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The IAA1 protein is encoded by *AXR5* and is a substrate of SCF^{TIR1}

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Summary

Recent studies of auxin response have focused on the functions of three sets of proteins: the auxin (Aux) response factors (ARFs), the Aux/IAAs, and the F-box protein TIR1. The ARF proteins bind DNA and directly activate or repress transcription of target genes while the Aux/IAA proteins repress ARF function. TIR1 is part of a ubiquitin protein ligase required for degradation of Aux/IAA proteins. Here we report the isolation and characterization of a novel mutant of Arabidopsis called *axr5-1*. Mutant plants are resistant to auxin and display a variety of auxin-related growth defects including defects in root and shoot tropisms. Further, the *axr5-1* mutation results in a decrease in auxin-regulated transcription. The molecular cloning of *AXR5* revealed that the gene encodes the IAA1 protein, a member of the Aux/IAA family of proteins. *AXR5* is expressed throughout plant development consistent with the pleiotropic mutant phenotype. The *axr5-1* mutation results in an amino acid substitution in conserved domain II of the protein, similar to gain-of-function mutations recovered in other members of this gene family. Biochemical studies show that IAA1/AXR5 interacts with TIR1 in an auxin-dependent manner. The mutation prevents this interaction suggesting that the mutant phenotype is caused by the accumulation of IAA1/AXR5. Our results provide further support for a model in which most members of the Aux/IAA family are targeted for degradation by SCF^{TIR1} in response to auxin.

Keywords: auxin, AUX/IAA, plant development.

Introduction

The plant hormone auxin has been implicated in virtually every aspect of plant development from embryogenesis to senescence (Davies, 1995). Genetic and molecular studies indicate that many of these processes depend on the action of members of a family of transcription factors called the auxin response factors (ARFs) (Gray and Estelle, 2000; Ulmasov *et al.*, 1999a). There are 23 ARF proteins encoded in the Arabidopsis genome. A typical ARF protein contains a highly conserved DNA-binding region near the N-terminus, a dimerization motif near the C-terminus and a divergent region in the middle. These proteins bind a DNA sequence called the Auxin Response Element (AuxRE) and depending on the ARF, either activate or repress transcription (Ulmasov *et al.*, 1999a,b). So far genetic studies have implicated individual ARF proteins in embryogenesis and vascular development (MONOPTEROS/ARF5) (Hardtke and Berleth, 1998; Przemeck *et al.*, 1996), tropisms (NONPHOTOTROPIC-HYPOCOTYL 4/ARF7) (Harper *et al.*, 2000), and floral development (ETTIN/ARF3) (Nemhauser *et al.*, 2000; Sessions *et al.*, 1997). In addition, the characterization of the *nph4* mutants gave direct evidence that NPH4 regulates auxin-mediated gene expression (Stowe-Evans *et al.*, 1998).

Members of a second large family of genes, called *Aux/IAA*, also participate in auxin-regulated gene expression (Reed, 2001). These genes were originally identified because transcription of some members of the family is rapidly induced in response to auxin (Abel and Theologis, 1996). Recent studies indicate that the transcriptional behavior of the family is complex. Individual *Aux/IAA* genes display qualitative and quantitative differences in their regulation by auxin (Abel *et al.*, 1995; Rogg *et al.*, 2001). The proteins themselves are found in the nucleus and have four

conserved regions called domains I–IV (Reed, 2001). Domains III and IV are similar to the dimerization domains present in ARF proteins and Aux/IAA proteins can form homodimers and heterodimers with either a second Aux/IAA or an ARF (Kim *et al.*, 1997; Ulmasov *et al.*, 1997, 1999a). Based on their rapid synthesis in response to auxin, the Aux/IAA proteins were originally thought to be positive regulators of auxin response. However, genetic and biochemical experiments indicate that most members of the family repress auxin response (Gray *et al.*, 2001; Rogg *et al.*, 2001; Tiwari *et al.*, 2001; Ulmasov *et al.*, 1997). Indeed, recent studies indicate that domain I functions as a transferable transcriptional repressor providing a likely mechanism of Aux/IAA regulation of ARF function (Tiwari *et al.*, 2004). The interaction between an Aux/IAA protein and an ARF protein through domains III and IV will bring domain I to the transcriptional apparatus, resulting in repression.

The Aux/IAA proteins are remarkably unstable with reported half-lives ranging from 6 to 80 min depending on the protein (Abel *et al.*, 1994; Gray *et al.*, 2001; Ouellet *et al.*, 2001; Worley *et al.*, 2000). Genetic and biochemical studies indicate that the sequences required for instability reside in domain II of these proteins. The fusion of a 13 amino acid sub-fragment of domain II to firefly luciferase (LUC) confers instability to LUC (Ramos *et al.*, 2001). Further, gain-of-function mutations in individual members of the *Aux/IAA* family result in a decrease in auxin response (Fukaki *et al.*, 2002; Hamann *et al.*, 2002; Nagpal *et al.*, 2000; Reed, 2001; Rogg *et al.*, 2001; Rouse *et al.*, 1998; Tatematsu *et al.*, 2004; Tian and Reed, 1999). In every case the mutation lies within domain II and in those instances where it has been tested, the mutation stabilizes the affected protein.

These results indicate that changes in Aux/IAA stability are a key aspect of auxin regulation. This has been confirmed by genetic and biochemical studies implicating the ubiquitin–proteasome pathway in auxin response (Dharmasiri and Estelle, 2002; Kepinski and Leyser, 2002). Ubiquitin is a small protein which is conjugated to other proteins through the sequential action of three enzymes called a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3) (Hershko and Ciechanover, 1998). The E3 enzyme is responsible for binding both the E2 and the substrate protein and facilitating formation of a isopeptide bond between the C-terminus of ubiquitin and a lysine on the substrate. Ubiquitin-conjugated substrate proteins are typically recognized and degraded by the 26S proteasome.

