

Auxin Action in a Cell-Free System

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Summary

The plant hormone auxin regulates diverse aspects of plant growth and development [1]. Despite its importance, the mechanisms of auxin action remain poorly understood. In particular, the identities of the auxin receptor and other signaling proteins are unknown. Recent studies have shown that auxin acts by promoting the degradation of a family of transcriptional regulators called the Aux/IAA proteins [2–4]. These proteins interact with another large family of plant-specific transcription factors called Auxin Response Factors (ARF) and negatively regulate their activity [5]. Auxin stimulates Aux/IAA degradation by promoting the interaction between a ubiquitin protein ligase (E3) called SCF^{TIR1} and the Aux/IAA protein [2]. In this report, we demonstrate that auxin promotes the interaction between the Aux/IAA proteins and SCF^{TIR1} in a soluble extract free of membranes, indicating that this auxin response is mediated by a soluble receptor. In addition, we show that the response is not dependent on protein phosphorylation or dephosphorylation but rather is prevented by an inhibitor of peptidyl-prolyl isomerases.

Results and Discussion

Auxin regulates gene expression through the action of two families of proteins called the Auxin Response Factors (ARF) and the Aux/IAA proteins [5, 6]. In general, ARFs bind directly to DNA and either activate or repress transcription depending on the ARF [7]. The Aux/IAA proteins interfere with ARF function, probably by heterodimerization [7]. Recent studies have shown that auxin promotes the degradation of the Aux/IAA proteins, thus permitting ARF function [2–4].

Despite these advances, many aspects of auxin action remain poorly understood. Foremost among these is the identity and location of the auxin receptor. The best-characterized candidate auxin receptor is a protein called Auxin Binding Protein (ABP1) [8]. ABP1 is a novel protein that is unique to plants and has no structural features that are strongly informative of its function or biochemical activity. ABP1 is localized primarily to the ER, although a small amount may be present at the plasma membrane [8]. Experiments with protoplast sys-

tems indicate that ABP1 is important for rapid electrophysiological responses, and genetic studies in *Arabidopsis* have shown that the protein is essential for viability [9]. There is no information on whether ABP1 is involved in rapid transcriptional responses to auxin.

Auxin Promotes the Interaction between the Aux/IAA Proteins and SCF^{TIR1} in Cell-Free Extracts

Auxin treatment of *Arabidopsis* seedlings stimulates binding of Aux/IAA proteins by SCF^{TIR1} [2]. To further investigate this response, we tested the effects of auxin added directly to protein extracts prepared from seedlings expressing *c-myc*-tagged TIR1 (TIR1-*myc*). A glutathione-S-transferase fusion protein (GST-IAA7) was added to extracts together with the endogenous auxin IAA or the synthetic auxin 2,4-D. GST-IAA7 was recovered, and the presence of TIR1-*myc* was assessed by protein blot. Figure 1A shows that both IAA and 2,4-D had a dramatic effect on the level of TIR1-*myc* recovered. The effect was evident at concentrations as low as 50 nM IAA or 5 μ M 2,4-D. We find that IAA is consistently more active than 2,4-D (or 1-NAA, see Figure 2A). In contrast, these auxins exhibit a similar level of activity when applied to *Arabidopsis* seedlings [10]. This difference is probably related to differences in the metabolism of the auxins when they are applied to seedlings.

To better understand the physiological relevance of this auxin response, we examined the effects of the *axr2-1* and *axr3-1* mutations on the response. These mutations cause amino acid substitutions in domain II of IAA7 and IAA17, respectively, resulting in stabilization of the affected protein, defects in auxin response, and a variety of auxin-related growth defects [2, 11]. GST-IAA7, GST-IAA17, GST-AXR2-1, and GST-AXR3-1 proteins were synthesized in *E. coli* and were added to plant extracts with the synthetic auxin 2,4-D. The hormone promoted recognition of GST-IAA7 and GST-IAA17 by SCF^{TIR1} (Figures 1B and 1C). In contrast, no detectable TIR1-*myc* was recovered when the mutant proteins were added to the extract.

The specificity of the cell-free auxin response was explored by testing the activity of several synthetic auxins and related compounds. The results in Figure 2A show that the active auxin 1-NAA promoted the interaction between IAA7 and TIR1, while the related compound 2-NAA had very little effect. Similar results were obtained with benzoic acid and tryptophan (Figure 2B). Since 2-NAA, benzoic acid, and tryptophan are not biologically active auxins, these results argue strongly that the effects of auxin in this assay are biologically relevant.

