*, *N–P*, and *R–T* X-Gluc were stained overnight. Samples *I*, *M*, *Q*, and *U–X* were stained for 5 h.

We found that in young seedlings and in flowers containing the *pTIR1:cTIR1-GUS*, *pAFB2:cAFB2-GUS*, and *pAFB3:cAFB3-GUS* transgenes, GUS staining was much more restricted than in the equivalent promoter:GUS fusion lines (Fig. 1, Fig. S6). In contrast, the GUS expression pattern was similar in seedlings containing either *pAFB1-GUS* or *pAFB1:cAFB1-GUS* transgenes.

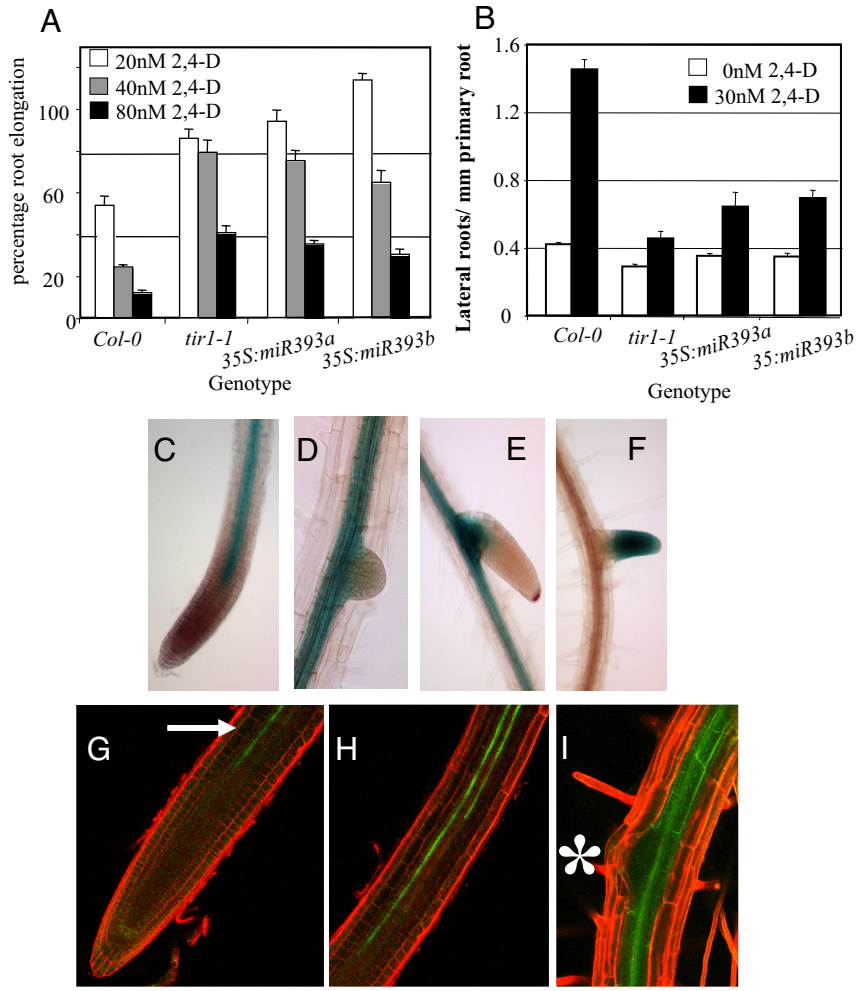

Fig. 2. miR393 influences the auxin response in the root. (*A*) Four-day-old seedlings grown on MS media were transferred to media containing 2,4-D. Root elongation was measured after an additional 4 days and expressed as a proportion of growth in the absence of auxin (Scale bars, SE). (*B*) Lateral root assay. Four-day-old seedlings grown on MS media were transferred onto media −/+ 2,4-D for an additional 9 days. The number of emerged lateral roots/mm primary root length was measured. (Scale bars, SE.) (*C–E*) Expression of *pmiR393*:*GUS* in the primary root tip (*C*) and lateral roots (*D* and *E*) of 10-day-old seedlings. (*F*) Expression of *pTIR1*:*cTIR1*:*GUS* in an emerging lateral root in a 10-day-old seedling. (*G–I*) Expression of *pimR393a*:*GFP* in the primary root tip region (*G*, arrow), root elongation zone (*H*), and beneath an emerging lateral root (*I*, asterisk) in 10-day-old seedlings.

