AXR2 Encodes a Member of the Aux/IAA Protein Family

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The dominant gain-of-function axr2-1 mutation of Arabidopsis causes gravitropic root and shoot growth, a short hypocotyl and stem, and auxin-resistant root growth. We have cloned the AXR2 gene using a map-based approach, and find that it is the same as IAA7, a member of the IAA (indole-3-acetic acid) family of auxin-inducible genes. The axr2-1 mutation changes a single amino acid in conserved domain II of AXR2/IAA7. We isolated loss-of-function mutations in AXR2/IAA7 as intragenic suppressors of axr2-1 or in a screen for insertion mutations in IAA genes. A null mutant has a slightly longer hypocotyl than wild-type plants, indicating that AXR2/IAA7 controls development in light-grown seedlings, perhaps in concert with other gene products. Dark-grown axr2-1 mutant plants have short hypocotyls and make leaves, suggesting that activation of AXR2/IAA7 is sufficient to induce morphological responses normally elicited by light. Previously described semidominant mutations in two other Arabidopsis IAA genes cause some of the same phenotypes as axr2-1, but also cause distinct phenotypes. These results illustrate functional differences among members of the Arabidopsis IAA gene family.

Auxin (indole-3-acetic acid [IAA]) mediates numerous aspects of plant growth and development including stem elongation, lateral root initiation, gravitropism, and apical dominance. Auxin is probably made in young leaves and possibly in the root meristem (Thimann, 1977), and is transported throughout the plant. This transport may result in gradients of auxin concentration (Sachs, 1991; Uggla et al., 1998), which then help to pattern embryonic, vascular, and meristem development (Goldsmith, 1977; Thimann, 1977; Sachs; Liu et al., 1993; Przemeck et al., 1996; Sabatini et al., 1999). Auxin has different effects on different tissues. It can stimulate cell elongation in stems, promote cell divisions leading to lateral root formation, or stimulate vascular, and meristem development (Goldsmith, 1977; Sachs, 1977). Auxin may be made in young leaves and possibly in the root meristem (Thimann, 1977), and is transported throughout the plant. This transport may result in gradients of auxin concentration (Sachs, 1991; Uggla et al., 1998), which then help to pattern embryonic, vascular, and meristem development (Goldsmith, 1977; Thimann, 1977; Sachs; Liu et al., 1993; Przemeck et al., 1996; Sabatini et al., 1999). Auxin has different effects on different tissues. It can stimulate cell elongation in stems, promote cell divisions leading to lateral root formation, or stimulate vascular, and meristem development (Goldsmith, 1977; Thimann, 1977; Sachs; Liu et al., 1993; Przemeck et al., 1996; Sabatini et al., 1999). Auxin has different effects on different tissues. It can stimulate cell elongation in stems, promote cell divisions leading to lateral root formation, or stimulate vascular, and meristem development (Goldsmith, 1977; Thimann, 1977; Sachs; Liu et al., 1993; Przemeck et al., 1996; Sabatini et al., 1999).

Auxin acts in part by regulating gene expression. Known auxin-regulated genes fall in several classes including the Aux/IAA family, the SAUR family, and the GH3 family (Abel and Theologis, 1996). Some of these are transiently expressed within minutes of auxin application, with subsequent desensitization of the response, implying that feedback controls dampen auxin responses (Dominov et al., 1992; Abel et al., 1995). Many of these genes can also be induced by the protein synthesis inhibitor cycloheximide (Franco et al., 1990; Abel et al., 1995; Gil and Green, 1997), suggesting that auxin may derepress transcription and/or regulate mRNA turnover. Some auxin-regulated genes have tissue-specific expression patterns, underscoring the diversity of auxin responses in different tissues (Gee et al., 1991; Lehman et al., 1996; Wong et al., 1996).

Many auxin-regulated genes have a 5′-TGCTCTC-3′ DNA sequence motif in their promoters; in studied cases this motif is required for auxin responsiveness (for review, see Guilfoyle et al., 1998). Proteins encoded by the ARF (auxin response factor) multigene family bind to this or similar DNA motifs (Ulmasov et al., 1997a, 1997b, 1999a, 1999b). ARF proteins have a conserved N-terminal DNA-binding domain, and (except for ARF3) share conserved C-terminal domains III and IV with proteins of the Aux/IAA family (see below). At least in some combinations, ARF proteins can dimerize with Aux/IAA proteins, probably through domains III and IV (Kim et al., 1997; Ulmasov et al., 1997b). Different ARFs can either repress or activate gene expression from auxin-regulated promoters depending on domains located between the DNA-binding and dimerization motifs (Ulmasov et al., 1999a).