The effect of auxin on SCF^{TIR1}-Aux/IAA binding does not require intact cells. However, the low-speed supernatants utilized in these experiments are likely to include membranes and associated proteins that may be required for auxin perception or signaling. To address this possibility, we subjected the protein extract to centrifugation at 160,000 \times g prior to performing the assay.

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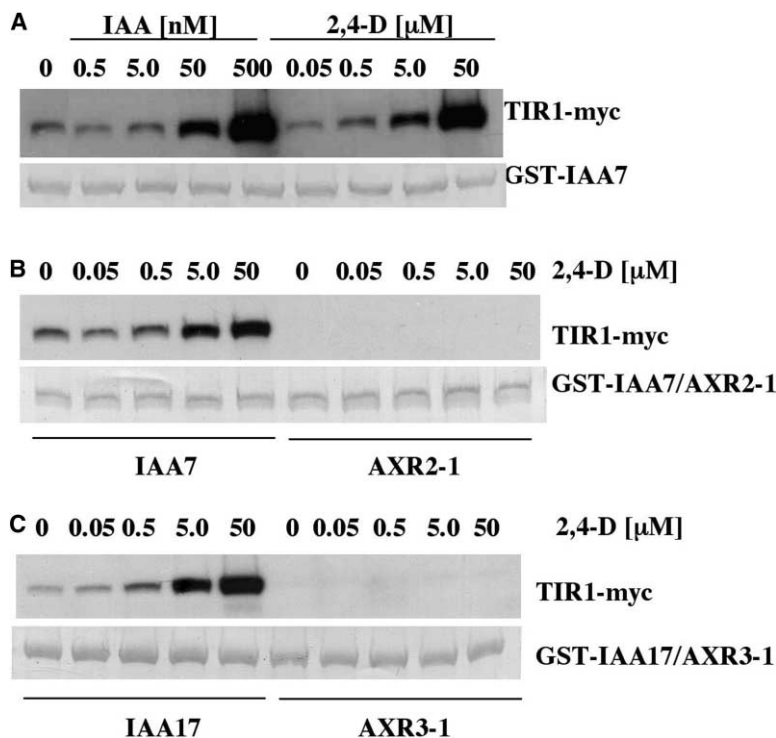


Figure 1. Auxin Stimulates the Interaction between SCF^{TIR1} and Aux/IAA Proteins in a Soluble Extract

(A) Protein extracts were prepared from *GVG::TIR1-myc* seedlings. GST-IAA7 (3–4 μ g) was added to each sample plus IAA or 2,4-D. GST pull-downs were analyzed by SDS-PAGE, and TIR1-myc was detected by immunoblotting. The lower panel shows imido black-stained GST-IAA7.

(B) 2,4-D was added to extracts together with GST-IAA7 or GST-AXR2-1. TIR1-myc was detected by immunoblotting.

(C) As in (B), except with GST-IAA17 or GST-AXR3-1.

Examination of OsO₄-treated low-speed and high-speed supernatants by TEM confirmed that this treatment removed lipids (see the Supplemental Data available with this article online). If a membrane component was nec-

essary for the auxin-dependent binding of Aux/IAA proteins, we expect that decreased membranes in the cell-free extract would cause a significant reduction in SCF^{TIR1}-Aux/IAA interaction. However, as shown in Figure 2C, high-speed centrifugation had no effect on the level of activity, indicating that this auxin response does not require cellular membranes. Based on these results, we propose that auxin regulation of Aux/IAA degradation involves an interaction between the hormone and one or more soluble proteins.

The auxin binding protein ABP1 is a candidate auxin receptor, and while it is a soluble protein, it is predominantly localized to the endomembrane compartment [8, 9]. To investigate a possible role of ABP1 in the cell-free assay, we used an antibody directed against *Arabidopsis* ABP1 to deplete ABP1 from extracts prior to the assay. This treatment did not affect the level of TIR1-myc recovered after GST-IAA7 pull-down (see the Supplemental Data). Similarly, the addition of 250 ng *Zea mays* ABP1 expressed and purified from insect cells did not affect the response. These results suggest that ABP1 is not required for auxin regulation of the SCF^{TIR1}-Aux/IAA interaction.