GUS staining in the *TIR1*-, *AFB2*-, and *AFB3*- *GUS* translational fusion lines is restricted to the primary and lateral root tips, young leaves, and the young flower buds, regions of the plant that are actively growing. However the AFB1:GUS fusion protein is found throughout the plant (Fig. 1, Fig. S6). We extracted RNA from flowers of *pTIR1*:*GUS* and *pTIR1*:*cTIR1*-*GUS* transgenic lines and showed by RT-PCR that RNA levels are similar in these lines even though the GUS staining is higher in *pTIR1*:*GUS* flowers (Fig. S6).

These data show that the *TIR1*, *AFB2*, and *AFB3* genes undergo significant posttranscriptional regulation. Based on the translational GUS fusions, these proteins are found in regions of cell division and/or expansion consistent with their role in auxin-dependent processes. Notably, differences in the apparent distribution of TIR1 and the AFB proteins do not appear to explain the differences in their relative contribution to auxin response.

The *TIR1/AFB* Genes Are Not Rapidly Auxin Regulated. Expression of many hormone-related genes is subject to negative and positive regulatory loops. For example transcription of the *Aux/IAA* genes, encoding negative regulators of auxin response, is rapidly induced by auxin. To determine whether auxin regulates expression of the *TIR1/AFB* genes in the root we performed qRT-PCR experiments using RNA isolated from auxin-treated roots. Somewhat surprisingly we did not detect any changes in *TIR1/AFB* RNA level even after treatment with high concentration of IAA (Fig. S4*B*). These data indicate that transcription of the auxin receptor genes does not change rapidly in response to auxin.

The microRNA *miR393* Regulates Auxin Response. Previous studies demonstrated that *miR393* plays a role in the regulation of *TIR1/AFB* expression (23). This RNA is produced from two loci called *miR393a* and *miR393b*. To assess the effect of increased levels of *miR393* we used the previously characterized *35S:miR393a* transgenic line (23) and also generated transgenic lines containing a *35S:miR393b* construct. We tested the response to 2,4-D in these transgenic seedlings by root elongation assay and showed that the roots of both *35S:miR393a* and *35S:miR393b* lines are auxin resistant. The level of resistance is similar to that observed in *tir1–1* seedlings (Fig. 2*A*).

Growth of seedlings on auxin stimulates lateral root formation. The number of lateral roots in wild-type seedlings increases

over threefold when grown on 30 nM of 2,4-D. However the roots of *tir1–1* and *35S:miR393* seedlings did not respond to auxin in the same manner with only a modest increase in the number of lateral roots (Fig. 2*B*). These results demonstrate that overexpression of *miR393* produces an auxin-resistant phenotype. However the phenotype is relatively weak when compared to that of the higher order *tir1afb* mutants. Further, neither overexpression line displays any of the severe growth defects exhibited by these mutants, indicating that increased levels of *miR393* has only a modest effect on TIR1/AFB levels.

To investigate the expression pattern of *miR393a* and *miR393b* we constructed promoter fusions lines in which 2.5 kb upstream of each gene was used to drive expression of the *GUS* reporter. Expression is identical in both *pmiR393a:GUS* and *pmiR393b:GUS* seedlings. In the primary root, GUS staining is observed throughout the central stele but not in the overlying tissues (Fig. 2*D*). Interestingly, the stelar GUS expression domain stops ≈0.5 mm from the root tip and is restricted from the root apical meristem (Fig. 2*C*). Similarly, expression is also restricted from lateral roots until later stages of development when expression is once again only observed in the stele (Fig. 2*E*). Strikingly, the pattern of expression of both genes in the roots is complementary to that seen in *pTIR1:cTIR1-GUS* roots (Fig. 1 *K–L*, Fig. 2*F*). *pTIR1:cTIR1-GUS* is also expressed in young leaves whereas *pmiR393a:GUS* and *pmiR393b:GUS* expression is absent from this region (Fig. S7 *A* and *B*). However in older leaves there is strong expression of *pmiR393a:GUS* and *pmiR393b:GUS* but not of *pTIR1:cTIR1:GUS* (Fig. S7 *C–E*).