Mutations in three different ARF genes have established that they are important for auxin-mediated...
development, and revealed that different ARF factors show specificity in the phenotypes they control. etlin/arf3 mutations affect floral patterning (Sessions et al., 1997), monopteros/arf5 mutations affect formation of vasculature (Przemeck et al., 1996; Hardtke and Berleth, 1998), and msg1/nph4/arf7 mutations decrease auxin sensitivity in the hypocotyl and leaf, and cause defective tropic responses and auxin-regulated gene expression (Liscum and Briggs, 1996; Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998; Harper et al., 2000).

Arabidopsis has at least 20 IAA genes encoding Aux/IAA proteins, and those that have been studied are regulated by auxin (Abel et al., 1995; Kim et al., 1997). Aux/IAA proteins share four conserved domains called I, II, III, and IV. Pea Ps-IAA4 and Ps-IAA6 proteins localize to the nucleus and have half-lives of 6 to 8 min, suggesting that the proteins play transient regulatory roles (Abel et al., 1994; Abel and Theologis, 1995). However, there is no evidence that Aux/IAA proteins bind DNA directly. Yeast two-hybrid screens and in vitro experiments with purified proteins have shown that Aux/IAA proteins can homo- and hetero-dimerize, and suggest that domains III and IV are required for dimerization (Kim et al., 1997; Ulmasov et al., 1997b; Soh et al., 1999). The same studies showed that Aux/IAA proteins can dimerize with ARF proteins, suggesting that Aux/IAA proteins might act by modifying transcriptional regulatory activity of ARFs. Aux/IAA proteins can antagonize auxin-dependent activation of genes in transfected carrot cells (Ulmasov et al., 1997b).

Partially dominant mutations in five Arabidopsis IAA genes, SHY2/IAA3, AXR3/IAA17, MSG2/IAA19, IAR2/IAA28, and SLR/IAA14, cause related but distinct phenotypes including slowed root growth (axr3, shy2, and iar2), few lateral roots (shy2, msg2, iar2, and slr), decreased gravitropism (axr3, shy2, msg2, and slr), and leaf formation in darkness (shy2; Kim et al., 1996; Leyser et al., 1996; Rouse et al., 1998; Rogg et al., 1999; Tian and Reed, 1999; M. Tasaka, personal communication; K. Yamamoto, personal communication). axr3-1 and shy2-2 mutations were deduced to cause a gain of function because they are partially dominant and can be suppressed by intragenic mutations having molecular characteristics of loss-of-function alleles (Rouse et al., 1998; Tian and Reed, 1999).

The axr2-1 mutant was isolated as having auxin-resistant root growth, and has several morphological phenotypes including a short hypocotyl, gravitropic root and shoot growth, and no root hairs (Wilson et al., 1990; Timpte et al., 1992, 1994). Genetic evidence using triploids showed that axr2-1 causes a gain of function (Timpte et al., 1994). We have now cloned AXR2 and find that it encodes another member of the Aux/IAA protein family, IAA7. We have also generated loss-of-function mutations in AXR2/IAA7, and we describe the corresponding mutant plants. We have compared several phenotypes of axr2-1 mutants with those of axr3-1 and shy2-2, and find that the phenotypes differ, implying that the AXR2/IAA7, AXR3/IAA17, and SHY2/IAA3 genes have distinct functions.

**RESULTS**

The axr2-1 Mutation Is in the IAA7 Gene

We mapped AXR2 to BAC T12C21 on chromosome 3 (Timpte et al., 1994; data not shown). Low-stringency hybridization with an IAA2 gene probe and public release of the genomic sequence of the BAC MXC7 (which overlaps with T12C21) revealed that two IAA genes, IAA2 and IAA7, are about 12 kb apart in this region. We sequenced the IAA2 and IAA7 genes from the axr2-1 mutant, and found a C to T missense mutation predicted to change a Pro to a Ser at codon 87 in IAA7 (Fig. 1A). This Pro residue is conserved in all known Aux/IAA proteins, and forms part of conserved domain II. As shown in Figure 1B, semidominant mutations in AXR3/IAA17 and SHY2/IAA3 also affect residues in this domain (Rouse et al., 1998; Tian and Reed, 1999), and in fact the shy2-2 mutation causes the identical change, from Pro to Ser, in the corresponding position of SHY2/IAA3.

![A](image1.png)

![B](image2.png)

**Figure 1.** A, Mutations in AXR2/IAA7. The box represents the coding part of the gene. Dark vertical lines within the box indicate positions of introns. Stippled boxes indicate conserved domains I, II, III, and IV. B, Mutations in conserved domain II of AXR2/IAA7, AXR3/IAA17, and SHY2/IAA3 proteins.