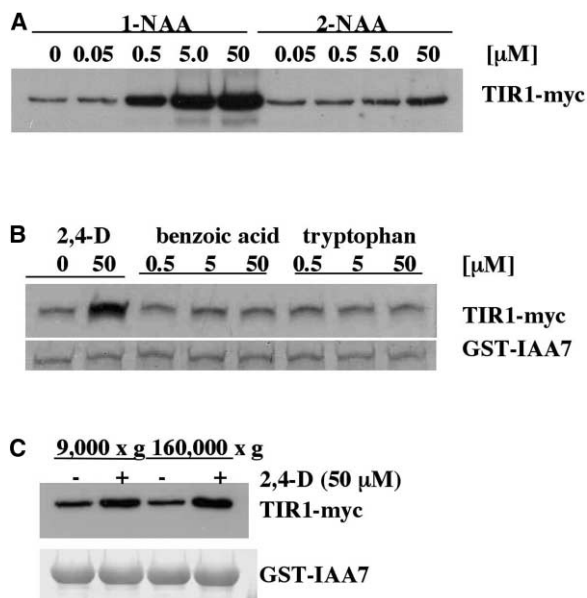


Figure 2. Specificity of the Cell-Free Auxin Response

(A) The active auxin 1-NAA and the inactive auxin 2-NAA were tested for activity in the in vitro assay as described in Figure 1.

(B) Benzoic acid and tryptophan do not promote the interaction between GST-IAA7 and SCF^{TIR1}.

(C) Protein extracts were prepared from *GVG::TIR1-myc* seedlings and were centrifuged at 9,000 \times g for 10 min or at 160,000 \times g for 1 hr. Supernatants were treated as described in Figure 1.

Role of Protein Phosphorylation in the SCF^{TIR1}-Aux/IAA Interaction

To determine if auxin stimulation of SCF^{TIR1}-Aux/IAA binding requires ATP, we added apyrase to the extract to hydrolyze the free ATP. Figure 3A shows that 10 U of apyrase had no effect on the auxin-induced SCF^{TIR1}-IAA7 interaction. In animal and fungal species, SCF substrate recognition requires substrate phosphorylation [12]. Previous studies indicate that phosphorylation within domain II of the Aux/IAA protein is not required

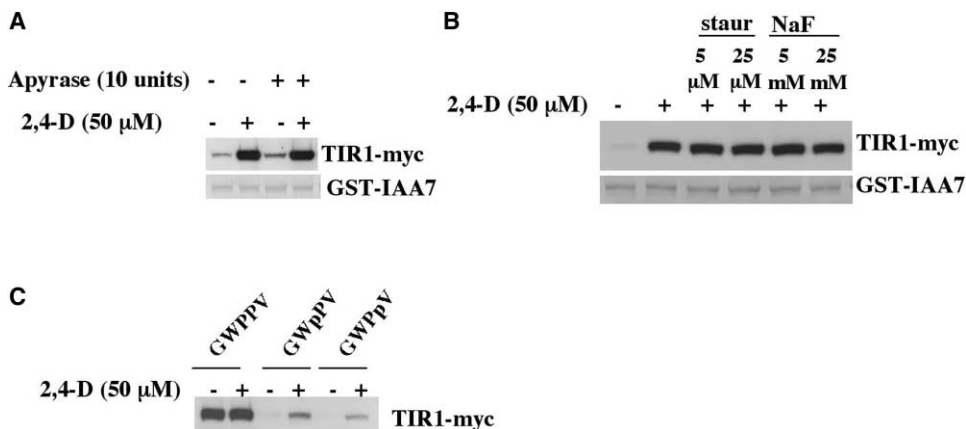


Figure 3. The In Vitro Auxin Response Does Not Require ATP, Protein Phosphorylation, or Hydroxylation of Conserved Prolines

(A) The addition of 10 U of apyrase has no effect on the auxin-stimulated interaction between GST-IAA7 and TIR1-myc. The assay was performed as described in Figure 1. ATP levels after apyrase treatment were zero, as measured by the ATP bioluminescence assay (Sigma). (B) Neither the broad specificity protein kinase inhibitor staurosporine (Staur) nor the protein phosphatase inhibitor NaF affects the response. (C) Hydroxylation of conserved prolines in Domain II of IAA7 does not enhance the interaction between Domain II peptides and TIR1-myc. A total of 4 μg biotin-tagged peptide was added to each reaction and was recovered with streptavidin beads. An upper case “p” (P) indicates proline, while a lower case “p” (p) indicates hydroxyproline.

