Auxin regulates SCF^{TIR1}-dependent degradation of AUX/IAA proteins

William M. Gray*†, Stefan Kepinski†‡, Dean Rouse‡§, Ottoline Leyser‡ & Mark Estelle*

* *The Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas 78712, USA*
‡ *Department of Biology, University of York, Box 373, York YO10 5YW, UK*
† *These authors contributed equally to the work*

The plant hormone auxin is central in many aspects of plant development. Previous studies have implicated the ubiquitin-ligase SCF^{TIR1} and the AUX/IAA proteins in auxin response. Dominant mutations in several *AUX/IAA* genes confer pleiotropic auxin-related phenotypes, whereas recessive mutations affecting the function of SCF^{TIR1} decrease auxin response. Here we show that SCF^{TIR1} is required for AUX/IAA degradation. We demonstrate that SCF^{TIR1} interacts with AXR2/IAA7 and AXR3/IAA17, and that domain II of these proteins is necessary and sufficient for this interaction. Further, auxin stimulates binding of SCF^{TIR1} to the AUX/IAA proteins, and their degradation. Because domain II is conserved in nearly all AUX/IAA proteins in *Arabidopsis*, we propose that auxin promotes the degradation of this large family of transcriptional regulators, leading to diverse downstream effects.

Plant development requires the coordinated regulation of cell division, expansion and differentiation. The plant hormone indole-3-acetic acid (IAA or auxin) is fundamental in regulating many of these processes.

Genetic studies in *Arabidopsis* indicate that regulated protein degradation is required for auxin response. Recessive mutations in *AXR1* and *TIR1*, both components of the ubiquitin-mediated proteolytic pathway, result in reduced auxin response[1]. *TIR1* encodes an F-box protein that interacts with the cullin AtCUL1 and a SKP1-like protein (ASK1 or ASK2) to form an SCF ubiquitin protein ligase (E3). On the basis of these results, we proposed that SCF^{TIR1} targets one or more repressors of auxin response for degradation[2,3]. AXR1 encodes a subunit of the enzyme that activates the ubiquitin-like protein RUB1 for conjugation to target proteins[4]. One target for RUB1 conjugation is the AtCUL1 subunit of the SCF^{TIR1} complex, and evidence suggests that modification of cullins by RUB1 is important in regulating activity of SCF ubiquitin-ligases[5–8].

Studies of the AUX/IAA family of transcriptional regulators have also implicated protein degradation in auxin response. The *Arabidopsis thaliana* genome contains at least 24 *AUX/IAA* genes, many of which were identified because of their rapid induction after auxin treatment[9]. The AUX/IAA proteins have a relative molecular mass of 20,000–35,000 (M_r 20K–35K) and share four conserved domains, designated I–IV. Domains III and IV mediate homo- and heterodimerization between AUX/IAA proteins and heterodimerization with members of a second large protein family called the auxin-response factors (ARFs), most of which also contain domains III and IV[10,11]. The ARF proteins are transcription factors that bind to auxin-response elements (AuxRE) located upstream of auxin-inducible genes[11]. Overexpression of some *AUX/IAA* genes was found to repress transcription of an AuxRE-reporter in transient transfection assays[12]. Because the AUX/IAA proteins are not known to bind DNA, this negative regulation may occur through interaction with ARF transcription factors.

Dominant mutations conferring auxin-related phenotypes have been isolated in several AUX/IAA genes[13–16]. These mutations all occur within the highly conserved core of domain II of each protein. Domain II has recently been demonstrated to act as a transferable protein degradation signal when fused to luciferase[17]. Furthermore, mutations in domain II equivalent to the dominant mutant alleles of *AXR2/IAA7*, *AXR3/IAA17* and *SHY2/IAA3* restored stability to the luciferase fusion protein. Consistent with this finding, pulse-chase experiments reveal that the mutant axr3-1 protein has a half-life about sevenfold greater than its wild-type counterpart[18]. These results indicate that rapid turnover of AUX/IAA proteins is essential for normal auxin response and that the biochemical basis for these dominant mutations is increased protein stability.

Here we show that both treatment with a proteasome inhibitor and mutations affecting the SCF^{TIR1} complex increase stability of AUX/IAA proteins. Furthermore, we demonstrate that SCF^{TIR1} physically interacts with AUX/IAA proteins. This interaction is mediated by domain II of the AUX/IAA proteins and is abolished by mutations within this motif. Auxin treatment stimulates the interaction between SCF^{TIR1} and AUX/IAA proteins and promotes their degradation. These data indicate that auxin promotes SCF^{TIR1}-dependent degradation of AUX/IAA proteins. Rapid changes in the levels of individual members of this large family of proteins are likely to result in the diverse downstream effects associated with auxin response.

Analysis of AUX/IAA stability with GUS fusions

To examine AUX/IAA protein stability, we generated transgenic plants expressing an AXR2–GUS (beta-glucuronidase) fusion protein under control of the cauliflower mosaic virus *CaMV 35S* promoter. Despite the presence of the *AXR2–GUS* transgene, we detected no, or in a few lines very weak, GUS activity by histochemical staining. The dominant *axr2-1* mutation results in an amino-acid substitution within the domain II motif known to be important for instability[13,17]. When plants expressing an axr2-1–GUS protein were examined, abundant GUS staining was detected in the nuclei of many cells, and was especially strong in root tips (Fig. 1a). These plants exhibited several auxin-related growth phenotypes, suggesting that the GUS fusion proteins retained AXR2 function (see below).

