ARTICLES

The F-box protein TIR1 is an auxin receptor

Nihal Dharmasiri[1], Sunethra Dharmasiri[1] & Mark Estelle[1]

The plant hormone auxin regulates diverse aspects of plant growth and development. Recent studies indicate that auxin acts by promoting the degradation of the Aux/IAA transcriptional repressors through the action of the ubiquitin protein ligase SCF^{TIR1}. The nature of the signalling cascade that leads to this effect is not known. However, recent studies indicate that the auxin receptor and other signalling components involved in this response are soluble factors. Using an *in vitro* pull-down assay, we demonstrate that the interaction between transport inhibitor response 1 (TIR1) and Aux/IAA proteins does not require stable modification of either protein. Instead auxin promotes the Aux/IAA–SCF^{TIR1} interaction by binding directly to SCF^{TIR1}. We further show that the loss of TIR1 and three related F-box proteins eliminates saturable auxin binding in plant extracts. Finally, TIR1 synthesized in insect cells binds Aux/IAA proteins in an auxin-dependent manner. Together, these results indicate that TIR1 is an auxin receptor that mediates Aux/IAA degradation and auxin-regulated transcription.

Since its discovery over 70 years ago, the plant hormone auxin or indole acetic acid (IAA) has been implicated in virtually every aspect of plant growth and development[1,2]. In some tissues auxin regulates cell elongation, while in others the hormone promotes cell division. Recent studies also indicate that auxin acts as a morphogen during embryogenesis and in the root meristem[3,4]. Despite the importance of auxin to the plant, many aspects of auxin signalling are poorly understood. In particular, the identity of the auxin receptor(s) is unknown. The best-characterized candidate receptor, the auxin binding protein 1 (ABP1), was identified by virtue of its auxin binding activity[5]. Although some characteristics of ABP1 are consistent with receptor function, the role of this protein in auxin signalling has not been determined.

In contrast, some aspects of auxin-regulated transcription are better understood. Two families of transcription factors, the auxin response factor (ARF) and Aux/IAA proteins, have been implicated in this process. The ARF proteins bind DNA directly and either activate or repress transcription depending on the ARF[6]. The Aux/IAA proteins exert their effects by binding to the ARF proteins through a conserved dimerization domain[7,8]. At least in the case of the activating ARFs, the effect of Aux/IAA binding is to repress transcription.

Auxin regulates transcription by stimulating the degradation of the Aux/IAA proteins[9–12]. Recent studies indicate that auxin acts by promoting an interaction between the Aux/IAA proteins and the ubiquitin protein ligase SCF^{TIR1}(ref. 10). In *Arabidopsis*, the Aux/IAA proteins are encoded by a family of genes comprised of 29 members[8]. Most of these proteins share four conserved regions designated domains I to IV. Domain II, including the conserved amino-acid residues GWPPV, has been implicated in the degradation of a luciferase reporter protein[13] and interacts with SCF^{TIR1}, suggesting that domain II is the auxin degron[14,15]. Mutations within domain II result in stabilization of the affected Aux/IAA protein and defects in auxin response[10–12,16].

How auxin promotes the interaction between Aux/IAA and SCF^{TIR1} is not known. Substrate recognition by many other cullin-based E3 ligases requires substrate modification, typically phosphorylation, although proline hydroxylation and glycosylation have also been reported[17–20]. In contrast, new evidence suggests that none of these mechanisms are likely to be involved in auxin-induced Aux/IAA degradation[14,15]. Similarly, the recent suggestion that a parvulin-type prolyl isomerase may be involved in the interaction[14] now appears unlikely with the discovery that the parvulin inhibitor juglone has a nonspecific effect on the Aux/IAA–SCF^{TIR1} interaction[15]. Instead, it has been suggested that substrate recognition requires an auxin-dependent modification of TIR1 or an associated protein, rather than the substrate[15]. To investigate this question, we adopted a biochemical approach based on the *in vitro* interaction assay we have previously described[14]. Here we report compelling evidence that auxin regulates degradation of the Aux/IAA proteins by binding directly to TIR1.

The TIR1–Aux/IAA interaction does not require a stable modification of either protein

We first asked whether the Aux/IAA–SCF^{TIR1} protein interaction is affected by temperature, reasoning that if stable modification of the TIR1 protein involves an enzyme, the reaction should be temperature-dependent. However, when pull-down assays were carried out at temperatures ranging from 4 to 25 °C, no differences in the recovery of TIR1–Myc were observed, suggesting that an enzyme-mediated modification is not involved (Fig. 1a). At 37 °C, the amount of TIR1–Myc in the pull-down decreased, possibly because the TIR1–Aux/IAA interaction is less stable at this temperature.