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IAA3. As described below, intragenic suppressing mutations confirm that this mutation in *IAA7* is responsible for the phenotypes of *axr2-1* plants. We refer to the gene as *AXR2/IAA7*, in keeping with the emerging nomenclature for genes of this family identified both by mutation and by sequence homology (Rouse et al., 1998; Tian and Reed, 1999).

Isolation of Loss-of-Function *axr2* Mutations

Previous genetic experiments demonstrated that *axr2-1* causes a gain of function (Timpte et al., 1994). To isolate loss-of-function alleles of *AXR2/IAA7*, we mutagenized *axr2-1* seeds, and screened among M2 progeny for reversion of the short hypocotyl phenotype of *axr2-1* plants (Timpte et al., 1994). We isolated several revertant individuals that proved to have one of two different mutations, one identical to that in the previously described *axr2-1-r3* mutant (Timpte et al., 1994), and another that we call *axr2-1-r4*. These mutations were each dominant to the wild-type *AXR2* allele and recessive to the *axr2-1* allele (Timpte et al., 1994; data not shown), and they were linked to *axr2-1*, suggesting that they were intragenic suppressors of *axr2-1*.

We sequenced the *AXR2/IAA7* gene from these mutants, and found mutations in each of them (Fig. 1A). In *axr2-1-r3*, a G to A mutation changed Arg at position 138 to Lys. In *axr2-1-r4*, a C to T mutation changed Leu at amino acid position 15 to Phe. This change also eliminated a BsmAI restriction site. Each of these mutated amino acids is highly conserved among all known Aux/IAA proteins. Leu-15 is in conserved domain I and Arg-138 is in conserved domain III. These intragenic suppressors of *axr2-1* confirm that the *IAA7* gene is indeed the same as *AXR2*.

Both intragenic suppressor mutants had phenotypes similar to each other and intermediate between those of wild-type and *axr2-1* plants (Timpte et al., 1994; data not shown). For example, *axr2-1-r3* and *axr2-1-r4* root growth was more sensitive to auxin inhibition than was root growth of *axr2-1* plants but less sensitive than was root growth of wild-type plants. Adult *axr2-1-r3* and *axr2-1-r4* plants had an intermediate stature, and their flowering stems grew at an altered angle—less upright than wild-type stems, but more upright than the weeping growth of *axr2-1* mutant plant stems. These phenotypes suggest that these mutations partially decrease the activity of *AXR2-1* protein.

In addition to these intragenic suppressors, we obtained a T-DNA insertion allele of *AXR2/IAA7*. Using a degenerate primer designed to hybridize to DNA encoding conserved domain IV of the IAA proteins, we screened for insertion mutations in a collection of T-DNA lines (Krysan et al., 1996). Among the mutations we found was an insertion in the *AXR2/IAA7* gene. We refer to this mutation as *axr2-5*. To define the end points of the insertion precisely, we PCR-amplified and sequenced a fragment flanking the T-DNA on the 3’ end of the gene. This sequence revealed that the insertion interrupts the second exon at a position corresponding to amino acid 124 in the coding sequence (Fig. 1A). We were not able to amplify the T-DNA junction on the 5’ end of the gene. However, we hybridized a Southern blot of digests of wild-type and *axr2-5* DNA with an *AXR2/IAA7*-specific probe, and found that a wild-type 0.5-kb *NsiI* fragment was shifted in size in the mutant, whereas all other *NsiI* fragments in the *AXR2/IAA7* gene were the same in both the mutant and wild-type DNA (Fig. 2A). This fragment contains the sequenced 3’ T-DNA insertion junction, and its shifted size shows that the *axr2-5* mutant lacks an intact *AXR2/IAA7* gene.

To assess whether the *axr2-5* mutation affects *AXR2/IAA7* transcript levels, we hybridized blots of mRNA from wild-type and *axr2-5* plants with a probe derived from an *AXR2/IAA7* cDNA (Abel et al., 1995). As shown in Figure 2B, this probe recognized two bands in wild-type mRNA, one at the predicted size of about 0.9 kb, and another larger band of about 1.3 kb. We presume that this larger band arose from cross-hybridization to a transcript from another *IAA* gene, as it did not appear when we used a probe derived from the 3′-untranslated part of the *AXR2/IAA7* cDNA (Fig. 2C). In the *axr2-5* mutant, only the 1.3-kb band appeared, indicating that *axr2-5* lacks *AXR2/IAA7* transcript. Together, the disrupted *AXR2/IAA7* gene structure and absence of *AXR2/IAA7* transcript in this mutant show that *axr2-5* is a null mutation.