We employed a similar approach to compare AXR3/IAA17 and axr3-1 protein levels. The amino-terminal domains I and II of AXR3 (AXR3NT) were fused to GUS and placed under the control of the soybean heat-shock promoter (*HS*)[19]. The resulting AXR3NT–GUS protein is non-functional but retains the bipartite nuclear localization signal spanning domains I and II. Wild-type plants expressing the *HS::AXR3NT–GUS* constructs were heat shocked at 37 °C for 2 h and stained for GUS activity 60 min after the end of the heat-shock period. Like the AXR2–GUS proteins, significantly more staining was detected with the HS::axr3-1NT–GUS construct than

§ Present address: CSIRO Plant Industry, GPO Box 1600, Australian Capital Territory 2601, Australia.

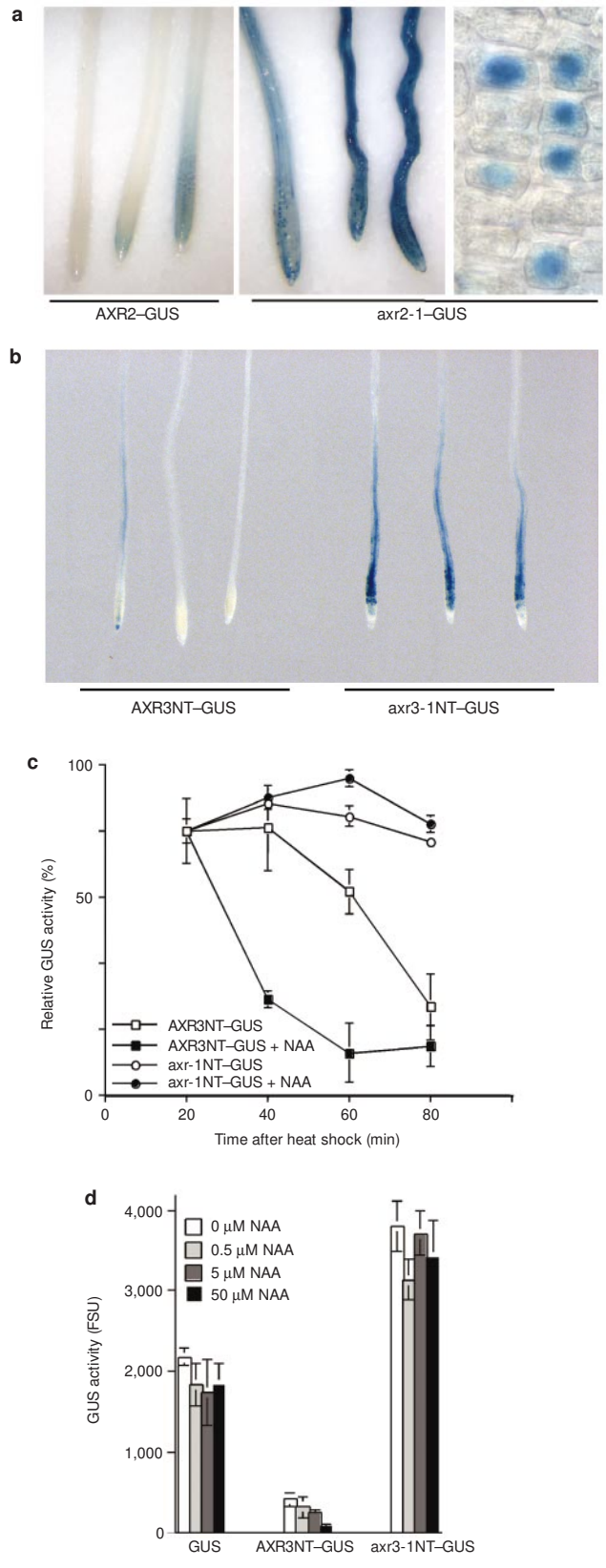

Figure 1 Analysis of AUX/IAA–GUS fusion constructs. **a**, Seven-day-old seedlings stained for GUS activity. Right, nuclear localization of axr2-1–GUS in root meristem cells. **b**, Seedlings were heat shocked for 2 h and stained for GUS activity 60 min after the end of the heat induction. **c**, Auxin destabilization of AXR3NT–GUS. Relative activity is expressed as percentage of the 20-min level. Error bars, s.e.m.; $n = 6$. **d**, HS::GUS, HS::AXR3NT–GUS, and HS::axr3-1NT–GUS seedlings were treated with NAA 20 min after the end of the heat-shock period. GUS activity was measured fluorometrically 50 min after NAA addition Error bars, s.e.m.; $n = 6$.

the wild-type derivative (Fig. 1b). For both wild-type and mutant proteins, staining was primarily nuclear (data not shown). These results support previous findings suggesting that the biochemical basis for the phenotypes conferred by dominant AUX/IAA mutations is increased stability of the mutant protein[17,18].

We examined the possibility that auxin regulates AUX/IAA degradation using *HS::AXR3NT–GUS* transgenic plants. Seedlings were treated with the synthetic auxin NAA (1-naphthalene acetic acid) 20 min after the end of the heat-shock period and assayed for GUS activity at succeeding 20-min intervals. Auxin treatment promoted degradation of AXR3NT–GUS, but had no effect on axr3-1NT–GUS levels (Fig 1c).