The importance of this pathway to auxin response was illustrated by the discovery of a ubiquitin-protein ligase called SCF^{TIR1} (Gray *et al.*, 1999, 2001). SCF-type E3s are composed of four subunits: a cullin, SKP1 (or ASK in Arabidopsis), RBX1, and an F-box protein. The cullin, SKP1, and RBX1 subunits are common among many different SCFs. The F-box protein interacts with the protein targets of the ubiquitin pathway and therefore confers specificity to the complex. In the case of SCF^{TIR1}, the F-box protein is called TIR1. Mutations in the *TIR1* gene confer resistance to auxin, suggesting that the substrates of SCF^{TIR1} are repressors of auxin response (Ruegger *et al.*, 1998). This was confirmed by studies showing that the Aux/IAA proteins are stabilized in a *tir1* background (Gray *et al.*, 2001). Further, three different Aux/IAA proteins, SHY2/IAA3, AXR2/IAA7, and AXR3/IAA17 directly interact with SCF^{TIR1} in an auxin-dependent manner (Gray *et al.*, 2001; Tian *et al.*, 2003). These results indicate that auxin response depends on the auxin-dependent degradation of Aux/IAA proteins.

By screening for Arabidiopsis mutants with resistance to exogenous auxin we have identified a number of genes that are required for auxin response. Here we report the isolation and characterization of the *axr5* mutant together with studies of the *AXR5* gene product. We show that *AXR5* encodes the IAA1 protein, another member of the Aux/IAA protein family. As for the other members of the family, an amino acid substitution in domain II results in a gain-of-function auxin-resistant phenotype as well as diverse defects in auxin-regulated growth and development. Biochemical studies indicate that AXR5/IAA1 interacts with SCF^{TIR1} in an auxin-dependent manner.

Results

Isolation and genetic characterization of the axr5-1 *mutant*

We have used screens for auxin-resistant Arabidopsis seedlings to identify numerous genes involved in auxin response. In one of these screens we recovered a single allele of a novel gene called *AUXIN RESISTANT 5* (*AXR5*). Genetic analysis showed that allele, designated *axr5-1*, is dominant over wild type, and maps to the long arm of chromosome 4 (data not shown). To obtain a more accurate map position we crossed homozygous *axr5* (*Col-0*) plants to homozygous *ga1* (*Ler*) plants. Auxin-sensitive F_2 seedlings were identified and analyzed with PCR markers. The results are summarized in Figure 1.

axr5-1 *plants display altered auxin responses*

To further investigate the effects of the mutation, we examined several auxin responses in both wild-type and *axr5-1* plants. First, the effects of various concentrations of auxin on root growth were examined. The results, shown in Figure 2(a), indicate that *axr5-1* seedlings are resistant to IAA. The concentration required for 50% inhibition of root growth is approximately 0.7 μM for *axr5-1* compared with approximately 0.05 μM for wild-type seedlings. This level of resistance is similar to that observed for the *axr1* mutants, but less than that of the *axr2* or *axr3* mutants (Leyser *et al.*, 1996; Lincoln *et al.*, 1990; Wilson *et al.*, 1990). Similar

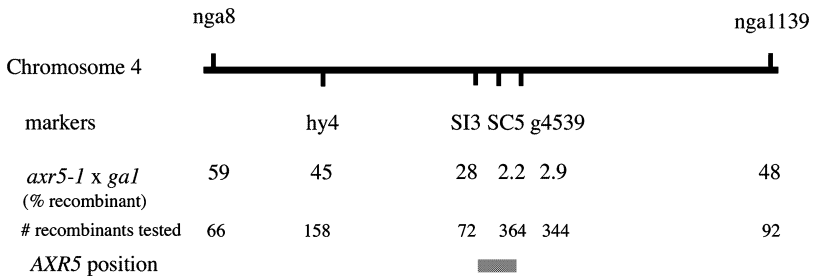

Figure 1. Mapping of the *AXR5* gene. A mapping population was derived from the cross *axr5-1* × *ga1-1* and characterized using the markers shown as described in Experimental procedures.

responses to the synthetic auxins 2,4-D and 1-NAA were also observed (data not shown). The 50% inhibition levels for 2,4-D were 0.04 and 0.15 μM in the wild type and *axr5-1*, respectively and for 1-NAA, 0.02 μM for wild type and 0.2 μM for *axr5-1*.

Exogenous auxin also inhibits hypocotyl elongation of dark-grown seedlings. To determine if *axr5-1* affects this response we grew wild-type and mutant seedlings on various concentrations of IAA in the dark. Hypocotyl length was measured after 7 days. When grown on medium with increasing levels of IAA, the length of both wild-type and mutant hypocotyls decreased (Figure 2b). However, wild-type seedlings were much more severely affected, indicating that *axr5-1* reduces auxin response in hypocotyls.

Auxin is an important regulator of lateral root initiation and growth (Casimiro *et al.*, 2003). Figure 3 illustrates the effects of auxin on lateral root formation in wild-type and *axr5-1* seedlings. On medium without auxin, both wild-type and mutant seedlings produce a similar number of lateral roots. However, when grown on media containing increasing concentrations of NAA, fewer lateral roots form on mutant seedlings compared with wild type, indicating that the mutation inhibits auxin induction of lateral roots.

One of the earliest effects of auxin is the rapid increase in transcription of auxin-regulated genes. To investigate the effects of *axr5-1* on auxin-regulated gene expression, we examined expression of three members of the *Aux/IAA* family in mutant and wild-type seedlings. The results shown in Figure 4(a,b) indicate that *axr5-1* plants display altered auxin-regulated gene expression although these differences are modest compared with the effects of some other *aux/iaa* mutations (Fukaki *et al.*, 2002; Park *et al.*, 2002; Rogg *et al.*, 2001; Tatematsu *et al.*, 2004; Timpte *et al.*, 1994). In the case of *IAA1* and *IAA5*, RNA levels were reduced in mutant plants in both buffer and auxin treated tissue. In contrast, the levels of *IAA2* RNA were similar in wild-type and mutant seedlings. To further analyze the effects of the mutation on auxin-regulated transcription, we crossed the auxin-responsive reporter *BA3*::GUS into *axr5-1* plants (Oono *et al.*, 1998). Wild-type and mutant seedlings were treated with 1 μM IAA for 2 h followed by staining for GUS activity. As reported earlier, staining was observed in the root elongation zone of wild-type seedlings after auxin treatment (Oono *et al.*, 1998).

