Developmental Cell, Vol. 9, 109–119, July, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.devcel.2005.05.014

Plant Development Is Regulated by a Family of Auxin Receptor F Box Proteins

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Summary

The plant hormone auxin has been implicated in virtually every aspect of plant growth and development. Auxin acts by promoting the degradation of transcriptional regulators called Aux/IAA proteins. Aux/IAA degradation requires TIR1, an F box protein that has been shown to function as an auxin receptor. However, loss of TIR1 has a modest effect on auxin response and plant development. Here we show that three additional F box proteins, called AFB1, 2, and 3, also regulate auxin response. Like TIR1, these proteins interact with the Aux/IAA proteins in an auxin-dependent manner. Plants that are deficient in all four proteins are auxin insensitive and exhibit a severe embryonic phenotype similar to the *mp/arf5* and *bdl/iaa12* mutants. Correspondingly, all TIR1/AFB proteins interact with BDL, and BDL is stabilized in triple mutant plants. Our results indicate that TIR1 and the AFB proteins collectively mediate auxin responses throughout plant development.

Introduction

The plant hormone indole-3-acetic acid (IAA or auxin) has been implicated in diverse aspects of plant growth and development (Davies, 1995). Recent studies of auxin signaling have focused on transcriptional regulation by members of the ARF and Aux/IAA protein families (Leyser, 2002). The ARF proteins (23 members in *Arabidopsis*) each contain conserved DNA binding and dimerization domains. ARF proteins bind a DNA element called the *AuxRE* and either activate or repress transcription, depending on the ARF (Hagen and Guilfoyle, 2002). Genetic studies have implicated individual ARF proteins in embryogenesis (MP/ARF5) (Hardtke and Berleth, 1998), tropisms (ARF2, NPH4/ARF7, and ARF19) (Harper et al., 2000; Li et al., 2004; Okushima et al., 2005), floral development (ETTIN/ARF3) (Sessions et al., 1997), and root and hypocotyl growth (ARF2, ARF7, ARF8, and ARF19) (Li et al., 2004; Okushima et al., 2005; Tian et al., 2004).

The Aux/IAA proteins (29 members in *Arabidopsis*) are small nuclear proteins that possess four conserved domains (I through IV). Domains III and IV are similar in sequence to the ARF dimerization domain. In yeast two-hybrid tests and in vitro, this sequence promotes the formation of diverse homo- and heterodimers among the Aux/IAAs and between Aux/IAAs and ARFs. Domain I is a transferable repressor domain that is dominant over the activation function of an ARF protein (Tiwari et al., 2004). Domain II contains a degron involved in auxin-dependent degradation of these proteins (Gray et al., 2001; Ramos et al., 2001; Zenser et al., 2001). Mutations within domain II act to stabilize the affected protein and result in a decrease in auxin response as well as diverse defects in growth and development (Gray et al., 2001; Liscum and Reed, 2002; Ouellet et al., 2001; Ramos et al., 2001; Tiwari et al., 2001). The most severe mutant, *bdl/iaa12*, has defects in embryogenesis that result in seedling lethality (Hamann et al., 1999; Hamann et al., 2002).

The biological functions of the ARFs and Aux/IAAs are complex. However, a number of lines of evidence indicate that the Aux/IAAs function as transcriptional repressors by binding to activating ARFs (Kim et al., 1997; Ulmasov et al., 1997; Ulmasov et al., 1999a; Ulmasov et al., 1999b). Although an interaction between a particular pair of Aux/IAA and ARF proteins has not been directly demonstrated in vivo, genetic studies suggest that MSG2/IAA19 and NPH4/ARF7 interact during hypocotyl growth and lateral root development while BDL/IAA12 represses MP/ARF5 function during embryogenesis (Hamann et al., 2002; Tatematsu et al., 2004).

Auxin stimulates degradation of the Aux/IAA proteins, suggesting that auxin acts, at least in part, by promoting the removal of these transcriptional repressors from the cell (Dharmasiri and Estelle, 2004; Leyser, 2002; Ouellet et al., 2001; Tiwari et al., 2001; Zenser et al., 2001). The F box protein TIR1 has been shown to directly interact with the Aux/IAA proteins and promote their degradation (Dharmasiri et al., 2003; Gray et al., 2001). Recently, we demonstrated that auxin directly binds SCF^{TIR1} and that TIR1 synthesized in insect cells interacts with recombinant IAA7 in an auxin-dependent manner (Dharmasiri et al., 2005). Similar results have also been obtained using TIR1 synthesized in *Xenopus* embryo cells (Kepinski and Leyser, 2005). These results imply that auxin interacts directly with TIR1 to facilitate the interaction with the Aux/IAA proteins. Thus, TIR1 appears to function as a novel auxin receptor. However, the *tir1* mutations have a very modest effect on auxin response and morphology, suggesting that SCF^{TIR1} has a limited role in auxin signaling.

Here, we describe three closely related genes called

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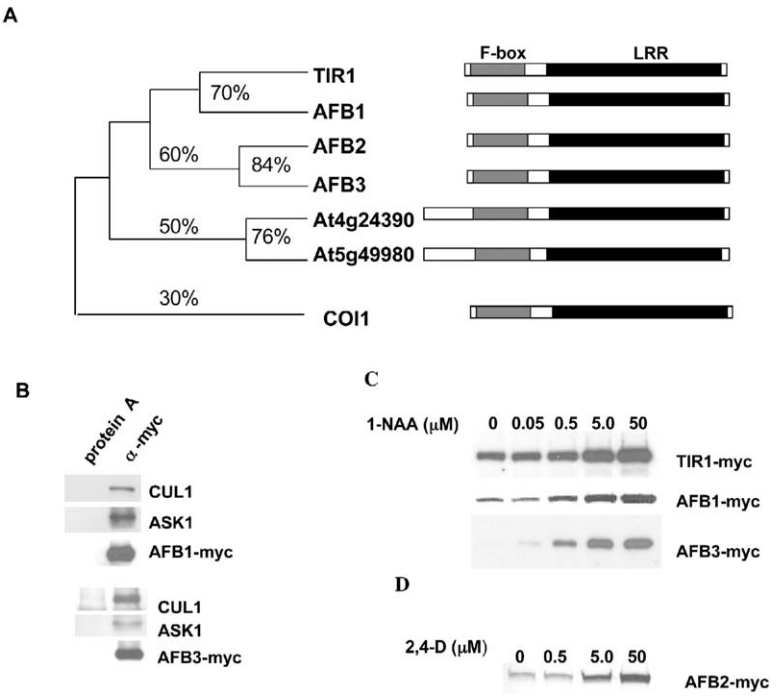

Figure 1. The AFB Proteins Are Subunits of SCFs that Interact with Aux/IAA Proteins in an Auxin-Dependent Way

(A) Phylogenetic tree of TIR1 and its closest relatives. Values represent % identical amino acids. The most distantly related protein in this subclade is COI1 implicated in jasmonic acid signaling.

(B) AFB1 and AFB3 form SCF complexes with ASK1 and CUL1. Crude protein extracts from *Arabidopsis* plants expressing AFB1-myc or AFB3-myc were immunoprecipitated with anti-myc antibody and immunoblotted with anti-CUL1 or anti-ASK1 antibody. The blots were then stripped and immunoblotted with anti-myc antibody.

(C) Aux/IAA proteins interact with TIR1, AFB1, and AFB3 in an auxin-dependent manner. Crude protein extracted from *Arabidopsis* seedlings expressing TIR1-myc, AFB1-myc, or AFB3-myc were used in pull-down assays with GST-IAA7 expressed and purified from *E. coli* in the presence of increasing concentration of auxin.

(D) A pull-down assay was performed as in (C) except that AFB2-myc was synthesized in a TNT wheat germ system (Invitrogen).