The effect of auxin on AXR3 stability was also measured in a dose-response assay. Activity of HS::AXR3NT–GUS progressively decreased as auxin concentration increased over a range of 0–50 μM. In contrast, GUS activities of the HS::axr3-1NT–GUS and the control HS::GUS reporters were unaffected by auxin treatment over the time course of this experiment (Fig. 1d). These data indicate that auxin rapidly destabilizes the AXR3 protein and that the *axr3-1* mutation prevents this auxin-mediated degradation.

Ubiquitin-mediated degradation of AUX/IAA proteins

Because the *AXR1* and *TIR1* genes encode proteins involved in ubiquitin-mediated degradation, we tested the possibility that the ubiquitin-proteasome pathway is involved in AUX/IAA degradation. Seedlings expressing either the AXR2–GUS or AXR3NT–GUS proteins were treated with the proteasome inhibitor MG132. Both fusion proteins were stabilized by MG132 (Fig. 2). Next, we examined whether MG132 could prevent the auxin-induced degradation of AXR3NT–GUS. Heat-shocked *HS::AXR3NT–GUS* seedlings were treated with MG132 for 60 min, followed by a 60-min treatment with 5 μM auxin. Histochemical staining revealed that preincubation with proteasome inhibitor largely blocked the auxin-mediated degradation of AXR3NT–GUS (Fig. 2b). In contrast,

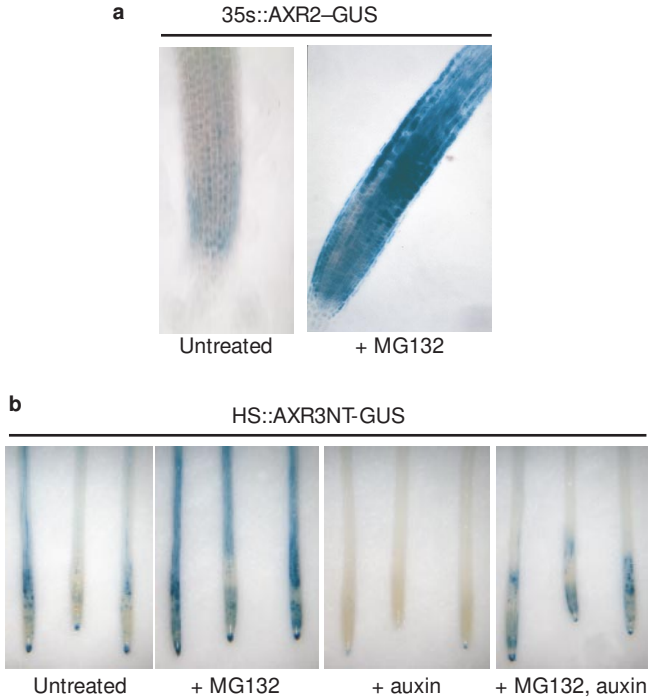

Figure 2 The proteasome inhibitor MG132 increases AUX/IAA protein stability. **a**, Seven-day-old seedlings were treated with 10 μM MG132 for 2 h and stained for GUS activity. **b**, Nine-day-old seedlings were heat shocked for 2 h. Where indicated, seedlings were treated with 10 μM MG132 after 1 h, and 5 μM 2,4-D was added at the end of the heat-shock period. Sixty minutes later, seedlings were stained overnight to detect GUS activity.

MG132 treatment had no observable effect on axr3-1NT–GUS levels over the course of the experiment (data not shown).

The effects of *axr1* and *tir1* mutations on AUX/IAA stability were investigated using the 35S::AXR2–GUS and HS::AXR3NT–GUS reporters. *tir1-1* mutants had higher AXR2–GUS levels compared with the wild type (Fig. 3a). Although the effect of the *tir1-1* mutation on AXR2–GUS levels was relatively modest, the *35S::AXR2–GUS* construct conferred auxin-related defects such as leaf curling and reduced apical dominance in *tir1-1* plants. The same construct had no effect on morphology in the wild-type background. The *tir1-1*[35S::AXR2–GUS] phenotype was similar, although less severe than the phenotype of wild-type plants expressing the stabilized axr2-1–GUS construct, suggesting that the *tir1-1* mutation results in increased AXR2 stability (Fig. 3b).

Expression of the 35S::AXR2–GUS protein in *axr1-3* plants resulted in dramatic changes in development. Most *axr1-3*[35S::AXR2–GUS] transformants developed a single cotyledon and lacked a root meristem (Fig. 3b). Less severe transformants had fused cotyledons and a rudimentary root. These seedlings displayed greater AXR2–GUS staining compared with wild-type controls (Fig. 3a). All of the recovered *axr1-3*[35S-AXR2–GUS] transformants (>100) arrested and died before or shortly after generating the first pair of true leaves.

HS::AXR3NT–GUS levels were also elevated in *tir1* and *axr1* mutants (Fig. 3c). To examine more precisely the effects of the *axr1-12* and *tir1-1* mutations, AXR3NT–GUS levels were measured at 20-min intervals after the end of the heat-shock period. Whereas AXR3NT–GUS levels decreased rapidly in wild-type seedlings, GUS activity remained high in both *axr1-12* and *tir1-1* seedlings (Fig. 3d). AXR3NT–GUS levels were significantly higher in *axr1-12* than in *tir1-1* seedlings. This is consistent with the more severe auxin response defect exhibited by *axr1-12* plants compared with *tir1-1* plants (Fig. 3c and data not shown).