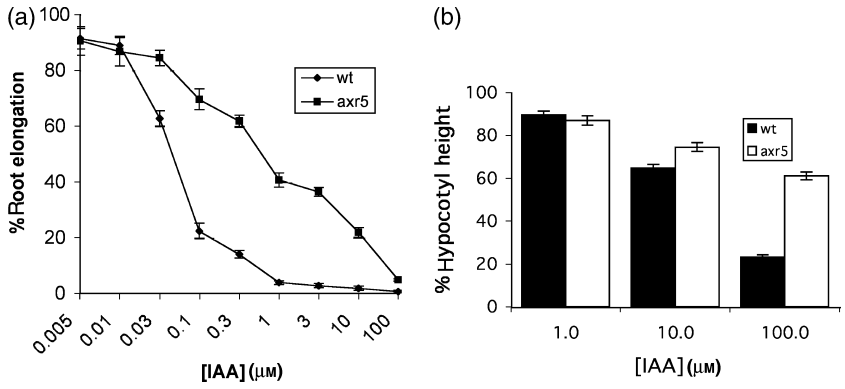

Figure 2. Effect of auxin on *axr5-1*and *Col-0* seedling growth.
(a) Effect of IAA on root elongation. Four-day-old seedlings were transferred from hormone-free plates to vertical plates containing different concentrations of hormone and the additional root elongation measured after 3 days of growth. At least 10 roots were measured for each point. Mean elongation on hormone-free plates for wild type was 20.1 ± 0.47 mm and for *axr5* was 25.7 ± 0.62 mm. Differences for all concentrations greater than 0.01 μM and less than 100 μM were highly significant ($P < 0.001$).
(b) Effect of IAA on hypocotyl elongation of dark-grown seedlings. Seedlings were germinated in the dark on *Arabidopsis thaliana* medium + 1% sucrose medium (Lincoln *et al.*, 1990) without sucrose containing the indicated concentrations of IAA and measured after 7 days ($n = 25$ for each point). Mean elongation on hormone-free plates in the same experiment for wild-type seedlings was 17.5 ± 1.7 mm and for *axr5* was 22.9 ± 2.9 mm. Differences at 10 and 100 μM were highly significant ($P < 0.001$).

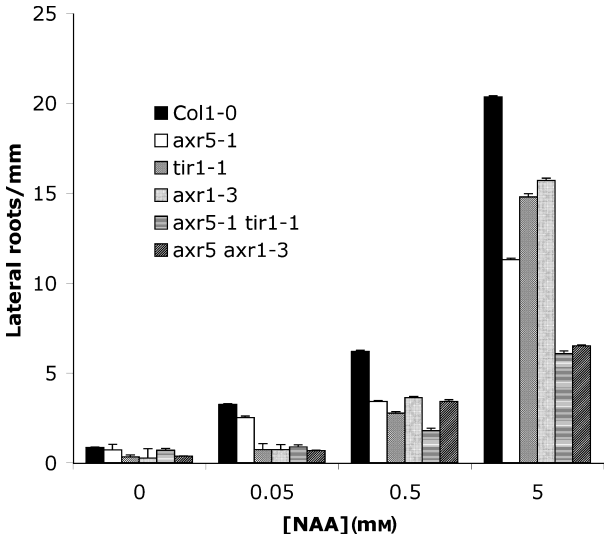

Figure 3. Lateral root formation in response to auxin. Four-day-old seedlings were transferred to media containing various concentrations of 1-NAA. After 5 days, the number of emerged lateral roots was counted and expressed relative to the length of the root. At least 20 seedlings were used for each treatment. Error bars represent standard errors.

No such staining was observed in the *axr5-1*mutant. Staining was also observed in the hypocotyl, the cotyledons and apical region of wild-type seedlings. Unlike the root expression, this staining was not affected by the *axr5-1* mutation.

The axr5-1 *mutants have a pleiotropic auxin-related phenotype*

The *axr5-1* mutant exhibits diverse defects in growth and development, some of which are illustrated in Figure 5. Mutant seedlings are normal in appearance except that the hypocotyls of dark-grown seedlings are slightly longer than the wild type and lack the characteristic apical hook (Figure 5a,b). Other auxin-resistant mutants including *axr1*, *axr2*, *axr3*, and *shy2*, also lack an apical hook. The rosette leaves of *axr5-1* plants are smaller than those of wild type and have shorter petioles (Figure 5c,d). When grown in continuous light, mutant plants flower at the same time as wild type (data not shown). However, the mature *axr5-1* inflorescence is shorter than the wild-type inflorescence (Table 1, Figure 5g,h). There are fewer lateral branches on the primary inflorescence of *axr5-1* plants, but more inflorescence branches growing from the rosette (Table 1). *axr5-1* flowers are normal in appearance but produce fewer seeds (Table 1).

Auxin plays an important role in both gravitropism and phototropism. To determine if the *axr5* mutants are deficient in these processes, we examined gravitropism in the root and shoot as well as shoot phototropism. Figure 5(i) reveals a modest but significant decrease in the gravitropic response of *axr5* seedling roots compared with wild type. The effect of the mutation on hypocotyl tropisms was more dramatic (Table 2). The response of two other auxin-related mutants, *axr1* and *nph4*, are shown for comparison. Both of these mutants have been shown previously to be affected in shoot tropisms (Stowe-Evans *et al.*, 1998; Watahiki *et al.*, 1999). The results in Table 2 show that *axr1*, *nph4*, and *axr5-1* are all strongly deficient in phototropism and gravitropism.

Mutations in AXR1 or TIR1 enhance the axr5 phenotype

The TIR1 protein is a component of SCF^{TIR1}, a ubiquitin protein ligase required for auxin response (Gray *et al.*, 1999). AXR1 is a subunit of a heterodimeric RUB-activating enzyme, required for normal SCF^{TIR1} function (del Pozo *et al.*, 1998). To investigate a possible role for SCF^{TIR1} in AXR5 function, we generated *axr5-1 tir1-1* and *axr5-1 axr1-3* double mutant plants. The effects of auxin on root growth were similar for all three single mutants and each double mutant (data not shown). However, additive effects were observed with respect to auxin induction of lateral root formation (Figure 3). Both *axr5-1 tir1-1* and *axr5-1 axr1-3* produced fewer lateral roots in response to auxin than the single mutants. In addition, the combination of *axr5-1* and *axr1-3* had a clear effect on rosette morphology (Figure 5e,f). The rosette leaves of double mutant plants were smaller and more distorted than either single mutant.