We also examined *pmiR393a:GFP* and *pmiR393b:GFP* transgenic lines (23). Both reporters display the same expression patterns as that observed in the *pmiR393:GUS* fusion lines. In the stele of the primary root, GFP signal is restricted to two strands of expression and is not found in either the root apical meristem or the emerging lateral roots (Fig. 2 *G–I*).

To determine whether *miR393* plays a role in *TIR1* regulation, we generated a *pTIR1:cTIR1-GUS* construct that includes four silent nucleotide changes within the *miR393* recognition site predicted to make the transgene resistant to *miR393*-directed regulation (Fig. S8*A*) (24). We evaluated over 20 independent T_2 lines containing the *pTIR1:mTIR1-GUS* transgene and found that the introduction of the *miR393* target site mutations had no effect on the pattern of GUS staining (Fig. S8 *B–G*). Similar results were observed for *pAFB2:mAFB2-GUS* and *pAFB3:mAFB3-GUS* transgenic lines.

AFB1 contains a single nucleotide mismatch in the *miR393* recognition site that is thought to cause resistance to *miR393* directed mRNA cleavage (23). This might explain why *pAFB1:AFB1-GUS* is expressed through the entire seedling. To test this hypothesis we created a *pAFB1:AFB1-GUS* transgenic line that included a nucleotide change that alters the *miR393* recognition site in AFB1 to be identical to that in TIR1 (Fig. S8*H*). These *pAFB1:AFB1^{TIR1}-GUS* transgenic lines exhibited the same expression pattern as in the wild-type *pAFB1:AFB1-GUS* seedlings (Fig. S8 *I* and *J*).

TIR1 and AFB1 Have Distinct Functions in Vivo. TIR1 and AFB1 are 70% identical at the amino acid level. To test whether these similar proteins have equivalent functions in planta we generated a promoter swap translational GUS fusion construct in which the *AFB1* coding region, fused to *GUS*, was cloned downstream of the *TIR1* promoter and introduced into *tir1–1* plants. We also generated a similar line in which the *TIR1* promoter was fused to the *AFB2* coding region. We assessed the GUS staining pattern in at least 10 independent lines and continued our work with lines that were representative.

RNA blot analysis showed that the level of *GUS* RNA was similar in each line (Fig. 3*A*). However when we examined GUS staining we found that levels were much higher in *tir1–1 pTIR1:cAFB1-GUS* than in *tir1–1 pTIR1:cTIR1-GUS* seedlings (Fig. 3 *B–E*), indicating that the higher level of expression in the *pAFB1:AFB1-GUS* line is related to the *AFB1* coding region and not the *AFB1* promoter (Fig. 1). In *tir1 pTIR1:cAFB2-GUS* seedlings we observed similar GUS expression to that in *tir1 pTIR1:cTIR1-GUS* (Fig. 3 *F* and *G*). This is reminiscent of the level of GUS expression in *pAFB2:cAFB2-GUS* seedlings (Fig. 1)