In a previous study, we showed that auxin promotes the interaction between TIR1 and the Aux/IAA protein within 5 min of addition to the pull-down reaction and that the response is saturated after 30 min (ref. 21). The results in Fig. 1b confirm that at both 0.5 and 50 μM 2,4-dichlorophenoxy acetic acid (2,4-D), the interaction is saturated after 20 min at 4 °C. To further explore the effects of temperature we performed pull-down experiments using 0.5 and 50 μM 2,4-D at 4 °C and 25 °C with an incubation time of 25 min. The results in Fig. 1c demonstrate that the kinetics of the response is similar at the two temperatures. Taken together, these results show

[1]Department of Biology, Indiana University, Bloomington, Indiana 47405, USA.

that the effect of auxin is largely temperature-independent, arguing against the involvement of an enzyme-based modification of TIR1 or any other protein.

The auxin receptor co-purifies with TIR1

The pull-down assay that we use to examine the interaction between TIR1 and the Aux/IAA proteins involves extensive washes in buffer lacking auxin. Because our results suggest that a stable protein modification is not required for Aux/IAA binding, we wondered if the interaction might depend on the continuous presence of auxin. As shown in Fig. 1d, the addition of auxin into the washing buffer greatly enhanced the recovery of TIR1–Myc from the pull-down reaction, suggesting that auxin acts to stabilize the interaction between TIR1 and the Aux/IAA protein and that the hormone is required continuously for this effect.

Next we investigated whether partially purified TIR1–Myc is capable of interacting with IAA7 protein in an auxin-dependent manner. TIR1–Myc was immunopreciptated from plant extracts in the presence or absence of 50 μM 2,4-D using anti-Myc antibody linked to sepharose beads (Covance). After the beads were washed with several bed volumes of buffer, TIR1–Myc was eluted with 50 mM Tris-Cl (pH 7.2), 300 mM NaCl, 0.5% Tween and 10% dioxane. Eluted TIR1–Myc was then used in pull-down assays with GST–IAA7. The results in Fig. 2a show that immunoprecipitated TIR1–Myc interacts with GST–IAA7 in an auxin-dependent manner similar to TIR1–Myc in crude plant extracts. Auxin treatment of the plant extract before immunoprecipitation did not affect the subsequent interaction, providing additional support for the hypothesis that auxin does not stably modify TIR1 or a protein associated with TIR1 (Fig. 2b). These results indicate that all the factors necessary for auxin-induced interaction, including the receptor, are present in the anti-Myc immunoprecipitate.

Because the Aux/IAA–TIR1 interaction depends on the continuous presence of auxin (Fig. 1d), it should be possible to purify TIR1–Myc by auxin-dependent binding to GST–IAA7. To investigate this possibility, pull-down assays were carried out with GST–IAA7 in the presence of auxin in both the pull-down buffer and the washing buffer. The TIR1–Myc protein was then eluted into washing buffer without auxin by vigorous agitation. Eluted TIR1–Myc was used in the pull-down reaction using GST–IAA7 in the presence or absence of auxin. TIR1–Myc recovered by this method was still responsive to auxin (Fig. 2c). Further, preincubation of GST–IAA7 with auxin either in the extraction buffer or in the plant extract did not affect the subsequent interaction in the pull-down assay (data not shown).

Auxin binds directly to SCF^{TIR1}

Because auxin does not appear to cause a stable modification of TIR1 or the Aux/IAA proteins, we hypothesized that auxin may regulate the interaction between Aux/IAA and SCF^{TIR1} by directly binding to the SCF^{TIR1} complex. To test this possibility, we carried out pull-down assays using GST–IAA7 and crude extracts from *GVG::TIR1-myc* seedlings in the presence of [^{3}H]-IAA. After washing the GST–IAA7 beads extensively, radioactivity retained with the GST–IAA7 beads was measured by scintillation counting. The results in Fig. 3a show that GST–IAA7 retained [^{3}H]-IAA, but GST–AXR2-1 did not. The AXR2-1 protein has an amino-acid substitution in domain II that prevents interaction with SCF^{TIR1} (ref. 10).