AFB1, *2*, and *3*. The *AFB* genes encode related F box proteins that assemble into SCF complexes. Genetic studies indicate that *TIR1* and the *AFB* genes function in a partially redundant fashion to mediate auxin response. The stepwise reduction in *TIR1* and *AFB* gene dosage results in a progressive decrease in auxin response and increasingly severe defects in development. The most severely affected plants resemble *bdl* or *mp* mutants, indicating that SCF-mediated degradation of BDL/IAA12 and perhaps other Aux/IAA proteins is an essential component of auxin signaling during embryogenesis. These results indicate that TIR1 and the AFB proteins constitute a family of F box protein/auxin receptors that collectively mediate auxin-regulated transcription throughout development.

Results

Members of the TIR1/AFB Family of F Box Proteins Interact with Aux/IAA Proteins in an Auxin-Dependent Manner

The *Arabidopsis* genome encodes nearly 700 F box proteins, most of which have not been characterized (Gagne et al., 2002). TIR1 is a member of a small subclade consisting of seven proteins (Figure 1A; Gagne et al., 2002). In addition to TIR1, this group includes COI1, a protein required for response to the plant hormone jasmonic acid (Xie et al., 1998; Xu et al., 2002), and five proteins of unknown function. We began our study of this family by focusing on the three proteins that are most closely related to TIR1: AFB1 (At4g03190), AFB2 (At3g26810), and AFB3 (At1g12820) for Auxin signaling F box protein 1, 2, and 3. We first asked if these proteins are present in SCF complexes together with CUL1 and the SKP1-related protein ASK1. Transgenic lines expressing c-*myc*-tagged versions of AFB1 and AFB3 under control of the *CaMV35S* promoter were generated and used for coimmunoprecipitation studies. The results in Figure 1B show that these two F box proteins interact with both CUL1 and ASK1, confirming that they are subunits in SCF complexes. Despite repeated attempts, we were unable to generate a line that expresses an epitope-tagged version of AFB2.

Previous studies have shown that SCF^{TIR1} interacts with members of the Aux/IAA family of proteins in the presence of auxin (Dharmasiri et al., 2003; Gray et al., 2001). To determine if AFB1, 2, and 3 also interact with Aux/IAA proteins, we performed GST pull-down experiments with GST-IAA7. In the case of AFB1 and AFB3, pull-downs were performed using extracts prepared from seedlings expressing c-*myc*-tagged versions of these proteins. A line expressing TIR1-myc was included for comparison. The results in Figure 1C show that both proteins interact with GST-IAA7 and that this interaction is promoted by auxin. In the case of AFB2, the F box protein was synthesized in a TNT wheat germ extract. This protein also interacted with GST-IAA7 in an auxin-dependent manner (Figure 1D). These results indicate that all three F box proteins interact with Aux/IAA proteins in vitro.

TIR1 and the *AFB* Genes Are Expressed in Largely Overlapping Domains

The *TIR1* gene is expressed throughout plant development including embryogenesis (Gray et al., 1999). To characterize expression of the *AFB* genes, we used a combination of RT-PCR and promoter-GUS analysis. Based on RT-PCR studies, the pattern of *AFB1* expression is very similar to that of *TIR1*, with the highest levels of expression in 12-day-old seedlings and flow-

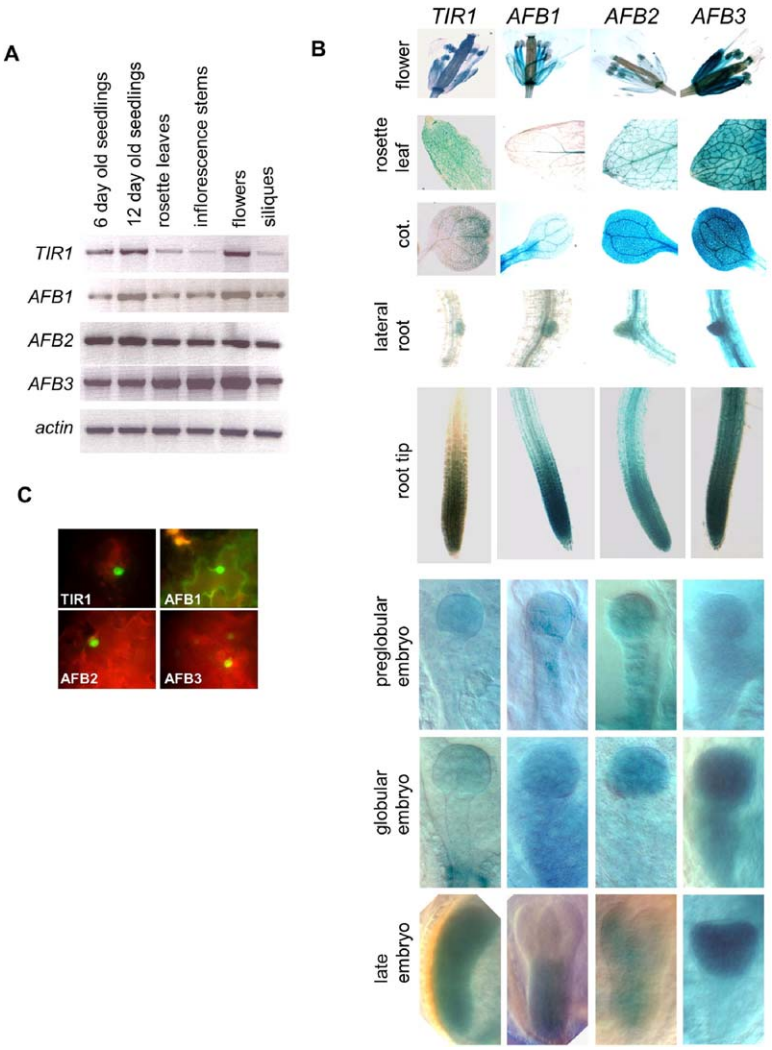

Figure 2. *TIR1* and the *AFB* Genes Are Expressed throughout the Plant

(A) Reverse-transcription PCR was performed using RNA extracted from different *Col-0* tissues. Roots were from 12-day-old plants and the rosette leaves from 27-day-old plants.

(B) Expression patterns of *TIR1*, *AFB1*, *AFB2*, and *AFB3* promoters, transcriptionally fused with GUS reporter gene. Each column represents a different promoter:GUS construct, and each row represents a different plant organ. The roots shown are from 6-day-old plants.

(C) N-terminal GFP-tagged TIR1 and the AFB proteins localized to the nucleus in *Nicotiana benthamiana* cells.

ers (Figure 2A). The *AFB2* and *AFB3* genes are more highly expressed than either *TIR1* or *AFB1*. *AFB2* RNA is slightly more abundant in seedlings, while *AFB3* is more highly expressed in the inflorescence and in flowers.

The analysis of *promoter::GUS* fusions for each gene also indicate that these genes are expressed in largely overlapping domains. A total of 10 independent transgenic lines was analyzed for each *promoter::GUS* fusion. Each gene is expressed in seedling root tips, emerging lateral roots, vascular bundles in cotyledons and mature leaves, and in mature floral organs (Figure 2B). In general, the activity of each promoter, as measured by GUS staining, corresponded to the level of RNA measured by RT-PCR. Thus, the *TIR1* and *AFB1* promoters are less active, while *AFB2* and *AFB3* are more highly expressed. In addition, each gene is expressed throughout embryogenesis as early as the preglobular embryo (Figure 2B). Our data clearly indicate that *TIR1* and the *AFBs* are widely expressed. However, it is important to note that recent computational studies identified a microRNA called *miR393* that may also regulate *TIR1/AFB* RNA levels (Adai et al., 2005; Jones-Rhoades and Bartel, 2004). So far the biological function of *miR393* has not been reported.

Previous studies indicate that the Aux/IAA proteins are nuclear localized. To determine if TIR1 and the AFBs are also nuclear proteins, we generated constructs that fused GFP to the N terminus of each protein and introduced these constructs into *Nicotiana benthamiana* leaves. The results shown in Figure 2C indicate that all four proteins are strongly localized to the nucleus in these cells.