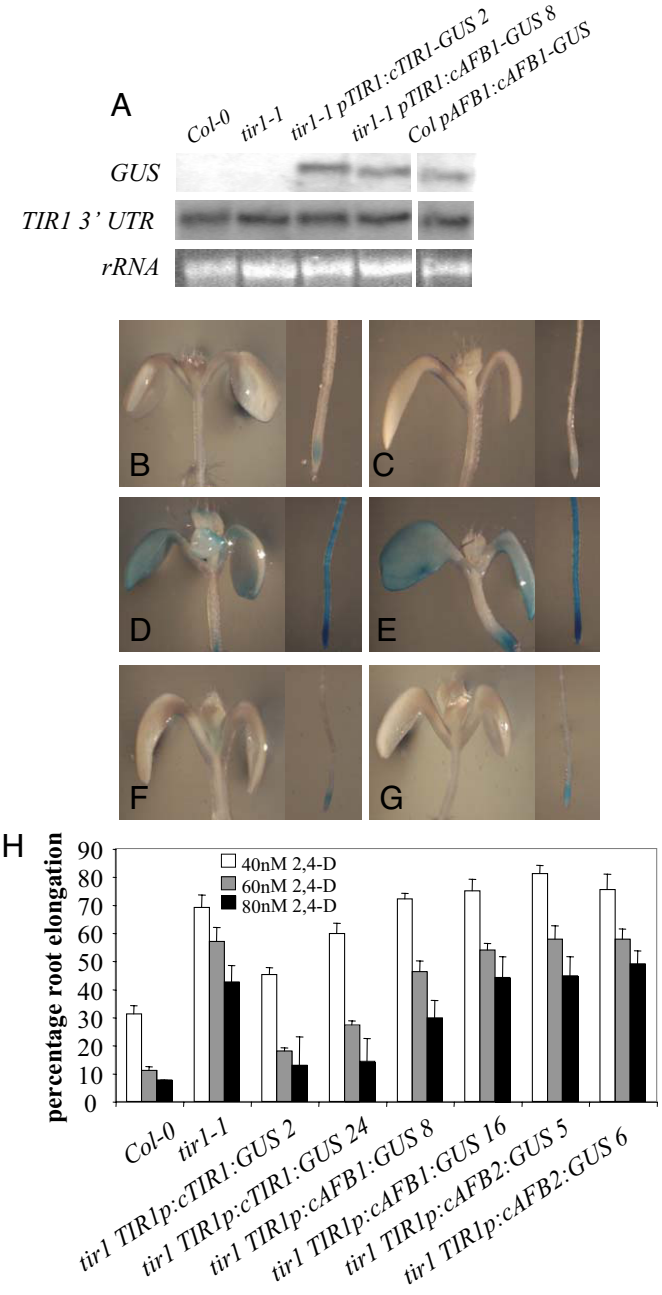

Fig. 3. AFB1 and AFB2 cannot replace TIR1 in *tir1–1* plants. (*A*) Northern blot of RNA isolated from 9-day-old *Col-O*, *tir1–1*, *tir1 pTIR1:cTIR1:GUS*, *tir1 pTIR1:cAFB1:GUS*, and *col pAFB1:cAFB1:GUS* seedlings. Expression evaluated using P^{32}-labeled probes that bind the GUS coding region or the 3′-UTR of the TIR1 gene. rRNA levels are stained using EtBr. (*B-G*) GUS expression in 8-day-old seedlings containing *tir1 TIR1p-cTIR1:GUS* line 2 (*B*), *tir1 TIR1p-cTIR1:GUS* line 24 (*C*), *tir1 TIR1p-cAFB1:GUS* line 8 (*D*), *tir1 TIR1p-cAFB1:GUS* line 16 (*E*), *tir1 TIR1p-cAFB2:GUS* line 5 (*F*), and *tir1 TIR1p-cAFB2:GUS* line 6 (*G*) transgenes. (*H*) Root elongation as performed in Fig. 2*A* with 4-day-old seedling grown in the presence of 2,4-D. Numbers denote independent lines.

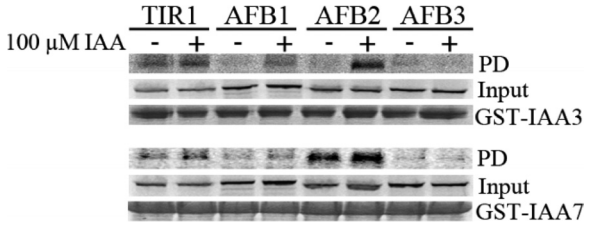

Fig. 4. TIR1 and AFB2 exhibit a stronger interaction with Aux/IAA proteins than AFB1 and AFB3 in vitro. TIR1, AFB1, AFB2, and AFB3 proteins were synthesized in vitro and incubated with GST-IAA3 and GST-IAA7 proteins in the presence or absence of 100 μM of IAA. After pulldown reactions (PD), recovery of the F-box proteins was assessed by autoradiography (*Top* panels). *Middle* panel shows the input of in vitro synthethized TIR1, AFB1, AFB2, and AFB3 in the pulldown reactions, whereas *Lower* panels show the coomassie staining of entire pulldown reactions as loading control for GST-Aux/IAA proteins.

indicating that *AFB2-GUS* undergoes the same mechanisms of posttranscriptional control as *TIR1-GUS*.