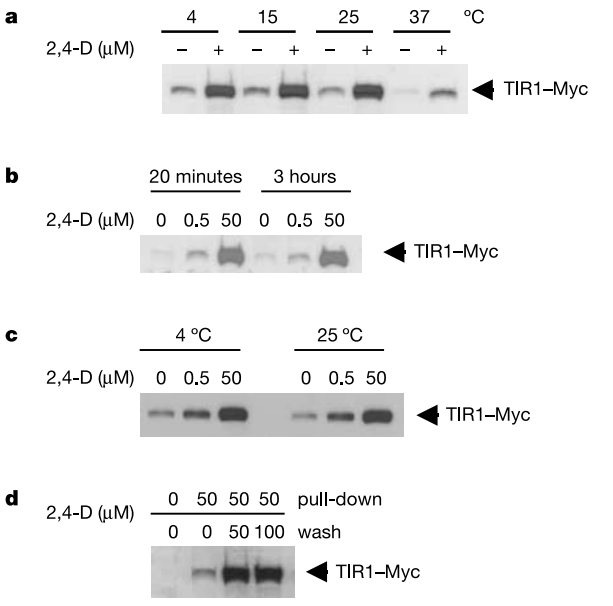

Figure 1 | Auxin-induced interaction between Aux/IAA and the SCF^{TIR1} is dependent on auxin concentration but not temperature. Pull-down reactions were carried out using recombinant GST–IAA7 and crude plant extracts prepared from *tir1-1*, *GVG-TIR1-myc* seedlings. Protein bound to GST–IAA7 was washed, separated on SDS–PAGE and immunoblotted with anti-Myc antibody. **a**, Pull-down reactions were carried out at indicated temperatures for 20 min in the presence or absence of 50 μM 2,4-D. **b**, Pull-down reactions were carried out at 4 °C for the indicated times with different 2,4-D concentrations. **c**, Pull-down reactions were incubated at the indicated temperatures and 2,4-D concentrations for 25 min. **d**, Pull-down reactions were incubated for 25 min at 4 °C and the glutathione beads were rinsed once with 1 ml of washing buffer without 2,4-D and then washed three times with washing buffer with or without 2,4-D.

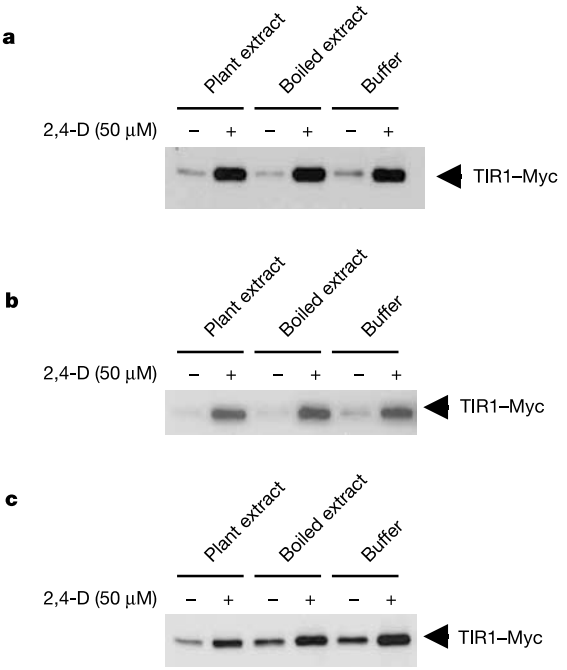

Figure 2 | Partially purified TIR1–Myc interacts with GST–IAA7 in an auxin-dependent manner. **a**, TIR1–Myc was immunoprecipitated from crude plant extracts with anti-Myc antibody. Eluted protein was used in a pull-down assay as described in Methods. 100 μg of wild-type plant extract (plant extract), boiled plant extract (boiled extract) or extraction buffer (buffer) was added to the pull-down reaction. **b**, Crude plant extract was incubated with 50 μM 2,4-D for 1 h and then TIR1–Myc was immunoprecipitated with anti-Myc antibody before the pull-down assay as above. **c**, TIR1–Myc was first pulled-down with GST–IAA7, washed in the presence of 50 μM 2,4-D and then eluted into washing buffer without 2,4-D. Eluted protein was used in the pull-down assay as described in **a**.

To explore further the biological relevance of this binding we performed competitive binding experiments with active auxins and related compounds. The active auxins IAA, 1-NAA and 2,4-D competed efficiently with [^{3}H]-IAA for binding while benzoic acid and tryptophan—related molecules with no auxin activity—did not compete effectively (Fig. 3b). These results suggest that the auxin receptor is localized to the SCF^{TIR1} complex. Scatchard analysis indicated that the apparent dissociation constant K_d of the receptor for IAA is 84 nM (data not shown). In addition, our results demonstrate that the natural auxin IAA has a higher affinity for the receptor than either 2,4-D or 1-NAA, consistent with our earlier results indicating that IAA promotes the interaction between Aux/IAA and TIR1 at nanomolar concentrations while 2,4-D and 1-NAA are active at micromolar levels[14]. The median inhibitory concentration IC_{50} for IAA, 1-NAA, and 2,4-D is 0.12, 1.3 and 1.4 μM, respectively, in this assay. It should be noted however that the K_d and IC_{50} values were obtained in binding assays with crude plant extracts containing unknown levels of endogenous IAA. In addition, it is likely that a fraction of the exogenous IAA is metabolized during the course of the experiment. Although the IC_{50} values provide information on the relative affinity of each compound for the receptor, it will be necessary to determine definitive K_d and IC_{50} values using purified proteins.

Because auxin is recovered with GST–IAA7 in the presence of crude plant extract, we tested whether GST–IAA7 itself interacts with [^{3}H]-IAA in the extraction buffer. The results in Fig. 3c show that [^{3}H]-IAA is recovered with GST–IAA7, but the amount recovered is not affected by addition of excess cold IAA. A similar level of binding was observed with the AXR2-1 protein (data not shown). Thus IAA binding to GST–IAA7 is nonspecific, suggesting that the Aux/IAA proteins do not function as receptors.