for degradation [13]. However, it is possible that an interaction between Aux/IAA proteins and TIR1 is mediated by one or more additional proteins that may be phosphorylated. To investigate this possibility, we included staurosporine, a broad-range protein kinase inhibitor [14, 15], and NaF, a phosphatase inhibitor, into our cell-free extract. Neither of these compounds had any effect on auxin activity, suggesting that protein phosphorylation is not directly involved in the SCF^{TIR1}-IAA7 interaction (Figure 3B).

Role of Domain II Prolines in Aux/IAA Recognition

Genetic studies indicate that two conserved prolines within domain II of the Aux/IAA proteins are required for interaction with SCF^{TIR1} [11]. In animals, recognition of the transcription factor HIF α by the E3 ligase VBC is known to be dependent upon hydroxylation of proline residues within HIF α [16]. Inhibitors of prolyl hydroxylase prevent this modification and stabilize HIF α [17, 18]. To investigate whether proline hydroxylation is important for Aux/IAA recognition, we determined the effects of various prolyl hydroxylase inhibitors, including Co²⁺ (1 mM), DMOG (4 mM), and DLP (10 mM), on the interaction between GST-IAA7 and TIR1-myc. None of these agents affected the interaction, suggesting that auxin does not regulate proline hydroxylation (data not shown). Alternatively, it is possible that a novel prolyl hydroxylase that is not affected by these compounds is involved in auxin response.

To further investigate the role of proline hydroxylation in auxin response, we synthesized three peptides encompassing residues 73–88 of IAA7. These peptides span most of domain II, including the two conserved prolines. The first peptide consisted of the wild-type sequence of amino acids, while the other two replaced pro-81 and pro-82, respectively, with hydroxyproline. All three peptides had a biotin tag on their N terminus. The peptides were added to protein extract containing TIR1-

myc with or without 2,4-D and were recovered on streptavidin beads. Figure 3C shows that the wild-type peptide interacted well with TIR1-myc, even in the absence of 2,4-D. In contrast, substitution of either proline with hydroxyproline dramatically reduced the amount of TIR1-myc recovered from the extract (Figure 3C). The interaction between the hydroxylated peptides and TIR1-myc was still auxin responsive, but the interaction was much less robust than with either the wild-type peptide or GST-IAA7. Similarly a peptide in which both prolines were replaced by hydroxyproline interacted weakly with SCF^{TIR1} (data not shown). These results are not consistent with a model in which auxin regulation of Aux/IAA proteins is directly dependent on proline hydroxylation.

The importance of the prolines in domain II led us to consider the possibility that a peptidyl prolyl *cis/trans* isomerase (PPIase) may be involved in auxin response. There are three known classes of PPIase [19]. Cyclophilins, FK506 binding proteins, and parvulins are inhibited by cyclosporin, rapamycin, and juglone, respectively [19]. We tested each of these compounds in our assay. Neither rapamycin nor cyclosporin had any effect on the in vitro auxin activity (data not shown). However, juglone dramatically inhibited the interaction between GST-IAA7 and SCF^{TIR1}, suggesting that a PPIase of the parvulin group might be required for the interaction (Figures 4A and 4B).

To investigate the physiological significance of the juglone effect, we examined auxin-regulated gene expression in vivo. Plants carrying the auxin reporter *BA3::GUS* were treated with 2,4-D with or without 20 μM juglone, cyclosporine, and rapamycin [20]. As expected, auxin treatment resulted in accumulation of GUS in the elongation zone of transgenic seedlings (Figure 4C). Juglone prevented this accumulation, suggesting that a PPIase is required for auxin-regulated gene expression. In contrast, cyclosporin and rapamycin had no effect on the auxin response.