We next evaluated whether the various constructs were able to restore wild-type 2,4-D response to *tir1–1* mutant roots. The *tir1–1 pTIR1:cTIR1:GUS* lines displayed a wild-type level of 2,4-D response showing that the TIR1-GUS protein is able to rescue the mutant phenotype. However neither AFB1-GUS nor AFB2-GUS rescued the *tir1–1* root growth phenotype even though AFB1-GUS is expressed at a high level throughout the seedling (Fig. 3*H*). These experiments demonstrate that AFB1 and AFB2 are unable to substitute for TIR1 protein even when regulated by the *TIR1* promoter. These results indicate that TIR1, AFB1, and AFB2 do not have identical activities and may have specialized function.

TIR1 and AFB2 Exhibit a Stronger Interaction with Selected Aux/IAAs Than AFB1 and AFB3. It is possible that differences in the function of TIR1/AFB family members are related to their binding to Aux/IAA substrates. In previous studies we showed that each of these proteins interact with IAA7 in an auxin-dependent manner (3). However, because some of the experiments were done using plant extracts and others with protein synthesized in TNT extracts, it was not possible to compare the strength of the interaction between different TIR1/AFB proteins and IAA7. To address this issue, we tested the ability of the Aux/IAA proteins IAA3 and IAA7 to bind each F-box protein in the absence and presence of auxin. The receptors were synthesized in TNT extracts and pulldown assays were performed with GST-tagged Aux/IAA proteins. Both TIR1 and AFB2 interact with each Aux/IAA protein in an auxin-dependent way (Fig. 4). In contrast, Aux/IAA binding to AFB1 and AFB3 was low or undetectable with this assay (Fig. 4). These results suggest that AFB1 and AFB3 may have a reduced affinity for Aux/IAA proteins compared to TIR1 and AFB2.

Discussion

The TIR1 protein has a critical role in auxin signaling. In *Arabidopsis*, TIR1 is a member of a small group of related proteins that includes AFB1 through AFB5 and the jasmonic acid receptor, COI1. Previous studies suggested that AFB1, AFB2, and AFB3 function together with TIR1 to regulate most aspects of auxin response throughout plant growth and development (3). However, the precise role of each member of the family is unclear. In this study we present evidence that the TIR1/AFB proteins have distinct biochemical and biological functions and that the regulation of these genes is complex.

Our phylogenetic studies indicate that the TIR1/AFB proteins are conserved among all land plants. The TIR1/AFB2, AFB4, and AFB6 lineages have been distinct since before the division of angiosperms and gymnosperms. Later, but before the separation of monocot and eudicot plants, the TIR1/AFB2 clade divided into two distinct clades (AFB1 and AFB2). The fact that these four clades have been maintained since these early events in plant evolution strongly suggests that the function of each clade is distinct. Consistent with this, our genetic studies indicate that TIR1 and AFB2 play a greater role in auxin response in the root than either AFB1 or AFB3. On the basis of the phenotype of single mutants, TIR1 appears to make the largest contribution followed by AFB2. As we reported in an earlier analysis of *Ws* alleles, both AFB1 and AFB3 contribute to auxin response, but this contribution is only apparent in higher order mutant combinations.

Interestingly, the phenotype of the *tir1–1 afb2–2 afb3–4* and *tir1–1 afb1–3 afb2–2 afb3–4* quadruple mutants is quite variable. Phenotypic variability was also observed in a previous study with a different combination of mutant alleles, indicating that this behavior is not related to the specific alleles involved (3). It seems likely that homeostatic mechanisms, perhaps related to regulation of auxin levels or response, contribute to this variability.

It is possible that the differences in relative contribution to auxin response exhibited by each TIR1/AFB protein are related to their expression level. However, an examination of transcript and protein levels indicates that this is not the case. *AFB1* is expressed at a much higher level than *TIR1*, *AFB2*, or *AFB3*. Further, we find that AFB1 and AFB2 do not rescue the *tir1* mutant even when regulated by the *TIR1* promoter. These results suggest that despite their close relationship, these proteins are biochemically distinct.