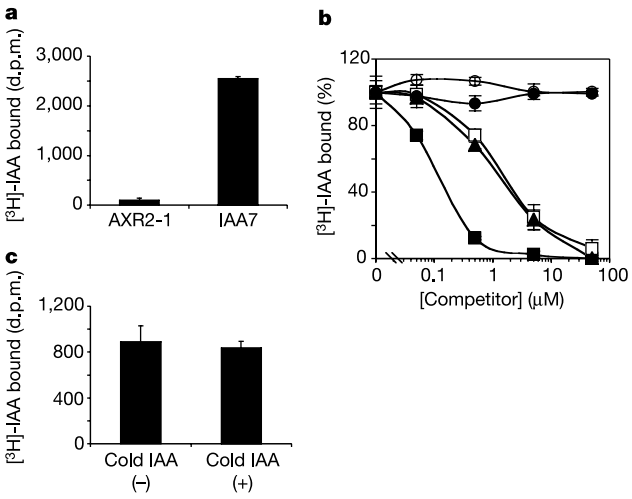

Figure 3 | [^{3}H] IAA interacts with the SCF^{TIR1} complex. a, Pull-down reactions were carried out with either GST–IAA7 or GST–AXR2-1 in the presence of 200 nM [^{3}H]-IAA. The retained fraction of [^{3}H]-IAA after washing the glutathione beads was measured by scintillation counting. Each point is the mean of three values ± standard deviation (s.d.). **b**, Competitive binding of [^{3}H]-IAA to SCF^{TIR1} in the presence of unlabelled IAA (black squares), 2,4-D (white squares) and 1-NAA (black triangles). The related compounds benzoic acid (white circles) and L-tryptophan (black circles) do not compete with [^{3}H]-IAA. Pull-down reactions were performed in the presence of 50 nM [^{3}H]-IAA and the indicated amount of competitor. Each assay was replicated three times and results were normalized relative to no competitor. Error bars represent s.d. **c**, GST–IAA7 was incubated in buffer containing [^{3}H]-IAA with or without cold IAA and recovered as described above. d.p.m., disintegrations per minute. Values are the mean of three experiments ± s.d.

TIR1 is an auxin receptor

SCF^{TIR1} contains Cullin homologue 1 (CUL1), arabidopsis skp1-like 1 (ASK1) and RING-box protein 1 (RBX1) in addition to TIR1 (ref. 19). Because these proteins are common to many different SCF complexes, it is likely that auxin interacts directly with either TIR1 or a protein tightly associated with TIR1. To distinguish between these two possibilities, we first synthesized TIR1 *in vitro* using a wheat germ extract (Promega). As shown in Fig. 4a, TIR1 synthesized in this way interacts with GST–IAA7 in an auxin-inducible manner. However, it is possible that endogenous proteins present in the wheat germ extract are facilitating this response. To eliminate this possibility we synthesized H6-TIR–Myc protein in insect cells. GST–IAA7 pull-downs were performed using extracts prepared from insect cells expressing H6-TIR1–Myc. The results in Fig. 4b show that the TIR1–Myc in the extract interacts with GST–IAA7 in an auxin-dependent manner whereas GST–AXR2-1 does not. TIR1 and GST–IAA7 are the only plant-derived proteins in the pull-down assay, so this result implies that auxin binds directly to TIR1.

Because CUL1, ASK1, and RBX1 are highly conserved between plants and animals it is possible that TIR1 synthesized in wheat germ extract or insect cells associates with endogenous ASK1 (SKP1 in insects) and CUL1. To begin to investigate the importance of SCF assembly for TIR1 function, we synthesized TIR1 protein lacking 75 amino acids near the amino terminus including the F-box motif (ΔFB-TIR1). This protein did not respond to auxin (Fig. 4a) in a pull-down assay. There are a number of possible interpretations of this result. One is that the deleted sequences directly mediate IAA and/or Aux/IAA binding. Alternatively, assembly of TIR1 into an SCF, or at least interaction with ASK1 may be required for TIR1 function. Additional studies will be required to resolve this issue.