AXR5 encodes the Aux/IAA protein IAA1

The phenotype and genetic behavior of *axr5-1* is similar in many respects to a number of other auxin-resistant mutants including *axr2*, *axr3*, *shy2*, *iaa28*, and *msg2* (Liscum and Reed, 2002). As each of these genes encodes a member of the Aux/IAA family of proteins, we wondered if *AXR5* might also be an *Aux/IAA* gene. There are two *Aux/IAA* genes in the vicinity of *AXR5, At4g14560* and *At4g14550*. *At4g14560* encodes the IAA1 protein, previously shown to be rapidly induced by auxin treatment (Abel *et al.*, 1995). The protein product of *At4g14550* is the SOLITARY-ROOT/IAA14 (SLR/IAA14) protein (Fukaki *et al.*, 2002). Sequencing of both genes revealed a C–T transition in *At4g14560* resulting in substitution of a serine for proline at position 61 of the protein (Figure 6a). No mutations were identified in *SLR/IAA14*. Proline 61 lies within the highly conserved domain II of the protein, the same region that is affected in gain-of-function mutations in other *Aux/IAA* genes.

To determine the pattern of *AXR5/IAA1* expression we performed RT-PCR analysis using RNA extracted from various plant tissues. The results in Figure 6(b) show that the gene is expressed in all tissues examined including seedlings, rosette leaves, inflorescence, and flowers. The highest level of *IAA1* expression is observed in flowers.

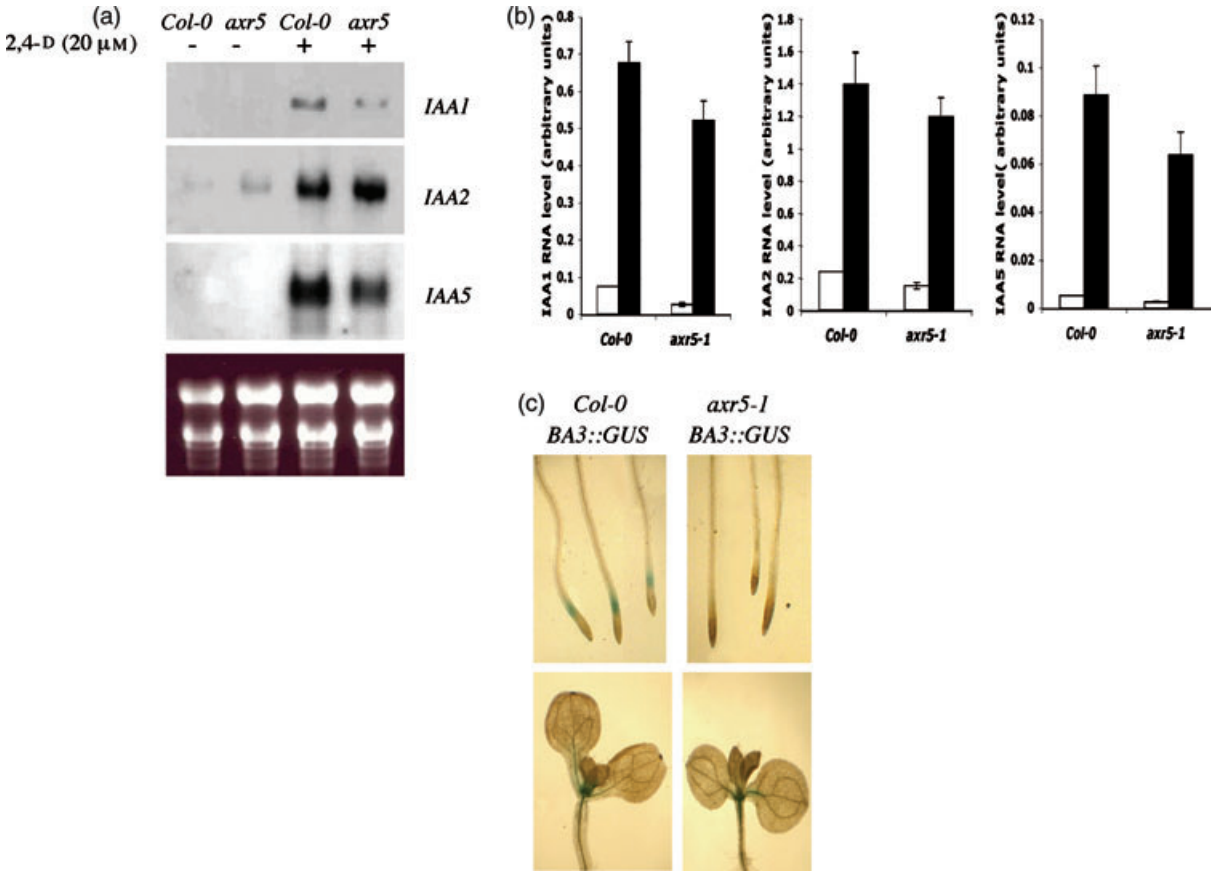

Figure 4. Auxin-regulated gene expression in *axr5-1* plants.
(a) RNA blot analysis of auxin-induced gene expression. *Col-0* and *axr5-1* seedlings were exposed to either buffer or 20 μM 2,4-D for 1 h prior to RNA extraction. Total RNA (10 μg) was loaded in each lane. The bottom panel shows ethidium bromide-stained rRNA to demonstrate equal loading.
(b) A different set of RNA blots was prepared as in (a) except that each blot was also hybridized to an actin probe. RNA levels were quantified by phosphorimaging and expressed relative to actin RNA levels. The experiment was repeated three times. Error bars represent SD. The differences between auxin treated *Col-0* and *axr5-1* are significant as assessed by Student's *t*-test ($P < 0.001$ for *IAA1* and <0.005 for *IAA5*). In the case of *IAA2*, the differences are not statistically significant.
(c) Six-day-old seedlings were treated with buffer or 1 μM IAA for 2 h prior to staining for GUS activity.