All Three *AFB* Genes Contribute to Auxin Response

To further investigate the function of the *AFB* genes in auxin response, we identified T-DNA insertion alleles in the Wisconsin collection (Figure 3A). We selected the *afb1-1*, *afb2-1*, and *afb3-1* alleles for further analysis. The positions of the T-DNA insertions for these alleles are nucleotides 1472, 812, and 1834, respectively (relative to the ATG for each gene), and the effect of each insertion on accumulation of *AFB* transcripts was determined by RT-PCR. No transcript was observed in the *afb2-1* mutant, suggesting that this allele is a null (Fig-

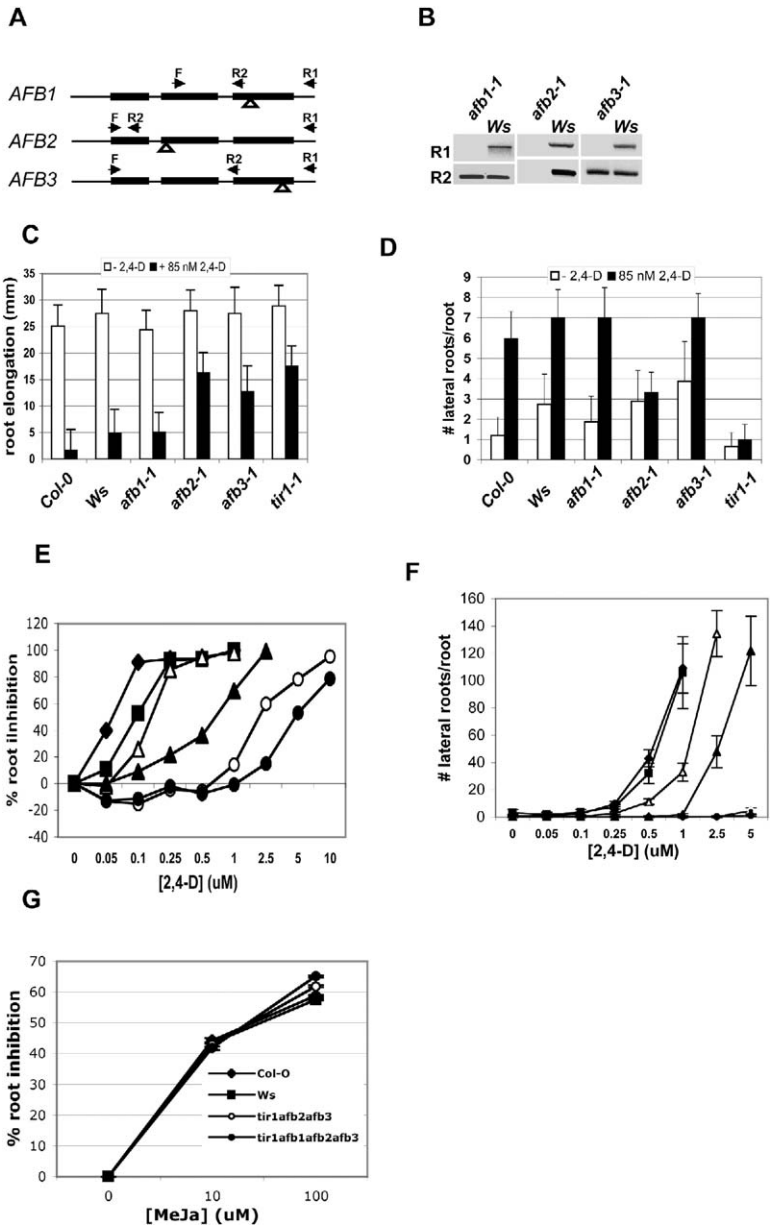

Figure 3. *TIR1* and the *AFB* Genes Each Contribute to Auxin Response

(A) Organization of the *AFB1, AFB2*, and *AFB* genes. Filled boxes represent exons. The position of T-DNA insertions is indicated with the open arrow. Horizontal arrows indicate the positions of forward (F) and reverse (R) primers that were used in reverse transcription PCR of mutant alleles.

(B) Products of RT-PCR for each allele. R1 amplifies the complete transcript while R2 amplifies a truncated transcript.

(C) Effect of auxin on root elongation in wild-type and mutant seedlings. 5-day-old seedlings were transferred from auxin-free medium onto media containing no auxin (white columns) or 85 nM 2,4-D (black columns), and root elongation was measured after 3 days.

(D) Effect of auxin on lateral root formation in wild-type and mutant seedlings. Seedlings were treated as in (C), and the total number of emerged lateral roots was counted 4 days after the transfer to new media.

(E) Mutations in *AFB* genes confer additive effects on auxin-resistant root elongation. Seedlings were treated as in (C) and the percent inhibition of root elongation relative to seedlings growing on medium without auxin was determined 3 days after transfer. *Col-0* (closed diamond), *Ws* (closed square), *tir1-1* (open triangle), *tir1-1 afb2-1* (closed triangle), *tir1 afb2-1 afb3-1* (open circle), and *tir1 afb1-1 afb2-2 afb3-3* (closed circle).

(F) Mutations in *AFB* genes confer additive effects on lateral root initiation. Seedlings were treated as in (C), and the number of lateral roots was counted 4 days after transfer to media containing the indicated concentrations of 2,4-D. In this case both emerged lateral roots and primordia were counted using a dissecting microscope. Symbols are as in (E).

(G) Effect of methyl jasmonate (MeJa) on root elongation of *tir1 afb* triple and quadruple mutants. Experiment was performed as described for (C) except that seedlings were transferred onto medium containing MeJa at the indicated concentration.

For (C)–(G), error bars represent the standard deviation.

ure 3B). Both the *afb1-1* and *abf3-1* insertions result in a truncated transcript (Figure 3B).

The role of the *AFB* genes in auxin response was assessed in *afb1-1, afb2-1*, and *afb3-1* seedlings. First we determined the effects of auxin on root elongation. Figure 3C shows that the *afb1-1* allele had no effect on auxin inhibition of root growth. In contrast, both *afb2-1* and *afb3-1* seedlings were resistant to auxin compared to the *Ws* control, with *afb2-1* displaying a slightly higher level of resistance than *afb3-1*. Next we examined auxin induction of lateral roots in the mutant lines. Both *afb1-1* and *afb3-1* were similar to *Ws*, but *afb2-1* seedlings were deficient in this response (Figure 3D). Similar results have been obtained with independent T-DNA mutants for each gene, confirming that these phenotypes are due to mutations in the *AFB* genes (data not shown).

To determine whether *TIR1* and the *AFB* genes function redundantly, we generated higher order mutants and examined auxin response in these lines. The data in Figures 3E and 3F show that loss of these genes results in a progressive decrease in auxin response during both root elongation and lateral root formation. The introduction of *afb2* or *afb3* into a *tir1-1* background resulted in an additive increase in auxin resistance, indicating that each of these genes contributes to auxin response (Figure 3E and data not shown). When we constructed the *tir1-1 afb2-1 afb3-1* triple mutant, we found that a large proportion of these plants arrested shortly after germination (see below). However, seedlings that did develop a root displayed a high level of auxin resistance with respect to both elongation and lateral root formation. Finally, the introduction of *afb1-1* into the triple mutant further enhanced the phenotype.