To confirm that the GUS fusion proteins accurately reflected protein stability, polyclonal antiserum was raised against AXR2 and used to examine protein levels. Although the antiserum detected recombinant AXR2 from *Escherichia coli* extracts, we were unable to detect AXR2 clearly in plant extracts on protein blots. As an alternative approach, [^{35}S]-methionine/cysteine was used to metabolically label seedling proteins. The AXR2 antiserum immunoprecipitated a 29K protein that co-migrated with the recombinant AXR2 protein (Fig. 3e). When immunoprecipitations were performed using extracts prepared from plants expressing an axr2-1–GFP (green fluorescent protein) fusion protein, an additional 59K species immunoprecipitated, suggesting that the antiserum does indeed recognize the AXR2 protein (Fig. 3e). Substantially more AXR2 protein was detected in *axr2-1* plants than in wild-type plants (Fig. 3e). Also consistent with the reporter analysis, more AXR2 protein was immunoprecipitated from *axr1-12* seedlings than in the wild type. In pulse-chase experiments (Fig. 3f), AXR2 exhibited a half-life of 10.8 ± 1.1 min in wild type compared with 28 ± 3.9 min in *axr1-12*. These findings validate the results obtained with the

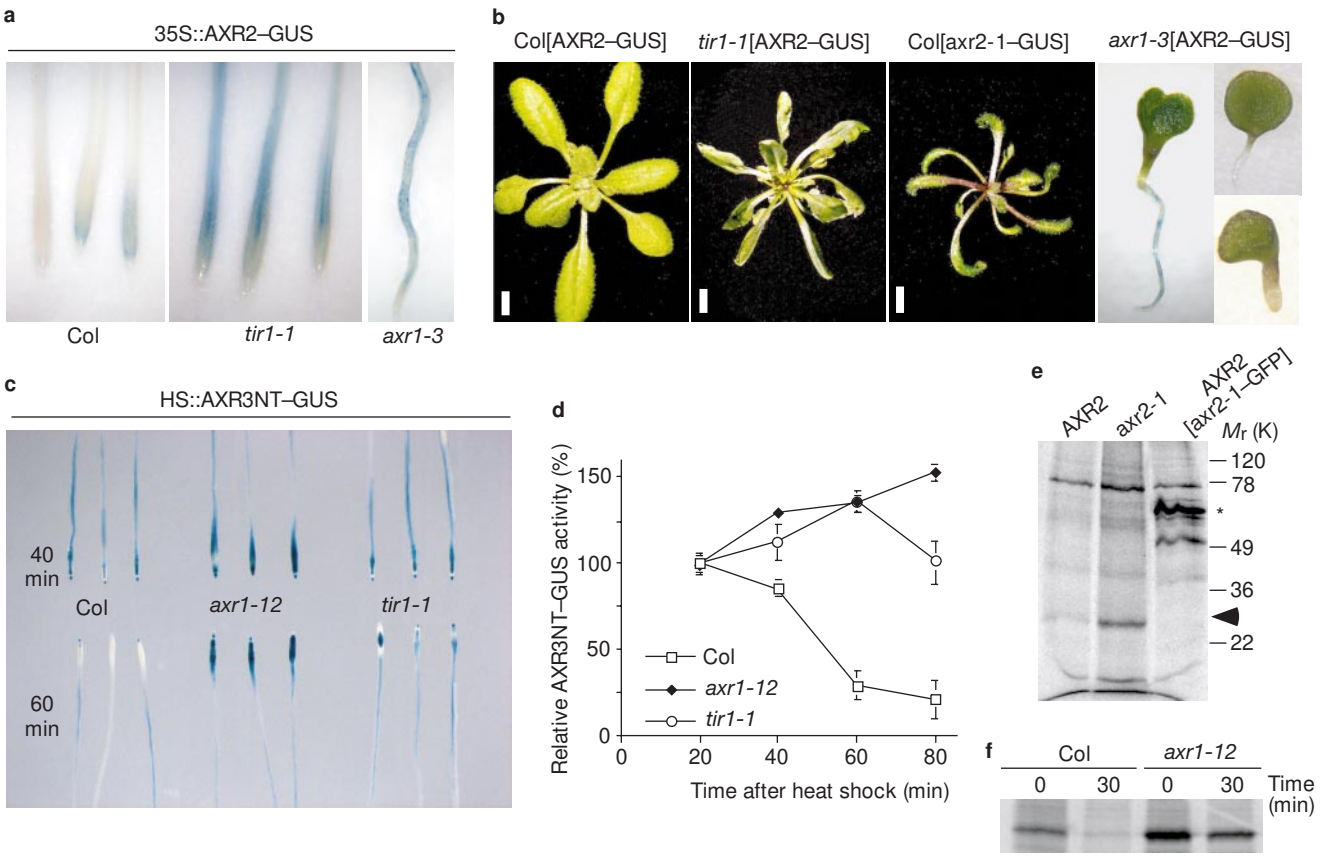

Figure 3 AUX/IAA proteins exhibit increased stability in *axr1* and *tir1* mutants. **a**, GUS histochemical staining of seedlings containing the *35S::AXR2–GUS* reporter. **b**, Twenty-five-day-old plants. *tir1-1* plants are indistinguishable from the *Col*[AXR2–GUS] plant shown. Scale bars, 5 mm. Right, 6-day-old primary transformants. The seedling on the left was stained for GUS activity. **c**, Seedlings were stained for GUS activity 40 (top) or 60 (bottom) min after the end of the heat-shock induction. **d**, AXR3NT–GUS activity at 20-min intervals after heat shock. Relative activity is expressed as percentage of the 20-min level. Error bars, s.e.m.; $n = 6$. **e**, Proteins were extracted from [^{35}S]-methionine/cysteine-labelled 7-day-old seedlings and AXR2 protein immunoprecipitated with the anti-AXR2 antibody. The arrowhead and asterisk indicate the positions of the AXR2 and axr2-1–GFP proteins, respectively. **f**, AXR2 was immunoprecipitated from metabolically labelled seedlings immediately after the labelling period (0 min) or 30 min after chasing with 1 mM methionine/cysteine.

GUS reporters and suggest that AXR2 and AXR3 are targeted for ubiquitin-mediated proteolysis by the SCF^{TIR1} ubiquitin-ligase.

AUX/IAA proteins interact with SCF^{TIR1}

Our results suggest that AUX/IAA protein turnover is dependent on SCF^{TIR1}. To examine whether SCF^{TIR1} physically interacts with AUX/IAA proteins, the AXR2 antibody was used in immunoprecipitation experiments with extracts prepared from seedlings expressing the c-*myc* epitope-tagged TIR1 derivative. TIR1–Myc was readily detected in anti-AXR2 immunoprecipitates but was absent from control precipitations using the AXR2 pre-immune serum (Fig. 4a).