There are 29 Aux/IAA proteins in *Arabidopsis*, 23 of which have the conserved domain II required for interaction with TIR1. It is possible that differences in TIR1/AFB function are related to the way that these protein interact with the Aux/IAAs. Consistent with this, we find that TIR1 and AFB2 exhibit a stronger interaction with several AuxIAA proteins than AFB1 or AFB3. At this point, the basis for this difference is unclear. Most of the residues believed to be important for IAA and Aux/IAA binding are conserved between TIR1 and AFB1. However, both AFB1 and AFB3 contain several nonconservative substitutions within loop 12 of these proteins, a region thought to be important for interaction with the Aux/IAA proteins (11). Further experiments will be required to determine whether these differences are responsible for changes in biochemical activity.

Regulation of TIR1/AFB Expression. Our results demonstrate that the *TIR1*, *AFB2*, and *AFB3* genes exhibit posttranscriptional regulation. The microRNA *miR393* is a prime candidate for this regulation. Previous studies indicate that *miR393* negatively regulates *TIR1*, *AFB2*, and *AFB3* in response to pathogen attack and that overexpression of *miR393* results in decreased levels of *TIR1* mRNA (23). We have extended this work by showing that *miR393* overexpression results in auxin-resistant root growth. Further, we show that expression of *miR39a* and *miR393b* is complementary to that of *pTIR1:TIR1-GUS* consistent with the hypothesis that *miR393* negatively regulates *TIR1* expression. On the basis of these results we were surprised to find that the introduction of mutations into the *miR393* target sequence of *TIR1*, *AFB2*, and *AFB3* did not affect expression of the respective fusion protein.

These results suggest that *miR393* may not contribute to the developmental regulation of the *TIR1/AFB* genes. However, recent studies of *ARGONAUTE 1* (*AGO1*) regulation suggest another possibility. Earlier work had shown that *AGO1* is negatively regulated by *miR168* (25). In addition to this regulation, recent studies show that *miR168* slicing results in the generation of numerous 21-nucleotide *siRNAs* that map in the vicinity of the slice site (26). These *siRNAs* result in further

silencing of the *AGO1* locus. Strikingly, examination of the *Arabidopsis* small RNA project (ASRP) database reveals the presence of numerous 21-nucleotide *siRNAs* in the vicinity of the *miR393* target site in *TIR1*, *AFB2*, and *AFB3*, but not in *AFB1* (27). Thus it is possible that silencing of the *pTIR1:mTIR1-GUS* transgene involves production of *siRNAs* from the endogenous *tir1* gene. Further experiments will explore this hypothesis.

The complementary expression pattern in the root of *TIR1/AFB* and *miR393* suggests that *miR393* might act in the formation of lateral roots. Interestingly the stelar expression of *miR393*:GFP is reminiscent of that observed in the GAL4 enhancer trap line J0121 (28). Transgenic lines that have an attenuated auxin response in this J0121 expression domain do not form lateral roots (28). Therefore in future work it will be interesting to discover whether the expression of *miR393* in these cell files represents a mechanism to control lateral root formation by modulating *TIR1/AFB* expression.

Methods

The details of plant growth assays, generation of transgenic lines, PCR, protein expression and pulldowns, and phylogentic analysis are described in the *SI Materials and Methods*. Briefly, plant growth assays were performed as described previously (3). RNA for RT-PCR experiments was isolated using QIAGEN RNeasy plant kit or Tri-reagent (Sigma). Biochemical studies of the TIR1/AFB protein were performed as described (9). Phylogenetic studies were conducted using MRBAYES 3.1.2 (29) with the parameters aamodelpr = mixed, nst = 6, and rates = invgamma.