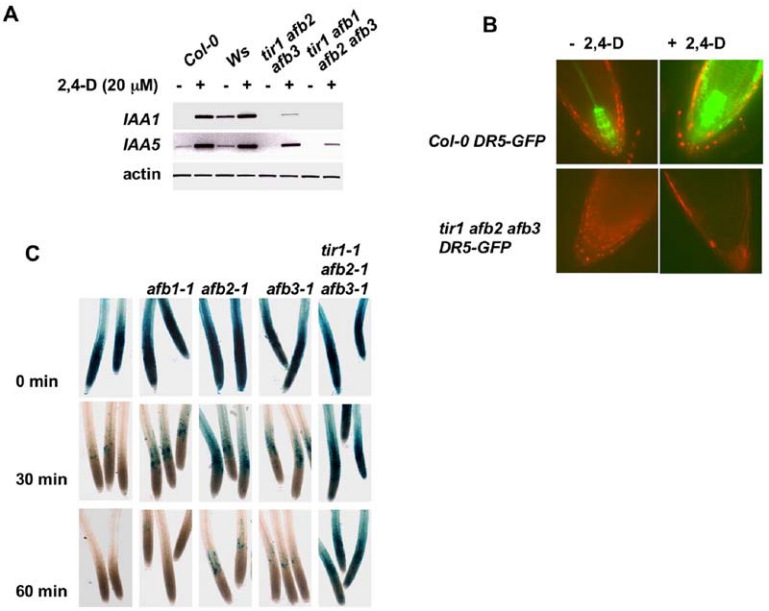

Figure 4. Auxin-Responsive Gene Expression and Aux/IAA Degradation Are Affected in *tir1 afb* Mutant Seedlings

(A) Auxin induction of *IAA1* and *IAA5* transcription. 6-day-old seedlings were treated with 20 μM 2,4-D for 60 min. For the triple and quadruple mutants, class I (see text) seedlings were selected for this analysis. RNA was extracted and RT-PCR was performed using primers specific for the *IAA1* and *IAA5* genes. PCR was performed for 25 cycles.

(B) Expression of the *DR5rev::GFP* auxin-responsive reporter is severely affected in *tir1 afb2 afb3* mutant seedlings. 6-day-old *Col-0* or *tir1 afb2 afb3* seedlings carrying the *DR5rev::GFP* reporter were transferred onto medium containing 1 μM 2,4-D for 24 hr. GFP fluorescence at the root tips was observed by confocal microscopy with propidium iodide counterstaining.

(C) AXR3/IAA17 degradation is delayed in *tir1 afb2 afb3* mutants. The *HS::AXR3NT-GUS* transgene was crossed into *afb1, afb2,* and *afb3* single mutants as well as the *tir1 afb2 afb3* triple mutant. 6-day-old seedlings were heat-treated for 2 hr, transferred into liquid growth media containing 5 μM 2,4-D, and stained for GUS activity after designated times.

Based on these results, we conclude that all four genes contribute to auxin response in the root. *TIR1, AFB2*, and *AFB3* appear to contribute equally to the response, while *AFB1* has a lesser role. However, it is important to note that the *afb1-1* and *afb3-1* alleles may not be nulls, leaving open the possibility of a larger role for these two genes in these processes.

Mutations in the closely related *COI1* gene are insensitive to jasmonic acid (JA), indicating that COI1 targets repressors of the JA response (Xie et al., 1998). To determine whether the *AFB* genes also function in this response, we examined the effects of exogenous JA on root growth in the *tir1-1 afb2-1 afb3-1* and *tir1-1 afb1-1 afb2-1 afb3-1* plants. The results in Figure 3G show that JA sensitivity is not altered in these mutants.

Since the Aux/IAA proteins are relatively stable in the *tir1* mutant, we would expect auxin-induced transcription to be reduced in this mutant (Dharmasiri et al., 2003). However, *tir1* plants are not obviously affected in the expression of known auxin-responsive genes (data not shown). To determine whether *TIR1* and the *AFB* genes cooperate to regulate gene expression, we examined expression of several members of the *Aux/IAA* gene family in *tir1-1 afb2-1 afb3-1* and *tir1-1 afb1-1 afb2-1 afb3-1* mutant plants. The results in Figure 4A show that expression of the *IAA1* and *IAA5* genes is significantly altered in triple mutant plants and further reduced in the quadruple mutant. To confirm these results, we crossed the auxin-responsive reporter *DR5rev::GFP* into *tir1-1 afb2-1 afb3-1* plants (Friml et al., 2003; Ulmasov et al., 1997). The results show that *tir1-1 afb2-1 afb3-1* seedlings are severely deficient in auxin-regulated expression of this reporter (Figure 4B).

Our results indicate that the AFB proteins are subunits in SCF complexes that interact with Aux/IAA protein IAA7. To determine whether the AFB proteins are required for Aux/IAA protein degradation, we introduced the *HS::AXR3NT-GUS* transgene into the *afb* mutants. This construct has been used to assess proteasome-mediated degradation of the AXR3/IAA17 protein (Gray et al., 2001). Seedlings were exposed to high temperature for 120 min and incubated in 5 μM 2,4-D thereafter. GUS staining was performed 0, 30, and 60 min after the end of the high temperature period. The results in Figure 4C show that each single mutant is deficient in degradation of AXR3NT-GUS. After 30 min, GUS staining is absent in the wild-type control but still present in each of the mutant lines. The stabilization of AXR3NT-GUS in the *afb1-1* mutant confirms that *AFB1* is involved in auxin response despite the lack of a mutant phenotype. GUS staining persists in *afb2-1* and *afb3-1* after 60 min, consistent with the stronger auxin response phenotype of these lines relative to *afb1-1*. The *tir1-1 aft2-1 afb3-1* genotype is dramatically altered with intense GUS staining, even after 60 min. These results demonstrate that $SCF^{AFB1/2/3}$ is required for auxin-dependent degradation of Aux/IAA proteins.

The TIR1/AFB Proteins Act Redundantly to Regulate Diverse Aspects of Plant Growth and Development

To investigate the role of the *AFB* genes in plant growth and development, we characterized the phenotype of the *afb* mutants as well as various combinations of these mutants. When grown either in the dark or the light, *afb1, afb2,* and *afb3* mutant seedlings were all similar to wild-type in appearance (data not shown). This was also true of all double mutant combinations involving the *afb* mutants and *tir1*. However, severe defects were observed in triple and quadruple mutants. When the progeny of homozygous *tir1-1 afb2-1 afb3-1*

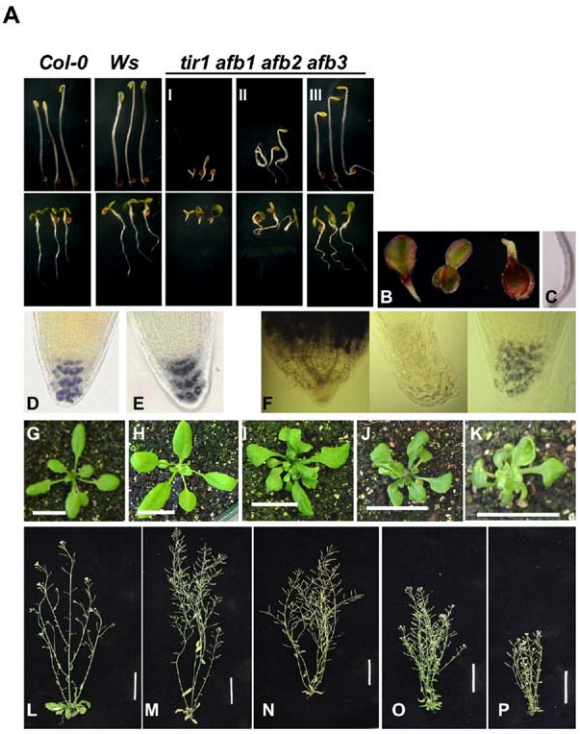

Figure 5. *TIR1* and the *AFB* Genes Act Redundantly to Regulate Diverse Aspects of Growth and Development