AXR5/IAA1 *interacts with SCF^{TIR1}*

The Aux/IAA proteins are degraded by the ubiquitin–proteasome pathway (Dharmasiri and Estelle, 2002). Three members of the family, SHY2/IAA3, AXR2/IAA7 and AXR3/IAA17, have been shown to interact directly with the ubiquitin-protein ligase SCF^{TIR1} in an auxin-dependent manner. To determine if AXR5/IAA1 is also a substrate for SCF^{TIR1}, we performed an *in vitro* pull-down experiment by adding recombinant AXR5-GST protein to extracts prepared from Arabidopsis seedlings expressing a TIR1-myc protein. The GST-pulldown was performed in the presence of 0, 0.5 μM, or 50 μM 2,4-D and analyzed by SDS-PAGE and immunoblotting. The results in Figure 7(a) show that GST-AXR5 associates with TIR1-myc, suggesting that like SHY2, AXR2 and AXR3, AXR5 is a substrate for SCF^{TIR1}. Treatment with auxin dramatically stimulated the amount of SCF^{TIR1} recovered in the pulldown. When a similar experiment was performed with the mutant GST-AXR5-1, little or no TIR1-myc was recovered in the pulldown, indicating that replacement of proline 61 with serine prevents the interaction between AXR5/IAA1 and the SCF.

To determine the rapidity of the auxin response, GST-pulldowns were performed in the presence of 50 μM auxin added at various lengths of time prior to recovery of AXR5-GST. The results in Figure 7(b) show a clear increase in the recovery of TIR1-myc after 5 min of auxin treatment. By 30 min the recovery of TIR1-myc had reached the maximum.

Discussion

The IAA1 *protein functions in diverse aspects of plant growth and development*

Genetic screens have resulted in the recovery of dominant (gain-of-function) mutations in nine members of the *Aux/IAA* gene family (Liscum and Reed, 2002; Tatematsu *et al.*, 2004). Although the primary screens have focused on

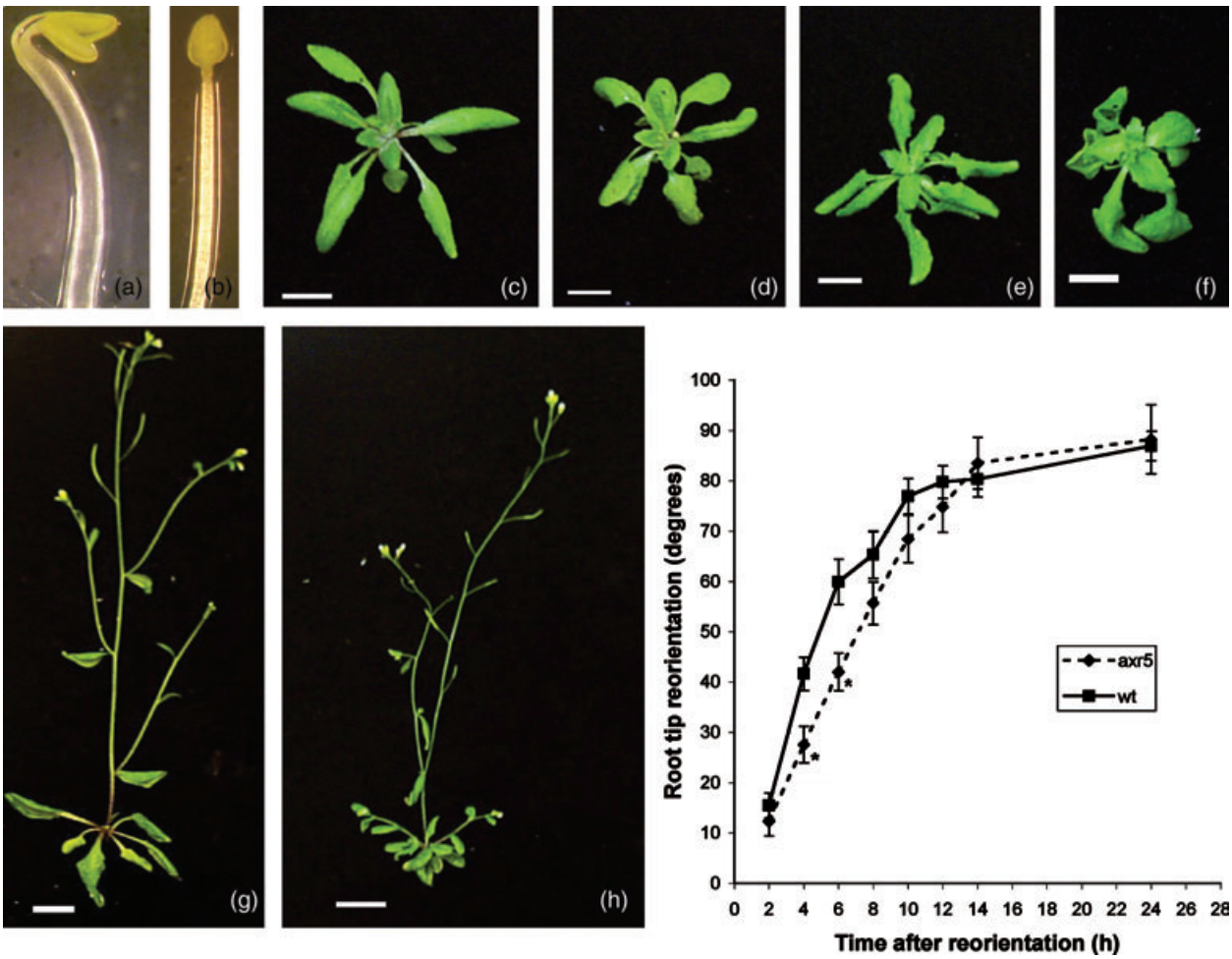

Figure 5. Morphology and root gravitropic response of *Col-0* and *axr5-1* plants.
(a,b) Four-day-old dark-grown *Col-0* (a) and *axr5-1* (b) seedlings.
(c–f) Twenty-day-old *Col-0* (c), *axr5-1* (d), *axr1-3* (e), and *axr5-1 axr1-3* (f) plants.
(g,h) Thirty-eight-day-old *Col-0* (g) and *axr5-1* (h) plants.
(i) Root tip reorientation. Seedlings on vertically oriented hormone-free plates were turned by 90° and the angle of the root tip measured subsequently. '90°' represents a root tip that is now growing directly downward.

different phenotypes, each of the mutants is characterized by a defect in auxin response. Most strikingly, each mutant protein has an amino acid substitution in conserved domain II of the protein. A variety of experiments have shown that domain II contains an auxin-dependent degradation signal (Dharmasiri *et al.*, 2003; Ramos *et al.*, 2001).