We explored the interaction between SCF^{TIR1} and the AUX/IAA proteins further using *in vitro* pull-down assays. Recombinant glutathione *S*-transferase (GST)–AXR2 was synthesized in *Escherichia coli* and purified with the GST tag. Purified protein was incubated with crude lysate prepared from *Arabidopsis* seedlings, repurified, and immunoblotted with c-*myc*, AtCUL1 and ASK2 antibodies. TIR1–Myc and AtCUL1 both co-purified with the GST–AXR2 fusion protein but were absent in control pull-down assays using GST alone (Fig. 4b, outer lanes). The Skp1-like proteins ASK1 and ASK2 were also present in GST–AXR2 pull-down assays. Because the ASK2 antibody cross-reacts with the co-migrating GST protein, we could not confirm that ASK1 and ASK2 were missing from the GST control.

We examined the effect of the *axr2-1* mutation on interaction with SCF^{TIR1} using a GST–axr2-1 mutant derivative. SCF^{TIR1} did not co-purify with the mutant protein, indicating that the single-base-pair *axr2-1* mutation prevents the protein from interacting with the SCF complex (Fig. 4b, centre lane).

To determine whether SCF^{TIR1} interacts with additional AUX/IAA proteins, we tested GST–AXR3 in pull-down assays. Similar to the results obtained with AXR2, GST–AXR3 co-purified with TIR1 protein and the *axr3-1* mutation substantially disrupted this interaction (Fig. 4c).

Because the *axr2-1* and *axr3-1* mutations disrupt interaction with SCF^{TIR1}, we tested whether domain II functions as a TIR1 interaction domain. A truncated derivative of the GST–AXR2 fusion protein containing only domains I and II was capable of interacting with TIR1 in a pull-down assay. Similarly, TIR1 was able to interact, albeit at a reduced level, with a GST–AXR2 fusion protein containing only domain II ($AXR2^{71-100}$). In contrast, when a short deletion was introduced into the highly conserved core of domain II, this mutant derivative of AXR2 ($AXR2^{\Delta 86-88}$) interacted very weakly with TIR1 (Fig. 4d). These data demonstrate that domain II is both necessary and sufficient to bind SCF^{TIR1}.

Because auxin promoted degradation of the AXR3–GUS fusion protein (Fig. 1), we examined the possibility that auxin regulates the interaction between AUX/IAA proteins and SCF^{TIR1}. We performed GST pull-down assays with AXR2 and AXR3 fusion proteins using crude *Arabidopsis* extracts prepared from seedlings treated with the synthetic auxin 2,4-D (2,4-dichlorophenoxy acetic acid) before protein extraction. Pull-down assays with extracts prepared from auxin-treated plants yielded more TIR1–Myc protein than control assays using untreated extracts. This increase was apparent after treatments as short as 5 min, increased until at least 60 min, and declined by 240 min (Fig. 5a; top, middle). Western blot analysis confirmed that this increase was not due to an increase in TIR1–Myc abundance in the extracts prepared from auxin-treated plants (Fig. 5a; bottom).

Applied auxin also promoted the SCF^{TIR1}–AUX/IAA interaction in a dose-dependent manner (Fig. 5b). Auxin treatment enhanced the SCF^{TIR1}–AXR2/AXR3 interaction at concentrations as low as 0.5 μM. This dose-response relationship correlates well with the effects of increasing concentrations of auxin on AXR3NT–GUS stability.