We also examined expression of *AXR2/IAA7* in light-grown *axr2-1*, *axr2-1-r3*, and *axr2-1-r4* mutant seedlings, and found that the transcript was present at a lower level in *axr2-1* seedlings than in wild-type seedlings (Fig. 2C). Previous data showed that the *AXR2/IAA7* transcript was also expressed at a lower level in dark-grown *axr2-1* mutant seedlings than in wild-type seedlings (Abel et al., 1995). Taken together, these results suggest that *AXR2/IAA7* participates in a negative autoregulatory feedback loop in both light- and dark-grown seedlings. *axr2-1-r3* and *axr2-1-r4* seedlings had an intermediate level of *AXR2/IAA7* transcript (Fig. 2C), consistent with their intermediate phenotypes.

Phenotypes of *axr2-5* Null Mutant Plants

To understand the role of *AXR2/IAA7* in development, we examined morphology of the *axr2-5* null mutant. *axr2-5* plants appeared very similar to wild-type plants at both seedling and adult stages. The kinetics of root growth in light-grown wild-type and mutant plants were almost identical (Fig. 3A). In response to auxin, root growth was inhibited to the same extent in both wild-type and *axr2-5* plants (Fig. 3C), and root gravitropism appeared normal in the
mutant (data not shown). Lateral root numbers were also the same in the two genotypes, 2.9 ± 0.3 versus 3.2 ± 0.2 (n = 57) for wild type versus axr2-5 in 2-week-old seedlings.

Shoots of axr2-5 plants also appeared similar to those of wild-type plants. However, in kinetic studies axr2-5 seedlings had slightly longer hypocotyls than wild-type seedlings did (Fig. 3B). Both hypocotyls and roots of dark-grown axr2-5 seedlings grew at the same rate as those of wild-type seedlings (Fig. 3, D and E), indicating that the long hypocotyls of light-grown axr2-5 seedlings reflect a decreased light response. In constant red or blue light, the increased elongation of axr2-5 seedling hypocotyls was less than in white light, and statistically significant in only a subset of experiments (data not shown). Thus, the phenotype in white light may reflect a combined effect of the mutation on both blue- and red-light response pathways.

**axr2-1 and axr3-1 Mutations Induce De-Etiolated Morphology in Dark-Grown Seedlings**

In contrast to the slightly elongated hypocotyls of axr2-5 seedlings, gain-of-function axr2-1 mutant seedlings have short hypocotyls (Timpte et al., 1994). This phenotype was more apparent in dark-grown than light-grown seedlings. Whereas wild-type seedling hypocotyls were about one-eighth as long as in the light as in the dark, axr2-1 hypocotyls were about one-half as long in the light as in the dark (Timpte et al., 1994). These results indicate that axr2-1 seedlings responded less than wild-type seedlings to light. Relative to elongation in darkness, axr2-1 seedling hypocotyls also responded less than wild-type seedlings to blue, red, or far-red light (Fig. 4A), and an axr2-1 phyB-9 double mutant had a hypocotyl length intermediate between those of the two corresponding single mutants (Fig. 4B). Thus axr2-1 probably does not interfere specifically with function of any particular photoreceptor.

These results suggested that in dark-grown seedlings the axr2-1 mutation might activate a developmental pathway normally activated by light. Consistent with this idea, dark-grown axr2-1 seedlings made leaves. After 6 d growth in the dark, wild-type seedlings have a long hypocotyl and no significant cotyledon or leaf growth. After 23 d in the dark, wild-type seedlings had open hooks, but still failed to develop visible leaves (Fig. 5). In contrast, 6-d-old dark-grown axr2-1 seedlings had short hypocotyls and open and slightly expanded cotyledons (Timpte et al., 1994), and 23-d-old axr2-1 seedlings had true leaves (Fig. 5). On occasion, we also observed floral buds after 23 d of dark growth.

The gain-of-function mutation axr3-1 affecting AXR3/IAA17 caused similar dark phenotypes as the axr2-1 mutation. Thus, axr3-1 seedlings had short hypocotyls in the dark (Leyser et al., 1996) and had a decreased response to red, far-red, or blue light (Fig. 4); and they made leaves in the dark (Fig. 5). The gain-of-function shy2-1, shy2-2, and shy2-3 mutations affecting SHY2/IAA3 also caused similar effects (Kim et al., 1996, 1998; Reed et al., 1998; Tian and Reed, 1999). These results suggest that AXR2/IAA7, AXR3/IAA17, and SHY2/IAA3 can each induce aspects of a de-etiolated developmental program when activated by mutation.
The de-etiolated phenotypes of axr2-1, axr3-1, and shy2-2 were less severe than those of the cop/det/fus class of mutants (Chory et al., 1989; Deng et al., 1991). Thus, whereas axr2-1, axr3-1, and shy2-2 mutants made leaves only after more than 1 week of dark growth, det1-1 plants had significant leaf expansion after 1 week, and had formed more (and larger) leaves than the other mutants after 3 weeks (Fig. 5).