Table 1 Morphometric characterization of mature *Col-0* and *axr5-1* plants

	Col-0	*axr5-1*
Height of main inflorescence (cm)	54.3 ± 2.6	36.5 ± 1.5
Number of branches on main inflorescence	4.4 ± 0.3	3.7 ± 0.3
Number of inflorescences	4.3 ± 0.2	5.3 ± 0.3
Number of siliques on main inflorescence	652 ± 62	1289 ± 100
Number of seeds/silique	57 ± 3	36 ± 5

Differences between the two genotypes are statistically significant ($P << 0.001$ for all categories except number of branches on the main inflorecence. In this case $P < 0.05$).

Table 2 Hypocotyl tropic responses in *Col-0* and mutant genotypes

Genotype	Phototropic curvature (degrees)	Gravitropic curvature (degrees)
Col-0	56.2 ± 2	55 ± 2
axr5-1	13 ± 3	13 ± 2
axr1-12	5 ± 3	38 ± 3
nph4-4	13 ± 3	13 ± 2

For phototropic measurement, 60-h-old dark-grown seedlings were exposed to unilateral blue light (0.01 μmol m^{-2} sec^{-1}) for 3 h. Gravitropic curvature was determined after 60-h-old dark-grown seedlings were rotated 90° and incubated for an additional 24 h.

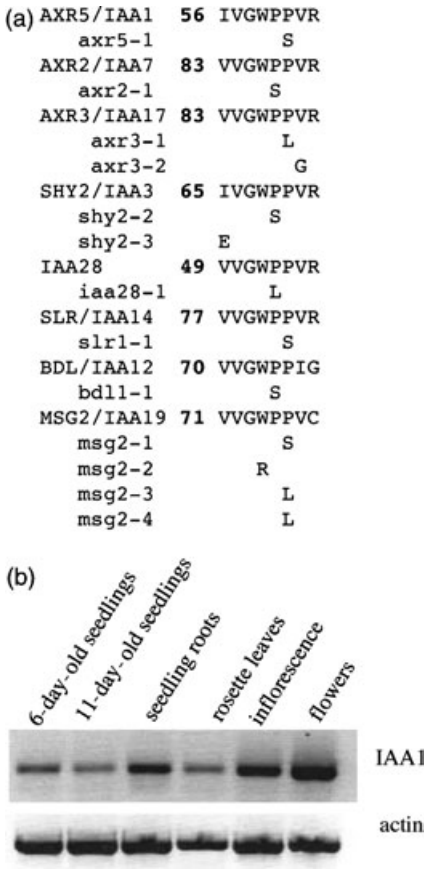

Figure 6. (a) Amino acid substitutions conferred by gain-of-function mutations in *IAA1* and other *Aux/IAA* genes (Fukaki *et al.*, 2002; Hamann *et al.*, 2002; Reed, 2001; Tatematsu *et al.*, 2004).
(b) *IAA* levels in various *Col-0* tissues as measured by RT-PCR.

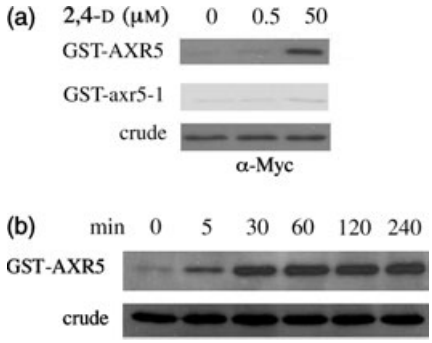

Figure 7. Interaction between AXR5/IAA1 and SCF^{TIR1}.
(a) Protein extracts were prepared from *GVG::TIR1-myc* seedlings. GST-IAA1 (3–4 μg) was added to each sample plus 2,4-D. GST-pulldowns were analyzed by SDS-PAGE and TIR1-myc was detected by immunoblotting. The lower panel shows TIR1-myc levels in the extracts.
(b) Experiment was performed as in (a) except that 50 μM 2,4-D was added to the extract at indicated times before recovery of GST-IAA by centrifugation.

Thus, the phenotypes of the dominant *aux/iaa* mutations are caused by the failure to degrade the mutant protein in response to auxin. In this report, we show that the auxin response mutant *axr5-1* is affected in the *Aux/IAA* gene *IAA1*. Like the other dominant mutants recovered in this family, the mutation results in an amino acid substitution within domain II of the protein. Based on these results we propose that IAA1/AXR5 is also a repressor of auxin response and that the mutation prevents auxin-dependent degradation. Consistent with our results, Park *et al.* (2002) have recently shown that transgenic plants expressing IAA1 proteins with amino acid substitutions in domain II exhibit diverse auxin-related defects.

Although the phenotype of each dominant *aux/iaa* mutant is distinct, many mutants have similar defects (Liscum and Reed, 2002). The most extreme mutant is *bdl/iaa12* with severe defects in embryogenesis (Hamann *et al.*, 2002). The other mutants do not display gross embryonic defects but exhibit a variety of other defects including effects on hypocotyl elongation, leaf development, tropisms, and repression of light-regulated development (Liscum and Reed, 2002). Presumably some of the phenotypic differences are related to differences in expression of the various genes. However, it is also possible that each Aux/IAA protein represses the activity of a specific group of ARFs resulting in specific effects on auxin-regulated transcription.