Discussion

Previous genetic and biochemical studies implicated SCF^{TIR1} as a positive regulator of auxin response in *Arabidopsis*[2]. We have proposed that SCF^{TIR1} promotes auxin response by targeting one or more negative regulators of the pathway for ubiquitin-mediated

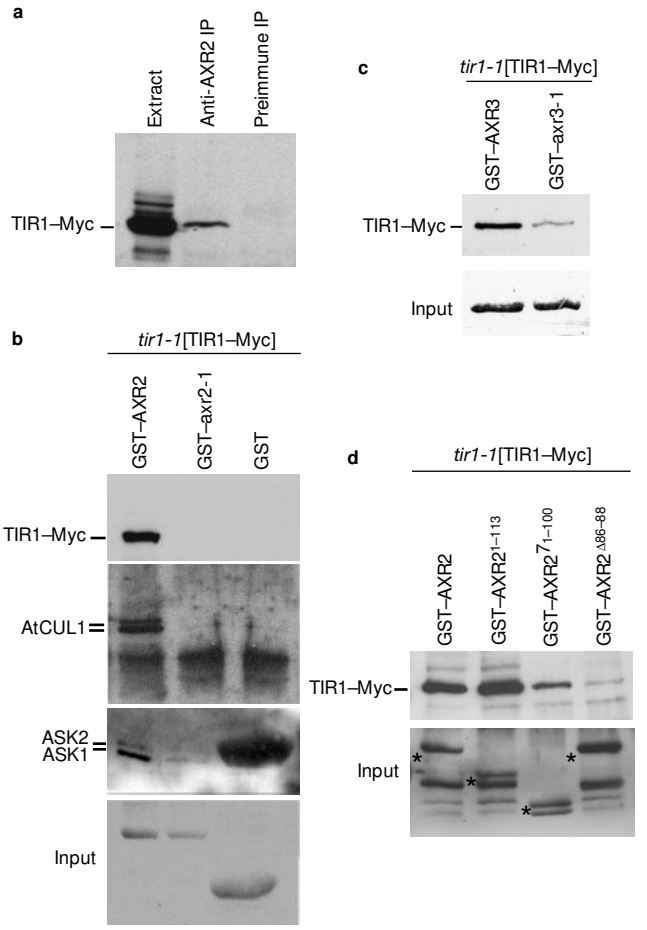

Figure 4 SCF^{TIR1} interacts with AUX/IAA proteins. **a**, Immunoprecipitates (IP) were blotted and probed with the anti-c-*myc* antibody. **b**, Recombinant GST–AXR2, GST–axr2-1, and GST proteins were used in pull-down assays with extracts prepared from *tir1-1*[TIR1–Myc] seedlings. Pull-down assays were immunoblotted with the indicated antibodies. The anti-ASK2 antibody detects both the ASK1 and ASK2 proteins. **c**, GST–AXR3 and GST–axr3-1 pull-down assays were probed with anti-c-*myc* antibody. **d**, Pull-down assays with full-length GST–AXR2 and deletion derivatives were immunoblotted and probed with anti-c-*myc* antibody. Input GST–AXR2 protein is visualized in the lower panel. The position of full-length protein is indicated with an asterisk in each lane.

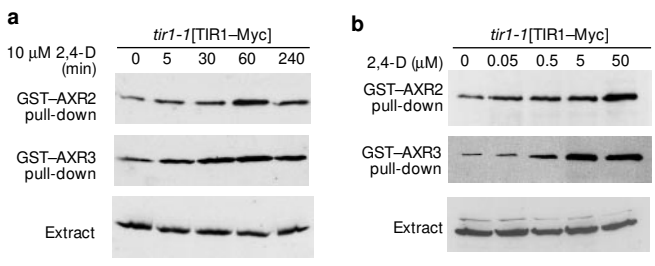

Figure 5 Auxin promotes the SCF^{TIR1}–AUX/IAA interaction. **a**, Pull-down assays were performed with extracts prepared from 6-day-old seedlings treated with 10 μM 2,4-D as indicated, and TIR1–Myc was detected by immunoblotting. Anti-c-*myc* western blots of the extracts confirmed that auxin treatment does not affect TIR1–Myc abundance (bottom). **b**, As in **a** except seedlings were treated with increasing concentrations of 2,4-D for 60 min.

degradation[1]. In this report we demonstrate that the AUX/IAA proteins are targeted for degradation by SCF^{TIR1} in response to auxin. Stabilization of the AUX/IAA proteins, either by recessive mutations that affect the SCF or by dominant mutations in the *AUX/IAA* genes, causes dramatic defects in auxin response and morphology. These results provide a mechanistic link between the genetically defined *AXR1–TIR1* pathway and two families of transcriptional regulators, the AUX/IAA and ARF proteins.

We suggest that AUX/IAA proteins are substrates of SCF^{TIR1} and the domain II of AUX/IAA proteins functions as a signal that targets these proteins for degradation[17,18]. Our reporter and immunological findings support this hypothesis because the *axr2-1* and *axr3-1* mutations resulted in increased protein stability. In addition, mutations in *TIR1* or *AXR1*, or treatment with the proteasome inhibitor MG132, caused increased AXR2 and AXR3 stability suggesting that SCF^{TIR1} ubiquitinates these proteins, marking them for degradation by the 26S proteasome.