If TIR1 functions as an auxin receptor, the level of saturable IAA binding should be reduced in the *tir1* mutant. However, the amount of binding in *tir1* extracts is indistinguishable from the wild type (data not shown). One possible explanation for this result is that TIR1 is a member of a family of related receptors. The *TIR1* gene is in a small subfamily of seven related F-box protein genes[22]. Three of these genes, *At4g03190*, *At3g26810* and *At1g12820* have been named *AFB1*, *AFB2* and *AFB3* respectively, for AUXIN SIGNALING F-BOX protein. In a previous report *At4g03190* and *At3g26810* were designated *LRF1* and *LRF2* (ref. 23). An alignment of TIR1, the AFB proteins, and the related F-box protein COI1 is shown in Fig. 5a. COI1 is required for jasmonic acid response and is in the same subclade as TIR1 and the AFB proteins[22,24]. To investigate the role of AFB1, AFB2 and AFB3 proteins in auxin binding, we performed GST–IAA7 pull-down experiments in the presence of [^{3}H]-IAA using quadruple mutant lines lacking TIR1, AFB1, AFB2 and AFB3. The results shown in Fig. 5b show that saturable [^{3}H]-IAA was

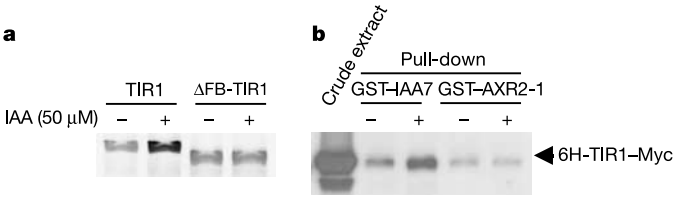

Figure 4 | TIR1 protein translated *in vitro* or expressed in insect cells interacts with GST–IAA7 in an auxin-dependent manner. a, TIR1 and ΔFB-TIR1 were translated *in vitro* in the presence of ^{35}S-methionine using the wheat germ system and directly used in pull-down assays in the presence or absence of 50 μM IAA. **b**, 6H-TIR1–Myc was expressed in High Five insect cells as described in the Methods. Crude protein extracts were used in pull-down reactions with either GST–IAA7 or GST–AXR2-1 in the presence or absence of 50 μM IAA.

undetectable in this line, indicating that auxin binding is dependent upon TIR1 and the AFB proteins.

Discussion

Our results provide compelling evidence that TIR1 is an auxin receptor that mediates rapid degradation of Aux/IAA proteins and consequent changes in expression of auxin-regulated genes. At this point it is not clear how auxin stimulates the interaction between SCF^{TIR1} and its substrates. It is possible that auxin binds to TIR1 and promotes a conformational change that favours Aux/IAA binding. Alternatively, auxin may bind cooperatively to both TIR1 and the Aux/IAA protein, thus stabilizing the SCF^{TIR1}-substrate complex. The site of auxin binding within TIR1 is also not known. The protein consists of the N-terminal F-box motif, a short spacer region of about 40 residues, 16 degenerate leucine-rich repeats (LRRs), and a C-terminal tail of approximately 70 residues (Fig. 5a). A comparison between TIR1, the three AFB proteins, and COI1 does not reveal any major TIR1/AFB specific domains that might be an auxin-binding pocket, although there are several short stretches of amino acids in the TIR1/AFB proteins that are not present in COI1. LRRs, which make up the bulk of TIR1, are typically thought to facilitate protein–protein interactions. However, brassinosteroids have recently been shown to bind to an LRR and adjacent sequences within the brassinosteroid receptor BRI1[25]. Thus it is possible that auxin binds one or more LRRs within TIR1. This and other possibilities will be assessed in future studies.

At this point it is not clear whether SCF^{TIR1} and related SCFs are the only targets of auxin action. In addition to changes in gene expression, auxin appears to regulate ion transport through the plasma membrane[26–29]. One of the best-characterized auxin responses is a rapid increase in plasma membrane H^+-ATPase activity, an effect that has been associated with cell elongation[29]. Because this response is too rapid to be mediated by transcriptional changes, it probably does not directly require TIR1-dependent degradation of Aux/IAA proteins. It is possible that TIR1 promotes the degradation of other proteins that regulate ion transport. Alternatively, and perhaps more probably, activation of ATPases and other ion transporters may be controlled by a different auxin signalling pathway.

Members of the SCF family of E3 ligases are known to play important roles in many aspects of cellular regulation in eukaryotes[17,19,30]. In addition, genomic analyses indicate that there are a large number of uncharacterized F-box proteins in both plant and metazoan genomes[19]. Among characterized SCFs, substrate recognition typically involves phosphorylation within the substrate degron[19]. Thus our discovery that the small ligand IAA regulates