(A) 6-day old *Col-0, Ws,* and *tir1 afb1 afb2 afb3* seedlings grown on vertically oriented plates in the dark (top) or light. Quadruple mutant seedlings are categorized as class I, II, or III.
(B) Class I *tir1 afb1 afb2 afb3* seedlings germinated in the light.
(C) Root of class III *tir1 afb1 afb2 afb3* seedling.
(D) Root tip region of *Col-0* seedling, stained with Lugol solution.
(E) Root tip region of *Ws* seedling, stained with Lugol solution.
(F) Root tip regions of class I, II, and III *tir1 afb1 afb2 afb3* seedlings stained with Lugol solution.
(G–K) 20-day-old *Col-0* (G), *Ws* (H), *tir1 afb2* (I), *tir1 afb2 afb3* (J), and *tir1 afb1 afb2 afb3* (K) rosettes. Scale bar equals 1 cm.
(L–P) 40-day-old *Col-0* (L), *Ws* (M), *tir1 afb2* (N), *tir1 afb2 afb3* (O), and *tir1 afb1 afb2 afb3* (P) plants.

or *tir1-1 afb1-1 afb2-1 afb3-1* plants are placed on agar medium, an array of phenotypes is observed that we have divided into three classes. Representative quadruple mutant seedlings are shown in Figure 5A. A similar range of phenotypes was observed among the triple mutant seedlings. The most severely affected seedlings (class I) lack a root and often have a single cotyledon (Figures 5A and 5B). The hypocotyl is either absent or rudimentary and does not elongate in the dark or light. The class I phenotype is remarkably similar to that of the *bdl/iaa12* or *mp/arf5* mutants (Berleth and Jürgens, 1993; Hamann et al., 1999). In the case of the triple mutant, 36% (n = 146) had this phenotype, while in the quadruple mutant, 49% (n = 129) were in this class. An intermediate class, called class II (11% of triple mutants and 15% of quadruple mutants), developed a short root with a gravitropic defect. These plants would occasionally form several small leaves before dying (Figure 5A). The hypocotyls of class II seedlings were shorter than wild-type in the light and dark and, like the roots, appear to have a defect in tropic response. In addition, these seedlings lacked the characteristic apical hook. The remainder of the seedlings (class III) produced a root with a clear defect in gravitropism and very few root hairs (Figures 5A and 5C). In the dark, these seedlings lacked an apical hook and many were shorter than wild-type. The relative sizes of the three classes were unchanged in both the triple and quadruple mutant through at least four generations.

Later in development, both the *afb1-1* and the *afb2-1* mutants are similar to the wild-type (*Ws*) in appearance (data not shown). The *afb3-1* mutant is slightly shorter than the wild-type line and has shorter siliques (data not shown). All double mutant combinations are also much like wild-type in appearance except that the *afb3-1* silique defect is present in all lines containing *afb3-1*. The one exception is the *tir1-1 afb2-1* line, which exhibits a reduction in rosette leaf size and inflorescence height (Figures 5I and 5N). Class III *tir1 afb2 afb3* and *tir1 afb1 afb2 afb3* seedlings continue to grow and form rosettes with small, highly curled leaves (Figures 5J and 5K). When the triple and quadruple mutants flower, they produce a highly branched dwarf inflorescence (Figures 5O and 5P). With respect to both rosette and inflorescence phenotype, the quadruple mutant is more severely affected than the triple mutant, indicating that *AFB1* contributes to growth and development at these stages.

Recent studies suggest that auxin has an important role in patterning of the root meristem. To characterize the root defects in the *tir1/afb* mutants in more detail, we stained wild-type and *tir1-1 afb1-1 afb2-1 afb3-1* seedling roots with Lugol solution to visualize the columella cells. Figure 5F shows three quadruple mutant seedlings representing, from left to right, class I, II, and III seedlings. All three seedlings have defects in root organization. Class I seedlings lack a recognizable root meristem. Class II seedlings have a highly disorganized meristem with a few cells showing faint Lugol staining, indicative of a columella fate. Class III seedlings have a columella, but the cells are not arranged in organized layers as in wild-type seedlings. We further characterized the meristems of class III quadruple mutants by determining the number of meristematic cells in 7-day-old seedlings. We found that *Col* and *Ws* had 59.4 ± 6.4 and 62.5 ± 6.1 meristem cells, respectively, while *tir1 afb1 afb2 afb3* seedlings had 45.7 ± 4.8 meristem cells (n = 10 for each genotype). Based on a Student's t test, the difference between the quadruple mutant and each wild-type line was significant with $p < .005$. These results indicate that even in those seedlings that develop a relatively normal root, the *tir1/afb* mutations affect cell proliferation in the meristem.

The TIR1 and AFB Proteins Regulate Degradation of BDL/IAA12 during Embryogenesis

The severe phenotype exhibited by *tir1 afb* triple and quadruple mutant seedlings as shown in Figure 5B strongly resembles *bdl* mutant seedlings, which have a gain-of-function mutation in the IAA12 protein (Hamann et al., 2002). The *bdl* phenotype can be traced to early embryo stages, and thus we examined whether the origin of the triple mutant phenotype corresponds to that

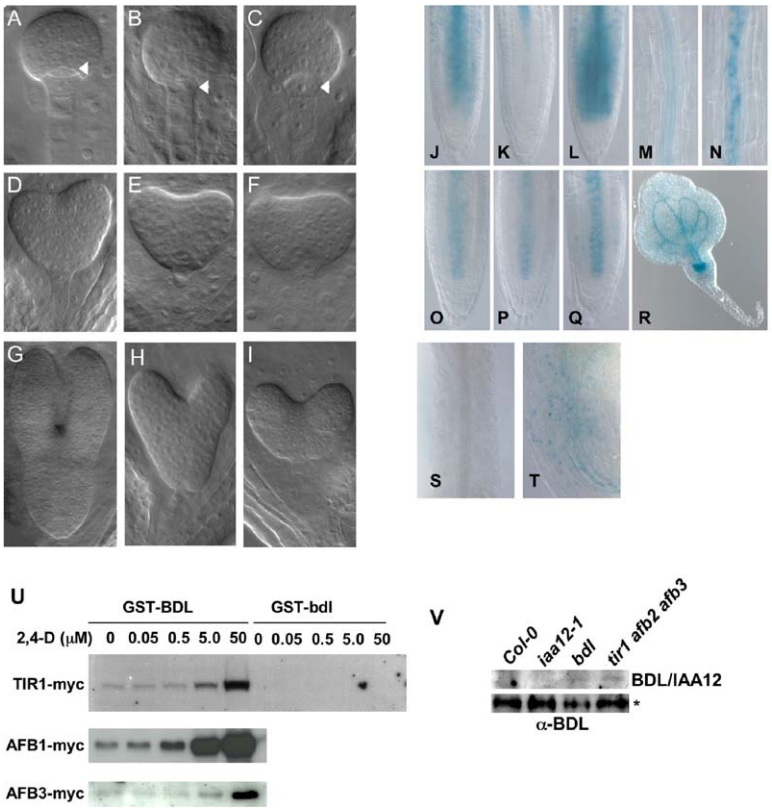

Figure 6. *TIR1* and the *AFB* Genes Regulate Embryogenesis by Promoting the Degradation of BDL/IAA12

(A–I) Embryos at globular (A–C), heart (D–F), and torpedo (G–I) stages from Columbia wild-type (A, D, G), *bdl* (B, E, H), and *tir1-1 afb2-1 afb3-1* (C, F, I) plants. Note that in *bdl* and *tir1-1 afb2-1 afb3-1*, hypophysis (arrowheads) division is aberrant, with either altered plane of division (B) or strongly delayed division (C). Eventually, the hypophysis derivatives, instead of giving rise to an organized cell group in wild-type (D, G), form unorganized structures in *bdl* (E, H) and *tir1-1 afb2-1 afb3-1* (F, I).