Our data suggest that domain II destabilizes AXR2 and AXR3 by targeting them to SCF^{TIR1}. Auxin causes reduced protein stability by promoting this interaction whereas the dominant *AUX/IAA* mutations confer increased protein stability by preventing the interaction between AUX/IAA proteins and the SCF. Although we have demonstrated SCF^{TIR1}–AUX/IAA interaction in crude extracts, we were unable to detect an interaction between recombinant AXR2 or AXR3 and immunopurified SCF^{TIR1}. This indicates that the plant extract provides a factor that facilitates the interaction. It is possible that SCF^{TIR1}–AUX/IAA binding is regulated by phosphorylation, as several yeast and mammalian SCF substrates must be phosphorylated to interact with their cognate SCFs[20]. Indeed, a MAP kinase activity was recently identified that is rapidly and transiently induced by auxin[21]. However, domain II, shown here to be necessary and sufficient for SCF^{TIR1} recognition, does not contain any conserved sites of phosphorylation[22]. It is possible that an additional protein, serving as a bridge between the SCF and substrate, is phosphorylated in response to auxin. Alternatively, domain II may be subject to a different type of post-translational modification. The identification of the modification and/or cofactor that is required for SCF^{TIR1} binding will provide important insight into the upstream events in the auxin-response pathway.

Our understanding of AUX/IAA protein function is based largely on genetic studies. The phenotypes of the gain-of-function *axr2*, *axr3*, *shy2* and *iaa28* mutations illustrate the consequences of failure to degrade individual members of the family. In general, accumulation of each protein results in decreased auxin response. For example, the *axr2-1* mutant is deficient in auxin induction of all members of the *AUX/IAA* gene family, indicating that stabilized AXR2 represses transcription of these genes[9]. In addition, transfection experiments demonstrate that some AUX/IAA proteins repress auxin-dependent gene expression[12]. However, it is important to note that some aspects of the *axr3-1* phenotype are more consistent with auxin hypersensitivity, suggesting that individual members of the family may have positive effects on auxin response.

Although the mechanism by which AUX/IAA proteins affect auxin response is unknown, one simple possibility is that they prevent the formation of ARF protein dimers. The ARF proteins seem to bind palindromic auxin response elements and activate transcription more efficiently as dimers[23,24]. Because AUX/IAA proteins can heterodimerize with ARFs, they may act by preventing formation of active ARF dimers[11]. In the case of ARFs that activate transcription, this will result in repression of transcription. For those ARFs that seem to act as repressors (for example, ARF1), interaction with an AUX/IAA protein could have a positive effect on transcription of target genes. This view is strongly supported by the effects of overexpression of the AXR2–GUS fusion protein in *axr1-3* plants. *axr1-3*[AXR2–GUS] transformants developed with single or fused cotyledons strikingly similar to loss-of-function mutants of the ARF transcription factor MP (ref. 25). Thus it is likely that increased AXR2–GUS levels in *axr1-3* plants repress the ability of MP to regulate auxin-responsive genes.

The *tir1* and *axr1* mutations probably have a global effect on AUX/IAA stability. Domain II is conserved in 24 of the 29 members of the family, so it is likely that most of these proteins are degraded in an auxin-dependent manner. In this context, it is important to note that the *Arabidopsis* genome encodes several proteins with high homology to TIR1 as well as one AXR1-like protein. These related gene products are likely to be at least partially redundant with TIR1 and AXR1. This would explain the relatively modest effect of the *tir1-1* mutation on AUX/IAA stability and auxin response in general.

On the basis of the results presented in this study, we propose the following model for auxin response (Fig. 6). Basal levels of AUX/IAA proteins repress the auxin-response pathway. Auxin derepresses the pathway by promoting AUX/IAA binding to SCF^{TIR1} and related SCF complexes, leading to their degradation. SCF^{TIR1} function requires AXR1-dependent RUB1 modification of the AtCUL1 subunit of the SCF. AUX/IAA proteolysis results in a transient derepression of the pathway until new AUX/IAA proteins can be synthesized and restore repression. According to this model, auxin-induced expression of the *AUX/IAA* genes is a negative-feedback loop that ensures tight regulation of the response similar to the rapid NF-κB activation of its inhibitor, IκB[26]. Auxin-induced destabilization of the AUX/IAA proteins would permit the formation of ARF–ARF dimers and hence a higher level of transcription of auxin-regulated genes. Auxin is known to elicit a diverse array of responses during the plant's life cycle. The key to this complexity may lie in the differences in expression of AUX/IAA family members, as well as differences in degradation kinetics. Indeed, the limited data available suggest striking differences in the instability of AUX/IAA proteins[18,27]. Consistent with this possibility, we find that AXR2 interacts with SCF^{TIR1} much more efficiently than AXR3 (W.M.G. and M.E., unpublished data), which may account for the shorter half-life of the AXR2 protein (Fig. 3f)[18]. Extrapolated to the