The *IAA1* gene is expressed throughout development with the highest level of RNA accumulation in roots, inflorescences, and flowers. In addition, the gene is induced rapidly by auxin and expression remains high after prolonged exposure to auxin. These observations suggest that IAA1 has a broad role in many auxin-regulated processes. This suggestion is supported by our characterization of the *axr5/iaa1* phenotype. Mutant plants are affected in root elongation, lateral root formation, hypocotyl elongation, root and shoot tropisms, leaf morphology, and inflorescence structure. The limitations inherent in the interpretation of gain-of-function mutations make firm conclusions difficult. However, we speculate that auxin regulation of these processes normally involves degradation of IAA1. At the very least, it is clear that preventing auxin-dependent degradation of IAA1 inhibits diverse processes in the plant.

It is noteworthy that *axr5* is deficient in hypocotyl phototropism. A similar phenotype was recently reported for the *iaa19/msg2* mutant, suggesting that degradation of Aux/IAA proteins is required for both gravitropic and phototropic responses (Tatematsu *et al.*, 2004). Loss of the ARF protein NPH4 results in defects in phototropism and IAA19 and NPH4 interact in a co-immunoprecipitation experiment suggesting that IAA19 represses NPH4-dependent gene expression (Tatematsu *et al.*, 2004). Although we have not tested for the interaction between IAA1 and NPH4, based on the mutant phenotype it is possible that both IAA1 and IAA19 repress NPH4 function. However, IAA1 and IAA19 are relatively divergent members of the Aux/IAA proteins family

(37% identical) and it is possible that IAA1 regulates other ARFs involved in tropic growth.

IAA1 *represses expression of some, but not all* Aux/IAA *genes*

Consistent with the visible phenotype of *axr5* plants, we find that expression of several auxin-regulated genes is altered in the mutant. Auxin-induction of *IAA1*, *IAA5*, and the *BA3*::GUS reporter was reduced in *axr5* seedlings. These results suggest that IAA1 normally represses its own synthesis as well as *IAA5* and presumably other auxin-regulated genes. In contrast, the *axr5* mutation does not dramatically affect expression of *IAA2* suggesting that this gene is not normally regulated by IAA1.

Park *et al.* (2002) also examined the effects of ectopic expression of a domain II mutant of IAA1. In their study, Pro 60 of IAA1 was replaced with Leu and the protein fused to the glucocorticoid hormone-binding domain (GR). The fusion protein was introduced into wild-type plants under control of the *CaMV 35S* promoter. Upon dexamethasone treatment the expression of all *Aux/IAA* genes tested was reduced including *IAA2*. The different behavior of *IAA2* in the two studies may reflect differences between endogenous versus *CaMV 35S*-driven expression of the mutant *IAA1* gene. Alternatively, it is possible that the Pro 60 substitution has a greater effect on IAA1 stability than Pro 61 substitution. Whatever the case both our study and that of Park *et al.* (2002), provide strong evidence that IAA1 is a repressor of auxin-dependent transcription.

IAA1 *is a substrate for* SCF^{TIR1}

Previous studies indicate that domain II of the Aux/IAA proteins contains an auxin-dependent degradation signal (Dharmasiri *et al.*, 2003; Gray *et al.*, 2001; Ramos *et al.*, 2001; Zenser *et al.*, 2001). Consistent with this we have recently shown that a peptide encompassing domain II will interact with SCF^{TIR1} (Dharmasiri *et al.*, 2003). Our current results indicate that like IAA2, IAA7 and IAA17, IAA1 interacts directly with SCF^{TIR1} in an auxin-dependent manner. The *axr5* mutation dramatically reduces this interaction confirming that domain II of IAA1 is required for interaction with the SCF.

The presence of a conserved domain II in 24 members of the Aux/IAA protein family suggests that each of these proteins is subjected to SCF-dependent degradation. However, how this degradation is regulated remains uncertain. In addition, it is not clear if SCF^{TIR1} is responsible for degradation of all Aux/IAA proteins or whether there may be other SCFs that also contribute to Aux/IAA regulation. This issue is addressed in part by our genetic data. The AXR1 protein, and the rest of the RUB conjugation pathway, is thought to be important for the function of many different SCFs (Hellmann and Estelle, 2002). The fact that the *axr1* mutation enhances the *axr5* phenotype to a greater extent than does *tir1* suggests that additional SCFs are regulating auxin response. TIR1 is part of a small subclade of proteins that includes six other proteins and it is possible that several of these are also involved in Aux/IAA degradation (N. Dharmasiri, S. Dharmasiri, M. Estelle, unpubl. Obs.). Further studies are required to determine if any of these also participate in IAA1 degradation.

Experimental procedures

Plant material and growth conditions

All lines used in this study were in the *Columbia* ecotype. The *BA3*::GUS line was obtained from *A. theologis*. Seeds were surface sterilized and grown on *Arabidopsis thaliana* medium + 1% sucrose (ATS) plates under 16 h light/8 h dark conditions at 22°C (Lincoln *et al.*, 1990). For root growth assays, 4-day-old seedlings were transferred onto ATS plates with or without various auxins. Root lengths and number of lateral roots were measured after 3–5 days depending on the experiment. Plants were grown as described previously (Hobbie *et al.*, 2000).

Phenotypic characterization

For morphometric measurements, seeds were sown directly into pots containing wetted MetroMix360 at a density of five seeds per pot. Seeds were cold-treated for 5 days, then transferred to a growth room, and subsequently fertilized with *A. thaliana* medium (without sucrose) once a week. Plants were grown at 20–23°C with continuous light at a photon fluence rate of 70–120 μmol m^{-2} sec^{-1}. For root gravitropism measurements, seedlings were germinated on vertically oriented hormone-free ATS plates and transferred after 4 days to square hormone-free ATS plates. After 3 additional days of growth in a vertical orientation, plates were turned by 90°, photographed, and then photographed at intervals thereafter. Photos were scanned and analyzed to determine root tip reorientation.