**axr2-1, axr3-1, shy2-2, and shy2-3 Plants Have Different Morphologies**

Despite having a mutation in a residue corresponding precisely or closely to dominant shy2-2 or axr3-1 mutations, the axr2-1 plants differ in several respects from shy2-2 and axr3-1 plants, as summarized in Table I. One possible explanation for the phenotypic differences between axr2-1 or axr3-1 mutants and shy2 mutants could be that, whereas axr2-1 and axr3-1 are each in the Columbia ecotype, shy2-2 and shy2-3 are in the Landsberg erecta ecotype. To test whether these ecotype differences might cause the different effects of the mutations, we generated Columbia/Landsberg erecta F1 hybrids that were heterozygous for each of these dominant or semidominant mutations, and examined their phenotypes. We found that all of the root and adult shoot phenotypes that differed among the mutants also differed among the F1 hybrid plants (Fig. 6). Thus roots of axr2-1/+ and axr3-1/+ plants grew agravitropically and had fewer root hairs than wild-type plants, whereas roots of shy2-2/+ and shy2-3/+ plants grew relatively normally and had abundant root hairs; axr3-1/+ plants, but not the other mutants, had extra adventitious roots growing from the hypocotyl (data not shown); and shoots of adult axr2-1/+ plants grew agravitropically, whereas shoots of adult axr3-1(+), shy2-2(+), and shy2-3/+ plants grew upright. We conclude that the different phenotypes of the mutants arise from functional differences among the mutant genes or proteins rather than from ecotypic background effects.

We also compared the de-etiolation phenotypes conferred by axr2-1, axr3-1, shy2-2, and shy2-3 mutations in these Columbia/Landsberg erecta F1 hybrid plants. We found that after growth for 23 d in the dark, the axr2-1/+ and axr3-1/+ plants had shorter hypocotyls and a greater frequency of cotyledon unfolding and leaf primordium growth than the shy2-2/+ or shy2-3/+ plants (Table II). These results show that axr2-1 and axr3-1 mutations cause more extensive de-etiolation than shy2-2 or shy2-3 mutations do, at least as heterozygotes.

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**Figure 3.** Growth phenotypes of the axr2-5 T-DNA insertion mutant. A, Kinetics of root elongation in light-grown seedlings; B, kinetics of hypocotyl elongation in light-grown seedlings; C, root growth in the presence of varying amounts of IAA; D, kinetics of root elongation of dark-grown seedlings; E, kinetics of hypocotyl elongation of dark-grown seedlings. Error bars indicate sds of measurements.
DISCUSSION

AXR2/IAA7 Controls Seedling Shoot Development in the Light

Plants carrying the *axr2-5* insertion allele lack an intact *AXR2/IAA7* gene as well as the corresponding mRNA transcript, indicating that this allele is a null mutation. Light-grown *axr2-5* seedlings had a slightly longer hypocotyl than wild-type seedlings, but had normal root growth, auxin inhibition of root growth, lateral root numbers, and root gravitropism.

These results suggest that AXR2/IAA7 contributes to shoot development, but is not essential for correct root development. *axr2-5* plants grew normally in the dark, suggesting that AXR2/IAA7 plays little or no role in development in the dark.

Conversely, plants carrying the gain-of-function mutation *axr2-1* had a short hypocotyl and made leaves in the dark. These results suggest that the activated AXR2/IAA7 protein in *axr2-1* promotes ectopic light response in dark-grown plants and that it may normally contribute to light responses. Photoreceptor-deficient mutants (Koornneef et al., 1980) have longer hypocotyls than the *axr2-5* mutant, indicating that if AXR2/IAA7 acts in a light response pathway, it probably acts redundantly with other factors. *elf3* mutants whose primary defect may be in interactions between light and the circadian rhythm also have elongated hypocotyls (Zagotta et al., 1996; Reed et al., 2000). However, the *shy2-2* mutation caused no discernible effect on circadian rhythms of CAB expression (D.E. Somers, personal communication), suggesting that these Aux/IAA proteins do not play a major role in circadian rhythms.

The de-etiolated morphology of *axr2-1, axr3-1, shy2-2*, and *shy2-3* mutant seedlings is less extreme than that of *det1-1* mutant. Moreover, dark-grown *shy2-2* mutant plants expressed the CAB and PSBA genes to a lesser extent than the *det1-1* or *cop1-1* mutants do (Chory et al., 1989; Deng et al., 1991; Kim et al., 1998). Therefore, the role of the Aux/IAA proteins in the de-etiolation response to light may be less global than that of the DET/COP/FUS group of proteins. One possibility is that the DET/COP/FUS genes repress one or more *IAA* genes as well as other genes involved in de-etiolation (Mayer et al., 1996).