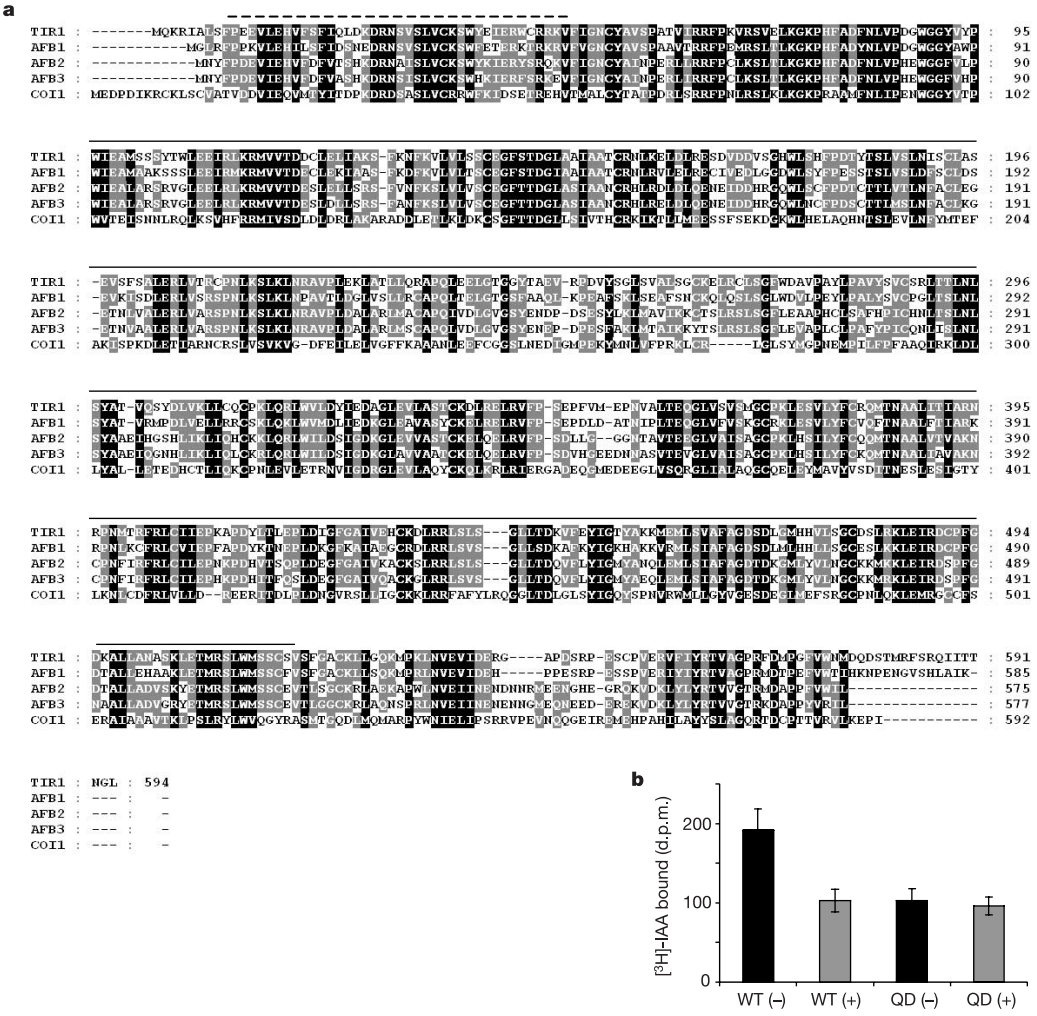

Figure 5 | A small family of F-box proteins contributes to auxin binding. **a**, TIR1 was aligned with three closely related F-box proteins called AFB1, AFB2 and AFB3. The COI1 protein is the most closely related F-box protein that does not function in auxin response. The dashed line above the sequence indicates the position of the F-box motif, and the solid line indicates the position of the LRRs. Residues shaded black are similar in all five proteins, while those shaded grey are similar in three of the proteins. **b**, [^{3}H]-IAA binding in the *Col* (wild type, WT) crude protein extracts and protein extracts from *tir1-1 afb1-1 afb2-1 afb3-1* (quadruple mutant, QD) seedlings. Values are the mean of three experiments ± s.d.

SCF^{TIR1}-substrate recognition represents a new paradigm for SCF regulation. Future studies will reveal exactly how IAA interacts with TIR1 and the Aux/IAA proteins and whether other plant and animal SCFs are also ligand regulated.

METHODS

Plant material. *Arabidopsis* (*Col*) *tir1*, *GVG::TIR1-myc* seedlings were grown under sterile conditions on vertically oriented ATS (*Arabidopsis thaliana* medium +1% sucrose) plates at 22 °C under constant light. Twelve-day-old seedlings were transferred into liquid ATS medium and TIR1–Myc expression was induced with 30 μM dexamethasone.

Pull-down assays. Total protein was extracted from seedlings in a 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 according to the manufacturer's instructions (Roche Diagnostics). Cell debris was removed by centrifugation at 10,000 *g* for 10 min. Total protein concentration was estimated by the Bradford assay (BioRad). Recombinant GST–IAA7 and GST–AXR2-1 were expressed in *Escherichia coli* and purified using glutathione beads according to standard protocols. GST–IAA7 or GST–AXR2-1 (3–4 μg) was incubated with 800 μg of total crude plant protein extract and incubated at 4 °C for 1 h unless otherwise specified. Glutathione beads were recovered by a brief centrifugation and washed three times with 1 ml of washing buffer (50 mM Tris-Cl (pH 7.2), 100 mM NaCl, 10% glycerol and 0.1% Tween 20. Where it is specified, 2,4-D was added to the washing buffer.