Measurement of hypocotyl tropisms

Surface-sterilized seeds were planted on 0.8% agar (w/v) medium containing 0.5-strength MS salts and placed in darkness at 4°C for 3 days. The cold-treated seeds were then allowed to germinate at 22°C in darkness for 60 h. For phototropism, the seedlings were grown in Magenta boxes and for gravitropism the seedlings were grown in vertically oriented Petri dishes so the hypocotyls grew along the surface of the agar. For phototropism experiments, the Magenta boxes with 60-h-old seedlings were placed in a black box that had a blue interference filter (450 ± 10 nm, 03FIV028; Melles Griot, Rochester, NY, USA) on one side. Light from a cool-white fluorescent bulb was passed through the blue interference filter to provide 0.01 μmol m^{-2} sec^{-1} of blue light at the center of the Magenta box. When red (660 ± 25 nm) or far-red light (730 ± 25 nm) was used as a pre-treatment the seedlings received 5 min red (3 μmol m^{-2} sec^{-1}) or far-red light (1.0 μmol m^{-2} sec^{-1}) from an LED array (QB1310CS; Quantum Devices, Barneveld, WI, USA) 1 h before receiving the unilateral blue light treatment. Curvature was measured from images of the hypocotyls captured after 3 h of exposure to the unilateral blue light. For gravitropism, the

Petri dishes were rotated 90° to gravistimulate the seedlings and then kept in darkness for 24 h. Images of the seedlings were then made and the angle of gravicurvature was measured. Each experiment was replicated at least three independent times with each replicate consisting of five to eight seedlings and data are presented as mean ± SE.

Mapping of the AXR5 *gene*

axr5 plants (Columbia background) were crossed to Landsberg erecta, auxin-sensitive F_2 plants identified, and DNA from 24 F_3 families scored with the ARMS set of RFLP markers (Fabri and Schaffner, 1994). Linkage was found to markers m448, m326, and d104, all on chromosome 4. For fine mapping, *axr5* was crossed to *ga1-1* (Landsberg ecotype) and 661 auxin-sensitive F_2s were isolated out of 4284 total F_2 seedlings. F_3 seedlings were rescreened to verify auxin sensitivity. DNA from F_3 families was scored with the following existing CAPS and SSLP markers from chromosome 4 (http://www.arabidopsis.org; Bell and Ecker, 1994): GA1, nga8, HY4, g4539, SC5, nga1139. Marker SI3 was developed based on an insertion/deletion from the Cereon SNP collection at position 8608454 on chromosome 4. Primers SI3-L (aaccatccgaggatccaaat) and SI3-R (tcaggtattatttgctttccatga) amplified a 150-bp band in Col and a 114-bp band in Ler.

GUS assays

Six-day-old seedlings were treated with ATS or ATS +1 μM IAA for 2 h followed by three washes with staining buffer lacking 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc, Sigma, St. Louis, MO, USA) (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.5 mM K_4 Fe[CN]$_6$, 0.5 mM K_3 Fe[CN]$_6$, and 0.1% Triton X-100), and then incubated for 18 h in staining buffer containing 1 mM X-gluc. To remove chlorophyll from green tissues, seedlings were incubated in 70% ethanol.

RNA blots and RT-PCR analysis

To study the expression of *IAA1*, total RNA was extracted from seedlings growing in liquid culture, or from adult tissues. To study the auxin regulation of the *IAA1*, *IAA2*, and *IAA5* genes, 6-day-old *axr5-1* and wild-type (*Col-0*) seedlings were treated with or without 20 μM 2,4-D for 60 min. Total RNA was extracted using Tri-reagent (Sigma), and 10 μg RNA was loaded on each lane. The entire coding region of *IAA1*, *IAA2*, or *IAA5* cDNA was used as a probe. For RT-PCR analysis, cDNA was synthesized from 10 μg of total RNA. The actin probe was prepared by amplifying a 643-bp fragment of a cDNA corresponding to At1g49240 using the following primers: 5′-gtggtcgtacaaccggtattgtgtt-3′ and 5′-cttagagatccacatctgctggaa-3′. Hybridized blots were exposed to Storage Phosphor Screen (Molecular Dynamics, Piscataway, NJ, USA), scanned using Typhoon 9200 Image scanner (Amersham Biosciences, Piscataway, NJ, USA) and the strength of the ^{32}P signals in each band was measured by the IMAGE QUANT program version 5.2 (Molecular Dynamics, Piscataway, NJ, USA).

IAA1 expression, GST-pulldowns, and protein blots

To generate the IAA1/AXR5 expression plasmid, a 500 bp fragment containing the *IAA1*cDNA was subcloned into the SmaI site of pGEX-2TK (Invitrogen, CA, USA). The *axr5-1* mutation was created in this construct by oligonucleotide-directed mutagenesis. To purify GST-IAA1 and GST-axr5-1, overnight cultures (25 ml) of *Escherichia coli* (DH5α) carrying the recombinant plasmids were inoculated into 250 ml of liquid LB and incubated at 30°C for 2 h. IPTG was then added to a final concentration of 1 mM and the culture was incubated at 30°C for a further 4 h. Bacteria were pelleted at 9500 ***g*** for 10 min. and the pellet was resuspended in 7 ml of PBS. The cells were lysed by sonication. PMSF and Tween 20 were added to the extract at 1 mM and 0.1% (v/v), respectively. Cell debris was removed by centrifugation at 9500 ***g*** for 10 min. Glutathione–agarose (Sigma) beads that were pre-equilbriated in PBS were added to the supernatant and incubated at 4°C for 3 h with gentle agitation. Beads were recovered by centrifugation and washed three times each for 15 min with 10 ml PBS containing 0.5% Tween 20. Washed beads were resuspended in 250 μl of PBS + 1 mM PMSF. Approximately 3–4 μg of GST-IAA1 were used in pull-down reactions. For pull-down assays, 3–4 μg of GST-IAA1 was incubated with 800 μg of crude Arabidopsis extract for 3 h at 4°C with gentle agitation. 2,4-D was added directly added to the reaction. At the end of incubation, glutathione beads were recovered by centrifugation and washed three times for 15 min with 1 ml of extraction buffer except for MG132 and protease inhibitors (Dharmasiri *et al.*, 2003). Beads were resuspended in 2× Laemlli buffer and proteins were resolved on 10% SDS-PAGE and transferred on to PVDF membrane (Bio-Rad, CA, USA). Proteins were detected by western blot analysis using α-myc antibody and enhanced chemiluminescence as described by the manufacturer (Amersham). For the time course experiment, all samples were incubated for a total of 4 h before recovery of the glutathione beads by centrifugation. Auxin was added at time intervals prior to centrifugation.

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