The gain-of-function *axr2-1* mutation causes additional dramatic phenotypes that reveal other potential roles of AXR2/IAA7 in development. Thus, in *axr2-1* mutant plants, roots and shoots each grow...
agravitropically, and roots lack epidermal hairs (Wilson et al., 1990; Timpte et al., 1992). These phenotypes reveal that AXR2/IAA7 may control responses to gravity and root hair specification or growth. The hypothesized redundancy among Aux/IAA proteins (see below) may explain why we have not observed defects in gravitropism or root growth in the null mutant.

AXR2/IAA7 May Act Redundantly with AXR3/IAA17 or SHY2/IAA3

The subtle nature of the axr2-5 mutant phenotype suggests that AXR2/IAA7 may act redundantly with other proteins, perhaps other members of the Aux/IAA family. A shy2 null mutation and axr3 loss-of-function mutations also caused only subtle phenotypes (Rouse et al., 1998; Tian and Reed, 1999), supporting this idea. Furthermore, gain-of-function mutations that activate AXR2/IAA7, AXR3/IAA17, or SHY2/IAA3 each cause short hypocotyls and leaf formation in the dark, suggesting that these proteins share regulatory targets. Double mutants carrying null alleles of axr2 and axr3 or shy2 will allow a more rigorous test of whether these three genes have redundant functions.

An alternative to redundancy to explain the similar shoot phenotypes of the gain-of-function mutants is that the Aux/IAA proteins regulate each other. Aux/IAA proteins can heterodimerize (Kim et al., 1997), providing one potential mechanism for such regulation. These proteins may also regulate their own or each others’ genes. Both SHY2/IAA3 and AXR2/IAA7 genes are expressed at a lower level in the axr2-1 mutant (Abel et al., 1995; see Fig. 2), suggesting that AXR2/IAA7 regulates expression of both its own gene and SHY2/IAA3. SHY2/IAA3 might also be upstream of AXR2/IAA7 in a regulatory cascade. Auxin application induced SHY2/IAA3 within minutes, but induced AXR2/IAA7 more slowly, suggesting that SHY2/IAA3 is part of a primary response to auxin, whereas AXR2/IAA7 is part of a secondary response (Abel et al., 1995). ARF proteins may mediate these putative regulatory interactions, as these can dimerize with Aux/IAA proteins (Kim et al., 1997; Ulmasov et al., 1997b), and can bind to auxin-responsive promoters (Ulmasov et al., 1997a, 1999a).

Different Aux/IAA Proteins Influence Arabidopsis Development Differently

Whereas axr2-1, axr3-1, shy2-1, shy2-2, and shy2-3 seedlings have quite similar seedling shoot phenotypes, they differ in root phenotypes and in adult shoot phenotypes (Timpte et al., 1992, 1994; Kim et al., 1996, 1998; Leyser et al., 1996; Reed et al., 1998; Table I.

Table I. Phenotypes of gain-of-function axr2-1, axr3-1, and shy2-2 mutants

<table>
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<th>shy2-2</th>
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<td>Extreme</td>
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<td>Normal</td>
<td>Reduced</td>
<td>Reduced</td>
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<td>Normal</td>
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<tr>
<td>Auxin sensitivity</td>
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<td>Reduced</td>
<td>Reduced</td>
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<td>More than w.t.</td>
<td>Fewer than w.t.</td>
</tr>
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<td>Fewer than w.t.</td>
<td>Fewer than w.t.</td>
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<tr>
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 AXR2 Encodes IAA7

Figure 6. Appearance of Columbia/Landsberg erecta hybrid plants heterozygous for axr2-1, axr3-1, shy2-2, or shy2-3. Seedlings were grown on vertically oriented MS/agar/2% (w/v) Suc plates for 7 d. As shown in the photograph, axr3-1/+ seedlings frequently grew in orientations other than upright.
Tian and Reed, 1999). Table I compares the morphological and auxin response phenotypes of the axr2-1, axr3-1, and shy2-2 mutants. Roots of axr2-1 and axr3-1 mutant plants grow extremely agravitropically, and they lack root hairs (Wilson et al., 1990; Leyser et al., 1996). In contrast, roots of shy2-2 and shy2-3 mutant plants have normal gravitropism, although they readjust to a shift in gravity more slowly than wild-type plants, and they have abundant root hairs (Tian and Reed, 1999). Furthermore, axr2-1 and axr3-1 mutant roots respond substantially less than wild-type roots to auxin, whereas shy2-2 or shy2-3 root growth is almost as sensitive to auxin as is root growth of wild-type plants. In an assay for adventitious root formation from the hypocotyl (Tian and Reed, 1999), we observed that axr2-1 plants made fewer adventitious roots than wild-type plants (0.3 ± 0.5 for axr2-1 and 2.8 ± 1.5 for wild type). In this respect, axr2-1 seedlings resemble shy2-2 and shy2-3 seedlings more closely than they do axr3-1 seedlings, which have extra adventitious roots (Leyser et al., 1996). Morphologies of adult shoots also differ significantly. axr2-1 shoots weep rather than growing upward as wild-type shoots do (Wilson et al., 1990). Adult axr3-1 plants have normal shoot orientation but fewer lateral shoot branches (Leyser et al., 1996). Finally, shy2-2 or shy2-3 shoots have apparently normal gravitropism and branching (Tian and Reed, 1999). Gain-of-function mutations in domain II of MSG2/IAA19, SLR/IAA14, and IAR2/IAA28 genes also cause related but distinct phenotypes (Rogg et al., 1999; M. Tasaka, personal communication; K. Yamamoto, personal communication). These phenotypic differences suggest that the IAA genes may not be truly redundant for all functions.