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1. Benjamins R, Scheres B (2008) Auxin: The looping star in plant development. *Annu Rev Plant Biol* 59:443–465.
2. Woodward AW, Bartel B (2005) Auxin: regulation, action, and interaction. *Ann Bot* (*Lond*) 95(5):707–735.
3. Dharmasiri N, et al. (2005) Plant development is regulated by a family of auxin receptor F box proteins. *Dev Cell* 9(1):109–119.
4. Mockaitis K, Estelle M (2008) Auxin receptors and plant development: A new signaling paradigm. *Annu Rev Cell Dev Biol* 24:55–80.
5. Overvoorde PJ, et al. (2005) Functional genomic analysis of the AUXIN/INDOLE-3-ACETIC ACID gene family members in *Arabidopsis thaliana*. *Plant Cell* 17(12):3282–3300.
6. Remington DL, Vision TJ, Guilfoyle TJ, Reed JW (2004) Contrasting modes of diversification in the Aux/IAA and ARF gene families. *Plant Physiol* 135(3):1738–1752.
7. Guilfoyle TJ, Hagen G (2007) Auxin response factors. *Curr Opin Plant Biol* 10(5):453–460.
8. Okushima Y, et al. (2005) Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in *Arabidopsis thaliana*: Unique and overlapping functions of ARF7 and ARF19. *Plant Cell* 17(2):444–463.
9. Dharmasiri N, Dharmasiri S, Estelle M (2005) The F-box protein TIR1 is an auxin receptor. *Nature* 435(7041):441–445.
10. Kepinski S, Leyser O (2005) The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature* 435(7041):446–451.
11. Tan X, et al. (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* 446(7136):640–645.
12. Ramos JA, Zenser N, Leyser O, Callis J (2001) Rapid degradation of auxin/indoleacetic acid proteins requires conserved amino acids of domain II and is proteasome dependent. *Plant Cell* 13(10):2349–2360.
13. Zenser N, Dreher KA, Edwards SR, Callis J (2003) Acceleration of Aux/IAA proteolysis is specific for auxin and independent of AXR1. *Plant J* 35(3):285–294.
14. Zenser N, Ellsmore A, Leasure C, Callis J (2001) Auxin modulates the degradation rate of Aux/IAA proteins. *Proc Natl Acad Sci USA* 98:11795–11800.
15. Szemenyei H, Hannon M, Long JA (2008) TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. *Science* 319(5868):1384–1386.
16. Tiwari SB, Hagen G, Guilfoyle TJ (2004) Aux/IAA proteins contain a potent transcriptional repression domain. *Plant Cell* 16(2):533–543.
17. Tiwari SB, Wang XJ, Hagen G, Guilfoyle TJ (2001) AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. *Plant Cell* 13(12):2809–2822.
18. Weijers D, et al. (2005) Developmental specificity of auxin response by pairs of ARF and Aux/IAA transcriptional regulators. *EMBO J* 24(10):1874–1885.
19. Tiwari SB, Hagen G, Guilfoyle T (2003) The roles of auxin response factor domains in auxin-responsive transcription. *Plant Cell* 15(2):533–543.
20. Cui L, et al. (2006) Widespread genome duplications throughout the history of flowering plants. *Genome Res* 16(6):738–749.
21. Schranz ME, Mitchell-Olds T (2006) Independent ancient polyploidy events in the sister families Brassicaceae and Cleomaceae. *Plant Cell* 18(5):1152–1165.
22. Alonso JM, et al. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 301(5633):653–657.
23. Navarro L, et al. (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312(5772):436–439.
24. Schwab R, Ossowski S, Riester M, Warthmann N, Weigel D (2006) Highly specific gene silencing by artificial microRNAs in Arabidopsis. *Plant Cell* 18(5):1121–1133.
25. Vaucheret H, Vazquez F, Crete P, Bartel DP (2004) The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev* 18(10):1187–1197.
26. Mallory AC, Vaucheret H (2009) ARGONAUTE 1 homeostasis invokes the coordinate action of the microRNA and siRNA pathways. *EMBO Rep* 10(5):521–526.
27. Backman TW, et al. (2008) Update of ASRP: The Arabidopsis Small RNA Project database. *Nucleic Acids Res* 36(Database issue):D982–985.
28. De Smet I, et al. (2007) Auxin-dependent regulation of lateral root positioning in the basal meristem of Arabidopsis. *Development* 134(4):681–690.
29. Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17(8):754–755.