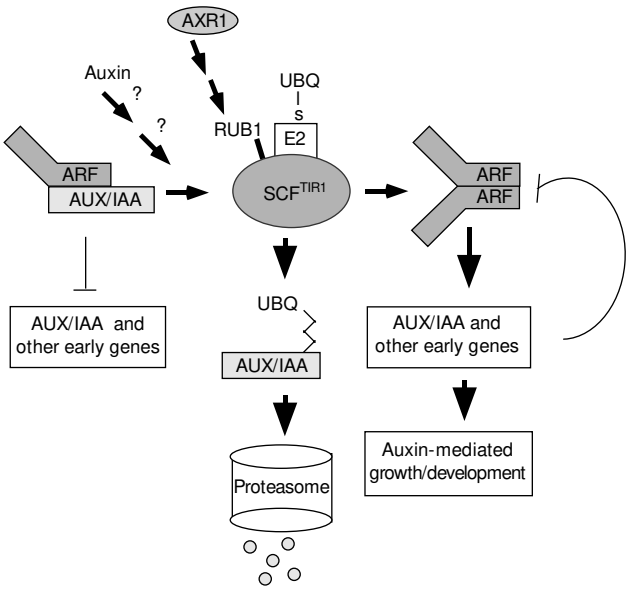

Figure 6 Model for auxin response. AUX/IAA proteins repress the auxin-response pathway by negatively regulating ARF transcription factors. Auxin promotes the ubiquitination of AUX/IAA proteins by targeting them to the SCF^{TIR1} ubiquitin-ligase. The subsequent degradation of AUX/IAA proteins results in activation of ARF and derepression of the auxin-response pathway. Because AUX/IAA genes themselves are rapidly induced by auxin, a negative-feedback loop exists with the newly synthesized AUX/IAA proteins restoring repression upon the pathway. Although the mechanism of AUX/IAA action is unclear, one possible mechanism is by preventing the formation of ARF–ARF dimers.

entire family, this would lead to considerable temporal variation in the relative abundance of individual AUX/IAA proteins in response to an auxin pulse. Such dynamics may account for the diversity of auxin responses observed in the plant. □

Methods

Plant material

All lines employed in this study were in the Columbia ecotype. Seedlings were grown under sterile conditions on vertically oriented ATS plates[28]. Seedlings used for protein extractions were grown for 5–7 d in liquid ATS media.

Reporter constructs

We fused the 400-base pair (bp) fragment of the soybean heat-shock promoter HS6871 (ref. 19) N-terminally to *GUS* (*HS::GUS*), domains I and II of *AXR3* and *GUS* (*HS::AXR3NT–GUS*), and domains I and II of *axr3-1* and *GUS* (*HS::axr3-1NT–GUS*) using the vector pB101.3 (Clontech). The AXR2 coding sequence was cloned into the *Bam*HI site of pBI121 (Clontech).

Heat induction and GUS assays

Seedlings were submerged in liquid ATS and heat shocked for 2 h at 37 °C. Plants were sampled at 20, 40, 60 and 80 min thereafter and stored in liquid nitrogen until protein extraction, or in the case of histochemical reactions, assayed immediately. Auxin treatments were performed by adding NAA 20 min after the end of the heat-shock period. GUS activity was measured as previously described[29]. Fluorometric assays were performed by incubating sample extracts in 2 mM MUG (4-methylumbelliferyl-β-D-glucoronide), 50 mM KPO_4 (pH 7.0), 0.1% Sarkosyl (BDH), 0.1% Triton X-100, 10 mM β-mercaptoethanol and 10 mM EDTA for 16 h followed by analysis with a Dynex MFX microtitre plate fluorometer. Extracts were prepared from ten seedlings and data were normalized against total protein levels.

Antibodies

The AXR2 coding sequence was cloned in-frame into the *Bam*HI site of the GST fusion vector pGEX-2T and introduced into *E. coli* strain MC1061. Stationary phase cells were diluted tenfold and grown for 1 h at 30 °C before induction with 0.1 mM IPTG. Cells were collected after 4 h of growth, resuspended in PBS buffer with 0.5% Triton X-100, and lysed by sonication. The GST–AXR2 fusion protein was purified and subjected to SDS–PAGE, excised from the gel and injected into a rabbit to generate anti-AXR2 antisera (Cocalico Biologicals). Crude antiserum was affinity purified against nitrocellulose-bound GST–AXR2 fusion protein[30]. Anti-c-*myc* monoclonal antibody was purchased from BabCo. The anti-ASK2 and anti-AtCUL1 polyclonal antibodies have been previously described[2].

Immunoprecipitations and pull-down assays

Immunoprecipitations were performed as previously described[2]. For GST–AXR2 and GST–AXR3 pull-down assays, 4 μg of purified fusion protein was added to 2.5 mg of crude *Arabidopsis* extract prepared from 7-day-old seedlings. Extracts were prepared by homogenizing seedlings in Buffer C (ref. 2) supplemented with 1 mM dithiothreitol, 10 μM MG132, 10 mM β-glycerolphosphate, 1 mM NaF and 1 mM orthovanadate. The resulting homogenate was cleared by microcentrifugation for 15 min. Where indicated, seedlings were treated with 2,4-D before extraction. Following addition of the glutathione–agarose-bound GST fusion protein, extracts were incubated at 4 °C with gentle agitation for 3 h. Glutathione beads were collected by brief centrifugation, washed three times in the above buffer, resuspended in SDS–PAGE sample buffer and subjected to SDS–PAGE electrophoresis and immunoblotting.

Metabolic labelling

Seven-day-old seedlings were transferred to 4 ml of ATS medium containing 200 mCi ^{35}S-Trans label (ICN) and grown for 3.5 h. Labelled seedlings were washed and proteins extracted immediately or after a 30-min chase in medium containing 1 mM methionine/cysteine and 100 μg ml^{-1} cycloheximide. AXR2 was immunoprecipitated with affinity-purified anti-AXR2 antibody as described above. AXR2 half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693t/\ln(N_0/N_x)$, where t is time in minutes. N_0 and N_x equal the amounts of AXR2 at $t = 0$ and $t = 30$ min, respectively. Values presented are the mean of three independent experiments (± s.d.)

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Correspondence and requests for materials should be addressed to M.E. (e-mail: mestelle@mail.utexas.edu).