Our experiments with F1 hybrids, and the change of Pro to Ser at the corresponding position in axr2-1 and shy2-2 mutations, show that differences in allele strength or ecotypic background do not account for these phenotypic differences. Instead, differences in functional properties of the proteins or in expression patterns may be more important. Northern-blot analyses showed that AXR2/IAA7 and SHY2/IAA3 were each expressed in similar tissues (Abel et al., 1995), suggesting that expression patterns may not account for the differences. However, the kinetics of induction of these genes were different (see above), and more detailed localization and kinetic studies will be required to resolve this question.

### Table II. Phenotypes of dark-grown Columbia/Landsberg erecta F1 hybrids heterozygous for gain-of-function mutations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hypocotyl Length</th>
<th>Fraction of Seedlings with Unfolded Cotyledons</th>
<th>Fraction of Seedlings with Leaf Primordia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>20.4 ± 1.0</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>axr2-1/+</td>
<td>5.4 ± 0.2</td>
<td>1.00</td>
<td>0.86</td>
</tr>
<tr>
<td>axr3-1/+</td>
<td>5.2 ± 0.6</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>shy2-2/+</td>
<td>8.6 ± 0.5</td>
<td>0.29</td>
<td>0.76</td>
</tr>
<tr>
<td>shy2-3/+</td>
<td>12.9 ± 1.6</td>
<td>0.38</td>
<td>0.50</td>
</tr>
</tbody>
</table>

The collected results from mutations in six different IAA genes suggest that domain II plays a key role in the function of various Aux/IAA proteins, and that it may be a target for regulatory action of auxin or some other signal. One model to explain the potency of mutations in domain II is that this domain causes Aux/IAA proteins to be degraded quickly. Pea Ps-IAA4 and Ps-IAA6 proteins have very short half-lives (Abel et al., 1994), and one way to increase the activity of Aux/IAA proteins may be to stabilize them. Indeed, fusion proteins with Ps-IAA6 are degraded rapidly in plant cells, and mutations in domain II appear to decrease this degradation (Worley et al., 2000). One attractive possibility is that Aux/IAA proteins are substrates for ubiquitin-mediated degradation. Mutations in components of a ubiquitin-protein ligase called SCFTIR1 reduce auxin response (Ruegger et al., 1998; Gray et al., 1999). Thus, it is possible that SCFTIR1 functions to ubiquitinate the Aux/IAA proteins, resulting in their degradation. Recent studies also suggest that SCFTIR1 is in turn regulated by modification by the ubiquitin-related protein RUB1 (del Pozo and Estelle, 1999). Mutations in genes that function in the RUB pathway also result in a defect in auxin response (del Pozo et al., 1998).

The axr2-1-r3 and axr2-1-r4 mutants have shorter hypocotyls than wild-type plants and altered adult shoot gravitropism; and are dominant to the wild-type allele for these phenotypes. Considering that the null allele axr2-5 confers much more subtle phenotypes, it seems likely that the axr2-1-r3 and axr2-1-r4 mutations cause just a partial loss of function, and that these phenotypes are caused by residual activity of AXR2-1 protein. axr2-1-r4 and axr2-1-r3 mutations affect conserved domains I and III of AXR2/IAA7. Intragenic suppressor mutations of axr3-1 in the corresponding domains of AXR3/IAA17 also conferred phenotypes intermediate between those of wild type and those of the starting gain-of-function mutant.
(Rouse et al., 1998). Thus it appears that domains I and III are important to the function of at least two members of the IAA family, as would be predicted based on sequence conservation.