For *in vitro* translation, the *TIR1* coding sequence was cloned into *Xho*1/*Xba*1 sites of the *pTNT* vector creating the plasmid *pNDS45*. To generate the *ΔFB-TIR1* mutant, a *Kpn1* site was introduced to the *TIR1* complementary DNA using QuikChange site-directed mutagenesis kit (Stratagene) with primers 5′-AGC GAATAGCCTTGTCGGTACCAGAAGAGGTACTAG-3′ and 5′-CTCTAGTA CCTCTTCTGGTACCGACAAGGCTATTCGC-3′. The resulting plasmid, *pNDS46*, was digested with *Kpn*1 to release a 228-base-pair (bp) *Kpn*1 fragment that includes the F-box domain, but leaving the N-terminal 8-amino-acid residues intact. The rest of the plasmid was religated to create *pNDS47* encoding ΔFB-TIR1 lacking amino acids 9 to 84. The full-length and ΔFB-TIR1 proteins were expressed in the coupled wheat germ extract system (Promega) in the presence of ^{35}S-trans label (1,175 Ci mmol^{-1}; MP Biochemicals). For pull-down assays, 20 μl of translated product was incubated with 3–4 μg of GST–IAA7 in 200 μl extraction buffer for 5 h and unbound proteins were washed as described above. Pull-down mixtures were separated on SDS–polyacrylamide gel electrophoresis (PAGE) and the bound TIR1 was detected by using the Phosphoimager (Typhoon 9200, Amersham Biosciences).

Auxin binding assays. To determine auxin binding, pull-down assays were done as described above using plant extract containing TIR1–Myc, except that [^{3}H]-IAA (specific activity 20 mCi mmol^{-1}) was added to the pull-down reaction. Each reaction had a final concentration of 50 nM [^{3}H]-IAA unless otherwise specified. After washing three times in the presence of excess unlabelled IAA, glutathione beads were resuspended in 100 μl of water and mixed with scintillation fluid. The radioactivity of the bound [^{3}H]-IAA was measured using a scintillation counter.

Expression of TIR1 in insect cells. The TIR–Myc coding sequence was amplified from *pGB28*[23] using the oligonucleotides 5′-CACCATGCAGAAGCGAA TAGCCTTGTC-3′ and 5′-AGCTTATCGATTTCGAACCCGGGGTAC-3′ and first cloned into the *pENTR-D/TOPO* and then into the *pDEST10* using the Gateway system according to the manufacturer's instructions (Invitrogen). The resulting plasmid, *pDEST10-H6-TIR1-myc* was then transformed into *DH10Bac* competent cells. *E. coli* colonies with recombinant bacmid were identified according to the manufacturer's instruction (Invitrogen). Initial viral production and amplification were done using Sf9 cells. Bacmid DNA (1 μg) diluted in 200 μl of Sf900 II SFM medium (Invitrogen) was mixed with 6 μl Cellfectin (Invitrogen) and incubated for 30 min at 22 °C. Two millilitres of Sf9 cells at mid-log phase (1×10^6 cells ml^{-1}) were infected and incubated at 28 °C for 96 h. The supernatant was collected by centrifugation at 2,000 r.p.m. for 5 min in a clinical centrifuge. This viral sample was diluted to 1/100 and infected into 50 ml Sf9 cells at mid-log phase (1×10^6). Virus was collected 96 h post-infection by centrifugation at 2,000 r.p.m. for 5 min. After amplifying the virus in sf9 cells, H6-TIR1–Myc protein was expressed in High Five cells. One millilitre of High Five cells at the mid-log phase (1×10^6) were infected with 1/500 dilution of the reamplified virus and cells were collected by centrifugation 24 h post-infection. Cells were washed once with the extraction buffer (described above) and then resuspended in 1 ml of the extraction buffer. Crude protein was isolated by sonication of the cells and the extract was cleared by centrifugation at 1,300 r.p.m. for 10 min at 4 °C. Total protein in the extract was estimated by the Bradford method. Approximately 100 μg total protein was used in the 250-μl pull-down reaction.

Received 10 January; accepted 11 March 2005.