(J–R) GUS activity in homozygous *BDL:GUS* (J–N) or hemizygous *bdl:GUS* (O–Q) seedling roots. Seedlings in (J) and (O) were mock treated, whereas seedlings in (K) and (P) were incubated in 20 μM IAA for 1 hr and seedlings in (L) and (Q) were treated with 50 μM MG132 for 1 hr. Panels (M) and (N) show regions higher up in the root of seedlings that were pretreated with mock medium (M) or medium containing 50 μM MG132 (N) for 1 hr, and then treated with 20 μM IAA for 1 hr. Panel (R) shows a seedling homozygous for the *bdl:GUS* transgene.

(S and T) BDL:GUS activity in the hypocotyls of phenotypically wild-type (S) and triple mutant (T) seedlings from an F2 population segregating *tir1-1 afb2-1* and *afb3-1*.

(U) IAA12/BDL interacts with TIR1 and AFB proteins in an auxin-dependent manner. Crude plant extracts from *Arabidopsis* seedlings expressing TIR1-myc, AFB1-myc, and AFB3-myc were used in pull-down assays with GST-BDL or GST-bdl expressed and purified from *E. coli*.

(V) BDL protein level in Columbia wild-type, *iaa12-1*, *bdl*, and *tir1-1 afb2-1 afb3-1* plants. The weak band at 26 kDa represents BDL and is visible only in *bdl* and *tir1-1 afb2-1 afb3-1* mutant backgrounds. The lower panel shows an unspecific cross-reacting band (asterisk) to demonstrate protein equal loading.

of the *bdl* mutant. The embryos of *tir1-1, afb2-1,* and *afb3-1* single mutants all develop normally (data not shown). In contrast, 48% (n = 186) of *tir1-1 afb2-1 afb3-1* embryos display early defects in embryogenesis, consistent with the occurrence of a similar proportion of defective seedlings. Embryogenesis appears normal up to the globular stage. At that point, while in wild-type embryos the hypophysis first divides asymmetrically (Figure 6A) and further cell divisions give rise to the embryonic root meristem (Figures 6D and 6G), in *tir1-1 afb2-1 afb3-1* embryos, defects in hypophysis division are apparent (Figure 6C) and are followed by the failure to establish an embryonic root meristem (Figures 6F and 6I). Like the seedling phenotype, this basal embryo phenotype is indistinguishable from that of the *bdl/iaa12* and *mp/arf5* at the globular and heart stages of embryogenesis (Figures 6B and 6E; Hamann et al., 2002; Berleth and Jürgens, 1993). At later embryonic stages, the triple mutant phenotype is slightly stronger than that of most *bdl* embryos (Figures 6H and 6I) and resembles more the phenotype of *mp/arf5* embryos (Berleth and Jürgens, 1993). These results suggest that TIR1/AFB-dependent degradation of BDL, and possibly other Aux/IAA proteins, is required for patterning of the early embryo.

The gain-of-function *bdl* mutation lies within domain II of IAA12, suggesting that the mutant phenotype is due to stabilization of IAA12 (Hamann et al., 2002). To determine whether BDL/IAA12 is degraded in an auxin-dependent manner, we generated *Arabidopsis* lines expressing BDL:GUS and bdl:GUS fusion proteins from the endogenous promoter and 5′ and 3′ UTRs. The BDL:GUS and bdl:GUS proteins are expressed throughout the plant in vascular tissue (data not shown), with the pattern at the distal root tip (Figure 6J) closely resembling the mRNA pattern in late embryos and the GUS pattern in previously described *pBDL::GUS* lines (Hamann et al., 2002). Plants carrying the *BDL:GUS* gene were wild-type in appearance, whereas plants containing a single *bdl:GUS* copy resembled *bdl* heterozygotes (not shown) and those containing two *bdl:GUS* copies were rootless (Figure 6R). Hence, the GUS fusion proteins reflect the activity of the endogenous *BDL* gene.

GUS staining experiments reveal that BDL:GUS is destabilized by a 60 min treatment with 20 μM IAA (Figures 6J, 6K, and 6M), while the bdl:GUS protein is unaffected by this treatment (Figures 6O and 6P). Treatments with the proteasome inhibitor MG132 showed that auxin-dependent BDL degradation requires the proteasome (Figures 6L). Thus, BDL behaves as a bona fide substrate for auxin-induced proteasome-dependent degradation, which makes it a likely substrate for $SCF^{TIR/AFB}$ during embryogenesis.

To investigate this possibility, we performed pull-down experiments with GST:BDL and GST:bdl using extracts prepared from *TIR1:Myc* seedlings. The results show that BDL/IAA12 interacts with SCF^{TIR1} in the presence of auxin, similar to IAA7 (Figure 6U). In contrast, GST:bdl does not interact with SCF^{TIR1}, confirming that domain II of BDL/IAA12 is involved in TIR1 binding. As expected from the contribution of multiple AFB proteins to embryo development (Figures 6C, 6F, and 6I), AFB1 and AFB3 also interact with BDL/IAA12 in the presence of auxin (Figure 6U). Finally, an antiserum raised against two peptides specific for BDL detects a protein the size of BDL (26 kDa) in *bdl* and *tir1-1 afb2-1 afb3-1* extracts, but not in extracts from wild-type or *iaa12-1* (T-DNA insertion line in the *IAA12* gene) plants (Figure 6V). Consistent with reduced degradation of BDL protein, the BDL:GUS fusion protein is more abundant in *tir1-1 afb2-1 afb3-1* (Figure 6T) than in wild-type seedlings (Figure 6S).

Taken together, these results strongly suggest that TIR1 and the AFB proteins regulate auxin response during embryogenesis by promoting the auxin-dependent degradation of BDL/IAA12 and perhaps other Aux/IAA proteins.

Discussion

The mechanism of auxin action has been the subject of intense investigation for decades (Leyser, 2002). Genetic studies have demonstrated that the F box protein TIR1, a subunit of the ubiquitin protein ligase SCF^{TIR1}, is required for auxin-dependent degradation of the Aux/IAA proteins leading to expression of auxin-regulated genes (Gray et al., 2001). Remarkably, recent experiments indicate that auxin binds directly to TIR1 to mediate Aux/IAA recognition, indicating that TIR1 functions as a receptor for this auxin response (Dharmasiri et al., 2005; Kepinski and Leyser, 2005).

Although these exciting findings provide important new insight into the mechanism of auxin action, the effects of loss of *TIR1* are quite mild compared to other auxin-related mutants, calling into question the importance of the SCF in auxin response (Liscum and Reed, 2002; Ruegger et al., 1998). In this report we show that the related F box proteins AFB1, AFB2, and AFB3 each contribute to auxin response by regulating degradation of the Aux/IAA proteins. Further, genetic studies indicate that the AFBs and TIR1 have overlapping and redundant functions in embryogenesis and throughout plant development.

Based on sequence, the TIR1 and AFB proteins can be divided into two groups with TIR1 and AFB1 in one group and AFB2 and AFB3 in the other. *TIR1* and *AFB1* are located in regions of segmental genome duplication, and based on their similar expression, activity, and mutant phenotype, they can be considered paralogs. *AFB2* and *3* are slightly more distantly related and are also diverged in their expression pattern. The grouping together of *TIR1* and *AFB1* and of *AFB2* and *AFB3* is reflected by global expression analysis using the Genevestigator program (not shown), where *TIR1* and *AFB1* have very similar expression in a range of conditions, whereas *AFB2* and *AFB3* behave differently. Nonetheless, each TIR1/AFB protein contributes to auxin response in a quantitative way and most cells express all four genes. The broad expression of *TIR1* and *AFB* genes implies that essentially all cells are capable of perceiving auxin.