MATERIALS AND METHODS

Genetic Material

The auxin-resistant mutants axr2-1 and axr3-1 and the null phytochrome B photoreceptor mutant phyB-9 are in the Columbia ecotype background (Wilson et al., 1990; Reed et al., 1993; Leyser et al., 1996). shy2-2 and shy2-3 mutants are in the Landsberg erecta ecotype (Reed et al., 1998). The insertion mutation axr2-5 is in the Wassilewskija ecotype.

Mapping, Sequencing, and Hybridizations

We mapped AXR2 relative to known cleaved amplified polymorphic sequence and RFLP markers and new polymorphic markers derived from BAC and YAC ends (data not shown). To sequence axr2 mutant alleles, we amplified the AXR2/IAA7 gene from axr2 mutants in 10 individual polymerase chain reactions, purified the pooled reactions through a Sephadex G50 column, and sequenced the products directly. Sequencing was performed by the University of North Carolina at Chapel Hill sequencing facility. Primers used were designed based on the genomic DNA sequence of the AXR2/IAA7 gene. We did not sequence all of the intron DNA. However, mRNA hybridizations showed that the mutants had AXR2/IAA7 transcript of the correct size (Fig. 2C). Genomic DNA was isolated as described previously (Krysan et al., 1996) from seedlings grown for 3 d in liquid MS (1/2 Gamborg's vitamin mix Sigma, St. Louis)/2% (w/v) Suc. Total RNA was isolated using oligo(dT)25 Dynabeads according to manufacturer's instructions (Dynal, Lake Success, NY). mRNA isolated from 50 g of total RNA was run on formaldehyde gels and blotted to nylon membranes (Hybond N, Amersham, Piscataway, NJ). 32P-Labeled probes were made using a random priming kit (Boehringer Mannheim, Indianapolis). For Southern hybridizations, probes were made from a full-length IAA7 cDNA clone (Abel et al., 1995) or from plasmid pD991 containing the T-DNA used to make the mutant population (a gift of Tom Jack, Dartmouth University, Hanover, NH). For northern hybridizations, probes were made from either a PCR product spanning domains II to IV of the AXR2/ IAA7 cDNA (Fig. 2B) or a 268-bp fragment including only the 3'-untranslated region of the cDNA after a BglII restriction site near the end of the coding sequence (Fig. 2C). Hybridizations were done as described (Church and Gilbert, 1984). Membranes were washed in 2× SSC once for 15 min, followed by 0.2× SSC, 0.1% (w/v) SDS twice for 15 min each at 65°C, and then exposed to x-ray films (Eastman-Kodak, Rochester, NY).

Isolation of an Insertion Mutation in IAA7

We designed a degenerate oligodeoxynucleotidite [IAAdIV30mer, 5'-ACTCCCA(A/T/C) GGAACATC(G/ A/T) CC(G/A/T) AC(G/A/C) AGCATCC-3'] predicted to hybridize to DNA encoding conserved domain IV of multiple members of the Arabidopsis IAA gene family. IAAAdIV30mer had 81-fold redundancy and was expected to hybridize to 18 of the known IAA genes with zero to three mismatches to any one gene. We amplified from template DNA derived from pools of T-DNA insertion lines, using IAAAdIV30mer and primers specific to the ends of the T-DNA as described previously (Krysan et al., 1996). Among the insertion mutations identified, one fell in IAA7. In this mutant, we could amplify a 0.6-kb fragment using the degenerate primer and the T-DNA left border primer, but we were unable to amplify the IAA7 gene using genespecific primers flanking the T-DNA insertion site. We will describe mutants in other IAA genes obtained in this screen elsewhere.

Growth Conditions and Phenotypic Measurements

Seedlings were surface sterilized and plated on MS/agar plates (1× MS salts [Gibco/BRL], 0.8% [w/v] phytagar [Gibco/BRL], and 1× Gamborg’s B5 vitamin mix [Sigma]) with or without 2% (w/v) Suc, stored overnight at 4°C, and moved to the appropriate light condition. Growth temperature was 21°C. For dark growth, seedlings were induced to germinate by treatment with 6 to 18 h of white light. For root growth response to exogenous auxin, seedlings were grown under white light at 21°C on vertically oriented MS/agar/2% (w/v) Suc plates for 5 d to allow the roots to grow along the surface, and 20 seedlings per treatment were transferred to new plates supplemented with various concentrations of IAA. The positions of root tips of all seedlings were marked, and the amount of new root growth was measured after 3 d. Hypocotyl and root lengths were measured with a ruler or with NIH Image software after taking an image of the seedlings with a CCD camera. Inhibition of hypocotyl elongation by red, far-red, or blue light was assayed as described previously (Reed et al., 1998). Red-, far-red-, and blue-light fluence rates were approximately 10 μmol m−2 s−1.

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