1. Davies, P. J. in *Plant Hormones Physiology, Biochemistry and Molecular Biology* (ed. Davies, P. J.) 1–12 (Kluwer Academic, Dordrecht, 1995).
2. Went, F. W. Wushstoff und wachstum. *Rec. Trav. Bot. Neerl.* **25,** 1–116 (1928).
3. Weijers, D. & Jurgens, G. Auxin and embryo axis formation: the ends in sight? *Curr. Opin. Plant Biol.* **8,** 32–37 (2005).
4. Willemsen, V. & Scheres, B. Mechanisms of pattern formation in plant embryogenesis. *Annu. Rev. Genet.* **38,** 587–614 (2004).
5. Napier, R. M., David, K. M. & Perrot-Rechenmann, C. A short history of auxin-binding proteins. *Plant Mol. Biol.* **49,** 339–348 (2002).
6. Hagen, G. & Guilfoyle, T. Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Mol. Biol.* **49,** 373–385 (2002).
7. Reed, J. W. Roles and activities of Aux/IAA proteins in *Arabidopsis*. *Trends Plant Sci.* **6,** 420–425 (2001).
8. Liscum, E. & Reed, J. W. Genetics of Aux/IAA and ARF action in plant growth and development. *Plant Mol. Biol.* **49,** 387–400 (2002).
9. Zenser, N., Ellsmore, A., Leasure, C. & Callis, J. Auxin modulates the degradation rate of Aux/IAA proteins. *Proc. Natl Acad. Sci. USA* **98,** 11795–11800 (2001).
10. Gray, W. M., Kepinski, S., Rouse, D., Leyser, O. & Estelle, M. Auxin regulates SCF^{TIR1}-dependent degradation of Aux/IAA proteins. *Nature* **414,** 271–276 (2001).
11. Tiwari, S. B., Wang, X. J., Hagen, G. & Guilfoyle, T. J. Aux/IAA proteins are active repressors, and their stability and activity are modulated by auxin. *Plant Cell* **13,** 2809–2822 (2001).
12. Tian, Q., Nagpal, P. & Reed, J. W. Regulation of *Arabidopsis* SHY2/IAA3 protein turnover. *Plant J.* **36,** 643–651 (2003).
13. Ramos, J. A., Zenser, N., Leyser, H. M. & Callis, J. Rapid degradation of Aux/IAA proteins requires conserved amino acids of domain II and is proteasome-dependent. *Plant Cell* **13,** 2349–2360 (2001).
14. Dharmasiri, N., Dharmasiri, S., Jones, A. M. & Estelle, M. Auxin action in a cell-free system. *Curr. Biol.* **13,** 1418–1422 (2003).
15. Kepinski, S. & Leyser, O. Auxin-induced SCF^{TIR1}-Aux/IAA interaction involves stable modification of the SCF^{TIR1} complex. *Proc. Natl Acad. Sci. USA* **101,** 12381–12386 (2004).
16. Ouellet, F., Overvoorde, P. J. & Theologis, A. IAA17/AXR3. Biochemical insight into an auxin mutant phenotype. *Plant Cell* **13,** 829–842 (2001).
17. Deshaies, R. J. SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu. Rev. Cell Dev. Biol.* **15,** 435–467 (1999).
18. Jaakkola, P. *et al.* Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science* **292,** 468–472 (2001).
19. Cardozo, T. & Pagano, M. The SCF ubiquitin ligase: insights into a molecular machine. *Nature Rev. Mol. Cell Biol.* **5,** 739–751 (2004).
20. Yoshida, Y. *et al.* E3 ubiquitin ligase that recognizes sugar chains. *Nature* **418,** 438–442 (2002).
21. Yang, X. *et al.* The IAA1 protein is encoded by AXR5 and is a substrate of SCF^{TIR1}. *Plant J.* **40,** 772–782 (2004).
22. Gagne, J. M., Downes, B. P., Shiu, S. H., Durski, A. M. & Vierstra, R. D. The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in *Arabidopsis*. *Proc. Natl Acad. Sci. USA* **99,** 11519–11524 (2002).
23. Gray, W. M. *et al.* Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. *Genes Dev.* **13,** 1678–1691 (1999).
24. Xie, D. X., Feys, B. F., James, S., Nieto-Rostro, M. & Turner, J. G. COI1: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. *Science* **280,** 1091–1094 (1998).
25. Kinoshita, T. *et al.* Binding of brassinosteroids to the extracellular domain of plant receptor kinase BRI1. *Nature* **433,** 167–171 (2005).
26. Frias, I. *et al.* A major isoform of the maize plasma membrane H(+)-ATPase: characterization and induction by auxin in coleoptiles. *Plant Cell* **8,** 1533–1544 (1996).
27. Blatt, M. R. & Thiel, G. K+ channels of stomatal guard cells: bimodal control of the K+ inward-rectifier evoked by auxin. *Plant J.* **5,** 55–68 (1994).
28. Philippar, K. *et al.* Auxin-induced K+ channel expression represents an essential step in coleoptile growth and gravitropism. *Proc. Natl Acad. Sci. USA* **96,** 12186–12191 (1999).
29. Luthen, H., Claussen, M. & Bottger, M. Growth: progress in auxin research. *Prog. Bot.* **60,** 315–340 (1999).
30. Pickart, C. M. Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* **70,** 503–533 (2001).

Acknowledgements We thank R. Dumas for technical assistance and C. Leblanc for help with insect cell culture. In addition, we thank S. Kepinski and O. Leyser for discussions. Research in the authors' laboratory is supported by grants from the NIH, the NSF and the DOE.

Author Information Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to M.E. (maestell@indiana.edu).