The analysis of auxin response in TIR1- and AFB-deficient seedlings indicates that these proteins have an essential role in auxin signaling. Their loss results in a progressive decrease in auxin response in several assays. With respect to root growth, the *tir1 afb1 afb2 afb3* mutant displays a very high level of resistance, greater than any mutant reported so far. Although the quadruple mutant plants used to assess root growth retain some auxin response, it is important to note that these seedlings were selected because they develop a root (class III). More severely affected seedlings (class I) completely lack a root. When auxin-regulated transcription is examined in these seedlings, auxin response is nearly absent. These results, together with the sequence similarity between TIR1 and the AFBs, suggest that the AFBs also function as auxin receptors. Our biochemical studies are consistent with this hypothesis. Each AFB protein interacts with the Aux/IAAs AXR2/IAA7 and BDL/IAA12 in an auxin-dependent manner. Further, we have recently shown that the AFB proteins contribute to auxin binding in *Arabidopsis* extracts (Dharmasiri et al., 2005). Thus, TIR1 and the AFB proteins constitute a new family of auxin receptors. Moreover, most cells appear to express as many as four functionally redundant auxin receptor genes, and a (near) complete loss of auxin responses is reached only in a quadruple mutant that lacks all these genes. Although two of the four mutant alleles used to construct the quadruple mutant line may have residual protein activity, the quadruple mutant shows an extremely strong phenotype.

With the generation of this quadruple mutant, it is possible to assess the consequences of a loss of auxin response on plant development. In the embryo, this leads to a failure in specifying the root meristem. In the seedling, loss of auxin response results in defects in hypocotyl elongation, apical hook formation, lateral root formation, tropic response, root hair development, and meristem organization. Later in development, the *tir1 afb* mutants exhibit defects in leaf morphology, overall stature, inflorescence architecture, and floral development. The quadruple mutant phenotype can be interpreted as the result of stabilization of many (or all) Aux/IAA proteins. Most *Aux/IAA* genes studied so far are expressed in a restricted set of cells or tissues, and stabilizing mutations reveal only a subset of auxin responses (reviewed in Reed, 2001; Weijers and Jürgens, 2004). Nonetheless, all phenotypes that have been described for *aux/iaa* mutants (Hamann et al., 2002; Hardtke et al., 2004; Liscum and Reed, 2002; Okushima et al., 2005; Park et al., 2002; Reed, 2001; Tatematsu et al., 2004; Yang et al., 2004) are found in the quadruple receptor mutant, supporting the conclusion that TIR1/AFB proteins mediate degradation of most, if not all, Aux/IAA proteins to allow developmental auxin responses. Stabilization of Aux/IAA proteins is expected to lead to constitutive inhibition of partner ARFs. Likewise, most reported *arf* mutant phenotypes (Hardtke et al., 2004; Harper et al., 2000; Okushima et al., 2005;

Berleth and Jürgens, 1993; Li et al., 2004; Tian et al., 2004) are found in the quadruple receptor mutant.

One of the best-characterized developmental auxin responses occurs during embryonic root formation where the activating ARF, MP/ARF5, and the Aux/IAA protein, BDL/IAA12, have been identified (reviewed in Weijers and Jürgens, 2005). In addition, a MP/BDL output is known: the DR5::GFP reporter requires normal activity of both (Friml et al., 2003). Since this promoter contains ARF binding sites and responds rapidly to auxin application, it is likely to be directly activated by MP. With the identification of the TIR1/AFB receptors as auxin-dependent effectors of BDL degradation, the entire auxin-dependent signaling pathway is known for this particular developmental process. Here, loss of auxin receptor activity (*tir1 afb1 afb2 afb3*), stabilization of its immediate substrate (*bdl*), or loss of the activating ARF (*mp*) leads to an identical phenotype: erroneous hypophysis division and failure to initiate a root meristem. By analogy to this relatively simple developmental auxin response, it is likely that other auxin responses also rely on the concerted action of TIR1/AFB auxin receptors and specific pairs of Aux/IAA inhibitors and ARF transcription factors. It remains a challenge for the future to determine whether this receptor system accounts for all auxin responses or whether there are other auxin signaling pathways that are independent of the TIR1/AFB proteins.

Experimental Procedures

Plant Material, Growth Conditions, and Treatments

Arabidopsis mutant lines *afb1-1*, *afb2-1*, and *afb3-1* are in the *Wassilewskija* (*Ws*) ecotype. All other mutants and transgenic lines used in this study were in the *Columbia* (*Col-0*) ecotype. The *iaa12-1* allele is a T-DNA insertion in the second exon (amino acid position 145) of the *BDL* gene and was kindly provided by A. Theologis (Plant Gene Expression Center, Albany, CA). The *GVG::TIR1-myc[tir1-1], DR5rev::GFP*, and *HS::AXR3NT-GUS* lines have been described elsewhere (Friml et al., 2003; Gray et al., 1999; Gray et al., 2001). To grow seedlings under aseptic conditions, seeds were surface sterilized and plated on *Arabidopsis thaliana* medium containing 1% sucrose (ATS) with 8 g agar per liter and placed vertically in a growth chamber at 22°C under continuous light. Where necessary, 8- to 10-day-old seedlings were transferred to soil and grown at 22°C under continuous light.

For root elongation and lateral root assays, 5-day-old seedlings growing on minimal medium on vertical agar plates were transferred onto media with or without hormone and grown vertically under constant light for designated times. Alternatively, 6-day-old seedlings were transferred into liquid ATS medium with or without 2,4-D and incubated for designated times with mild shaking.

To examine auxin-regulated expression of the *DR5rev::GFP* reporter, seedlings were grown on ATS medium for 6 days and transferred onto ATS with or without 1 μM 2,4-D for 24 hr. Roots were counterstained with propidium iodide (10 μg/ml) and observed under the UltraVIEW LCI confocal microscope (Perkin Elmer).

Generation of Transgenic Lines

The *AFB1* cDNA was cloned into the *Bam*HI and *Pst*I sites of *pBluescript SK* vector. *AFB2* was cloned into the *Eco*RI site of the *pBluescript SK* vector, and the *AFB3* cDNA was amplified from a *Col-0* cDNA library and cloned into the *pCR 2.1* vector.

To express c-*myc*-tagged versions in plants, *AFB1* and *AFB3* cDNAs carrying the c-*myc* epitope were placed behind a *35S CaMV* promoter in the binary vector *pROKII* and introduced into the *Agrobacterium tumefaciens* strain *GV3101*. Transgenic *Arabidopsis* plants were generated by transforming *tir1-1* mutant plants using the floral dip method to generate the *tir1-1 35S::AFB1-myc* and *tir1-1 35S::AFB3* lines. The *AFB2* cDNA carrying the c-*myc* epitope at the C terminus was cloned into *pTNT* vector (Invitrogen) between *Eco*RI and *Kpn*I restriction sites. To express GST-BDL, the *BDL* cDNA was cloned into the pGEX-2T vector.

Promoter::GUS transcriptional fusion constructs were created by cloning 1.8 kb regions upstream of the *AFB1*, *AFB2*, and *AFB3* genes into the *pBI101* binary vector. Transgenic plants were generated as described above.

To create *BDL:GUS*, first an *Spe*I restriction site was introduced 1 amino acid position upstream of the *BDL* stop codon in a pGreenII/BAR binary vector (Hellens et al., 2000) containing a genomic fragment spanning 4.5 kb of the *BDL* gene (Hamann et al., 2002). Then, a PCR-amplified *GUS* open reading frame with in-frame *Spe*I sites on both ends was introduced into this vector to create *pGreenII/BAR BDL:GUS*. The *bdl* (P72S) mutation was introduced into the *BDL:GUS* gene by PCR-mediated mutagenesis. Both *BDL:GUS* and *bdl:GUS* transgenes were introduced into wild-type Columbia plants.