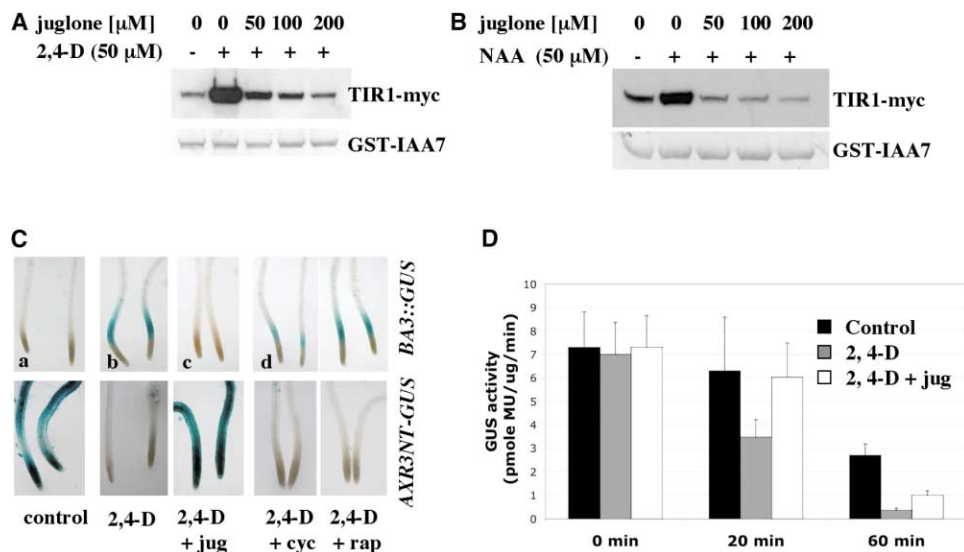


Figure 4. The Prolyl-Isomerase Inhibitor Juglone Inhibits the Auxin Response Both In Vitro and In Vivo

(A and B) Increasing concentrations of juglone were added to extracts containing TIR1-myc, GST-IAA7, and either (A) 2,4-D or (B) 1-NAA. GST pull-downs were analyzed as in Figure 1.

(C) Juglone inhibits auxin-regulated transcription and degradation of AXR3NT-GUS. BA3::GUS seedlings were treated with 20 μM 2,4-D alone or together with 20 μM juglone, cyclosporine, or rapamycin. HS::AXR3NT-GUS seedlings were heat shocked for 2 hr at 37°C and were transferred into media containing 20 μM 2,4-D alone or 20 μM 2,4-D + 20 μM PPIase inhibitor. Control seedlings were treated in a similar way. Treated seedlings were stained for GUS activity after 60 min. The roots of 9-day-old seedlings are shown for each treatment.

(D) HS::AXR3NT-GUS seedlings were treated as in (C). GUS activity was measured by a flourometer. Each data point is the mean of three experiments. The error bars represent standard deviations. The differences between 2,4-D and 2,4-D + juglone are statistically significant for both 20 min and 60 min (t tests: for 20 min, $P = 0.003$; for 60 min, $P = 0.0001$).

Juglone inhibits the auxin-dependent interaction between the Aux/IAA proteins and SCF^{TIR1}. If the compound has the same effect in vivo, we expect that this will result in stabilization of the Aux/IAA proteins. Previous studies have shown that the AXR3NT-GUS fusion protein is degraded in response to auxin [2]. Transgenic seedlings were shifted to 37°C for 2 hr to induce synthesis of the fusion protein. At time 0, seedlings were treated with 2,4-D or 2,4-D plus PPIase inhibitor. After 60 min, seedlings were stained for GUS activity. Figures 4C and 4D show that auxin caused a decrease in the amount of GUS staining compared to the control, and this finding is consistent with earlier results. In contrast, seedlings treated with juglone, but not cyclosporin or rapamycin, exhibited much stronger GUS staining, indicating that juglone inhibits auxin-dependent degradation of AXR3NT-GUS.

During the last several decades, the identity and cellular location of the auxin receptor has been a subject of intense interest and speculation. The results described in this report provide clear evidence that soluble proteins mediate auxin induction of transcription. Further, we provide evidence that the auxin response requires a parvulin-type PPIase. We speculate that prolyl isomerization within domain II of the Aux/IAA proteins is required for recognition by the SCF. Alternatively, a PPIase may be required for a different step in auxin signaling. The *Drosophila* PPIase Dodo has been implicated in the degradation of the transcription factor CF2 [21]. However, this is the first report of a potential role for a PPIase in SCF-substrate recognition. In addition, these results

will considerably simplify our future efforts to identify the auxin receptor and other signaling proteins.

Supplemental Data

Supplemental Data including the Experimental Procedures and two figures are available at <http://www.current-biology.com/cgi/content/full/13/16/1418/DC1/>.

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