Isolation of T-DNA Insertion Mutant Lines

T-DNA insertional mutants of *AFB1, AFB2*, and *AFB3* were isolated by screening the University of Wisconsin lines (*WS*). Gene specific primers 5AFB1, 5′-CGATTCCCACCTAAGGTGTTGGAACATAT-3′; 5AFB2, 5′-GGAATCTTGCTGGTGAAGTTAGAGATGAA-3′; and 5AFB3, 5′-CCAGACGAGGTTATAGAGCACGTGTTTGA-3′, together with the T-DNA-specific left border primer JL202, 5′-CATTTTATAATAA CGCTGCGGACATCTAC-3′ were used for mutant screening. After selecting the mutants, T-DNA insertion sites were confirmed by sequencing the PCR products using the JL202 primer.

Two independent T-DNA insertion lines each were identified in this screen for *AFB1*, *AFB2*, and *AFB3*. Of these, *afb1-1*, *afb2-1*, and *afb3-1* alleles were selected for further analysis. The genotypes of single, double, triple, and quadruple mutants were confirmed by PCR. Similarly, the *DR5rev*::*GFP* and *HS::AXR3NT-GUS* transgenes were introduced into *tir1 afb2 afb3* plants by crossing. The appropriate genotypes were identified in the F2 populations and confirmed by PCR-based genotyping and antibiotic resistance.

Localization of TIR1 and the AFB Proteins

The *TIR1* and *AFB* cDNAs were introduced into the *pENTR/D-TOPO* vector (Invitrogen). The *GFP-TIR1* and *GFP-AFB* fusions were obtained after LR recombination (Invitrogen) between the entry clones and pVR-GFPNt (kindly provided by X.W. Deng) (Rubio et al., 2005). The GFP fusion proteins were transiently expressed in agro-infiltrated leaves of *Nicotiana benthamiana* as previously described (Voinnet et al., 1998). Three days after infection, the infiltrated leaves were peeled and observed by epifluorescence microcscopy using a NIKON E800.

Northern and RT-PCR Analysis

To determine expression of the *Aux/IAA* genes, 6-day-old mutant and wild-type seedlings were transferred from vertical agar plates into liquid ATS medium, washed for 10 min, and transferred into fresh ATS medium with or without 20 μM 2,4-D. The seedlings were incubated with mild shaking for 60 min, washed with DEPC-treated water, and ground in liquid nitrogen. To analyze expression of the *AFB* genes at different stages of development, 100 mg of plant material was collected from *Col-0* seedlings grown on vertical plates or from mature tissues of soil-grown plant. Total RNA was extracted using Tri-Reagent (Sigma), and 10 μg total RNA was used in reverse transcription reaction using SuperScript II reverse transcriptase (Invitrogen) and oligo-dT primer. 1 μl of the reverse transcription reaction was amplified in 25 μl PCR reaction volume with specific primers.

Protein Expression, Immunoprecipitation, and Pull-Down Assays

tir1-1 35S::AFB1-myc and *tir1-1 35S::AFB3* seedlings were grown on ATS plates under continuous light at 22°C for 10–12 days. The *tir1 GVG::TIR1-myc* line is described elsewhere (Gray et al., 1999). Crude protein extracts were prepared in buffer containing 50 mM Tris-Cl (pH 7.2), 100 mM NaCl, 10% glycerol, 1 mM PMSF, 10 μM MG132, and complete mini protease inhibitors per manufacturer's

instructions (Roche Diagnostics GmbH). Cell debris was removed by centrifugation at 10,000 × g for 10 min. Total protein concentration was determined by the Bradford assay (BioRad).

For immunoprecipitation, plant extract containing 1 mg of protein was incubated with α-myc antibody (1:150 v/v) for 1 hr at 4°C on a rotory shaker. Then, 20 μl of Protein A agarose was added and incubated for another 3 hr at 4°C. Agarose beads were recovered after a brief spin, and the immunoprecipitate was washed 3 times with 1 ml washing buffer (extraction buffer without MG 132 or protease inhibitors). The immunoprecipitate was resuspended in 2× sample buffer and separated on 12.5% SDS-PAGE. The presence of CUL1 and ASK1 in the immunoprecipitate was determined by immunoblotting with α-CUL1 and α-ASK1 antibody.

For pull-down assays, GST-IAA7 or GST-BDL was expressed in *E. coli* and purified using glutathione beads according to the manufacturer's instructions. Pull-down assays were carried out using crude plant extracts as described elsewhere (Dharmasiri et al., 2003) in the presence of different concentrations of auxin. After washing the pull-down reactions with washing buffer three times, proteins were separated on SDS-PAGE. AFB-myc proteins were detected by immunoblotting with α-myc antibody and anti-mouse IgG as secondary antibody. Proteins were visualized using the ECL kit (Pierce).

To analyze BDL protein levels, floral buds and young flowers were collected from Columbia, *bdl*, *iaa12-1*, and *tir1-1 afb2-1 afb3-1* plants, homogenized in ice-cold buffer (50 mM Na-phosphate [pH 7.5], 150 mM NaCl, 0.5% Triton-X100 containing 50 mM MG132, 1 mM PMSF, and complete plant protease inhibitor cocktail [Sigma]), and centrifuged twice at 10,000 × g for 15 min at 4°C. Equal amounts of protein were loaded onto 12% PAA gels and blotted onto Immobilon P PVDF membranes (Millipore). Membranes were incubated with a 1:1500 dilution of crude rabbit serum raised against two synthetic peptides RGVSELEVGKSNLPA (aa 2–16) and CPRRQEQKDRQRNNPV (aa 225–239) of BDL (Eurogentec, Belgium). A horseradish peroxidase-coupled goat-anti-rabbit secondary antibody was used to detect signals using ECLplus reagent (Amersham).

Embryo Analysis

For the analysis of embryo phenotypes, siliques containing immature seeds were collected from Columbia, *bdl*, *tir1-1*, *afb2-1*, *afb3-1*, and *tir1-1 afb2-1 afb3-1* plants, slit along both sides of the septum, fixed, and mounted in chloral hydrate as described (Berleth and Jürgens, 1993).

GUS Staining and Analysis

For *promoter::GUS* studies, plant material was washed with 100 mM Na_2HPO_4 and stained as described previously (Yang et al., 2004) except that the duration of staining was 4 hr. For embryo staining, siliques of various developmental stages were collected, slit along both sides of the septum, fixed in 90% acetone at –20°C for 30 min, and stained for GUS activity as described (Weijers et al., 2001) for several hours. After staining, siliques were fixed and mounted in chloral hydrate as described (Berleth and Jürgens, 1993).

4-day-old seedlings from representative *BDL:GUS* (homozygous) and *bdl:GUS* (hemizygous) transgenic lines were transferred into either control liquid 1/2MS medium or the same medium containing 20 μM IAA or 50 μM MG132 and incubated for 1 hr at room temperature. Alternatively, seedlings were pretreated for 1 hr in control medium or medium containing 50 μM MG132. Then, IAA was added to a final concentration of 20 μM, and seedlings were incubated for another hour. After treatment, seedlings were stained for GUS activity during 3 hr as described (Weijers et al., 2001).

6-day-old *HS::AXR3NT-GUS* seedlings were heat shocked at 37°C for 120 min in liquid ATS medium. The seedlings were collected by filtration and transferred into new medium containing 5 μM 2,4-D, and samples of at least 12 seedlings were taken out at designated times to stain for GUS activity.

A selected BDL:GUS line was crossed with a homozygous *tir1-1 afb2-1 afb3-1* plant, and triple mutant seedlings carrying the transgene were selected in the F2 generation and stained for GUS activity along with wild-type siblings from the same F2 population.

Acknowledgments

We thank Jiri Friml for the *DR5rev::GFP* line and Ryan Dumas for technical assistance. This work was supported by grants from the DOE (De-FG02-02ER15312), the NIH (GM-43644), and NSF (DBI-0115870) to M.E., from the Deutsche Forschungsgemeinschaft (SFB 446/B8) to G.J., and from the European Molecular Biology Organization (ALTF 582-2001) to D.W.

Received: April 6, 2005
Revised: May 19, 2005
Accepted: May 20, 2005
Published online: June 2, 2005

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