AXR2 Encodes a Member of the Aux/IAA Protein Family[1]

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The dominant gain-of-function *axr2-1* mutation of Arabidopsis causes agravitropic 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, 1991; 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 strand differentiation. The diverse effects of auxin in different cells may reflect differential mechanisms of auxin response, differential exposure to auxin, or a common auxin response system coupled to diverse outputs.

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′-TGTCTC-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

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development, and revealed that different ARF factors show specificity in the phenotypes they control. *ettin/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, agravitropic 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/

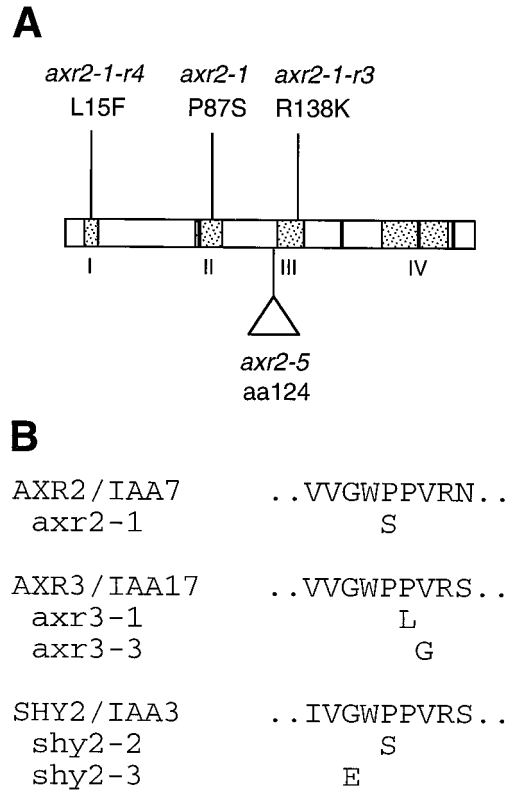

Figure 1. A, Mutations in *AXR2/IAA7*. The box represents the coding part of the gene. Dark vertical lines within the box indicate positions of introns. Stippled boxes indicate conserved domains I, II, III, and IV. B, Mutations in conserved domain II of AXR2/IAA7, AXR3/IAA17, and SHY2/IAA3 proteins.

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 *Bsm*AI 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 *Nsi*I fragment was shifted in size in the mutant, whereas all other *Nsi*I 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

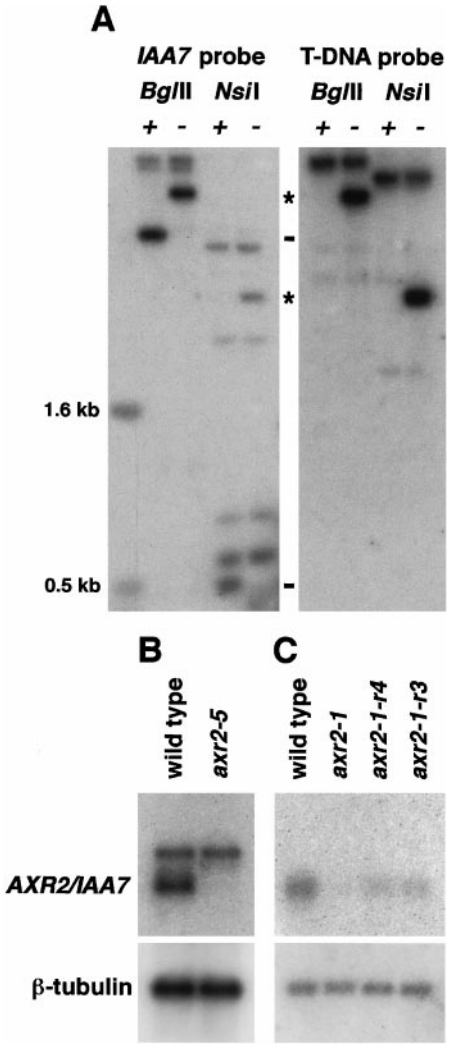

Figure 2. A, Southern hybridization of wild-type (+) and *axr2-5* (−) DNA cut with *Bgl*II or *Nsi*I restriction enzymes and probed with an *AXR2/IAA7* probe (left) or a T-DNA probe (right). Horizontal bars indicate bands present in wild-type DNA but missing in the mutant, and asterisks indicate bands that are unique to the mutant and hybridize to both probes. B and C, Northern blots of mRNA from *axr2* mutants probed with *AXR2/IAA7* cDNA probes. B, mRNA from wild-type Wassilewskija and *axr2-5*, probed with a PCR product derived from an *AXR2/IAA7* cDNA; C, mRNA from wild-type Columbia, *axr2-1*, and intragenic suppressors derived from *axr2-1*, probed with the 3′-untranslated region of the *AXR2/IAA7* cDNA. Hybridizations of the same two blots with a β-tubulin probe show that the amount of mRNA loaded was roughly equal in each lane (lower panels).

mutant (data not shown). Lateral root numbers were also the same in the two genotypes, 2.9 ± 3.7 ($n = 57$) for wild type versus 3.2 ± 2.3 ($n = 57$) for *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 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 white 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.

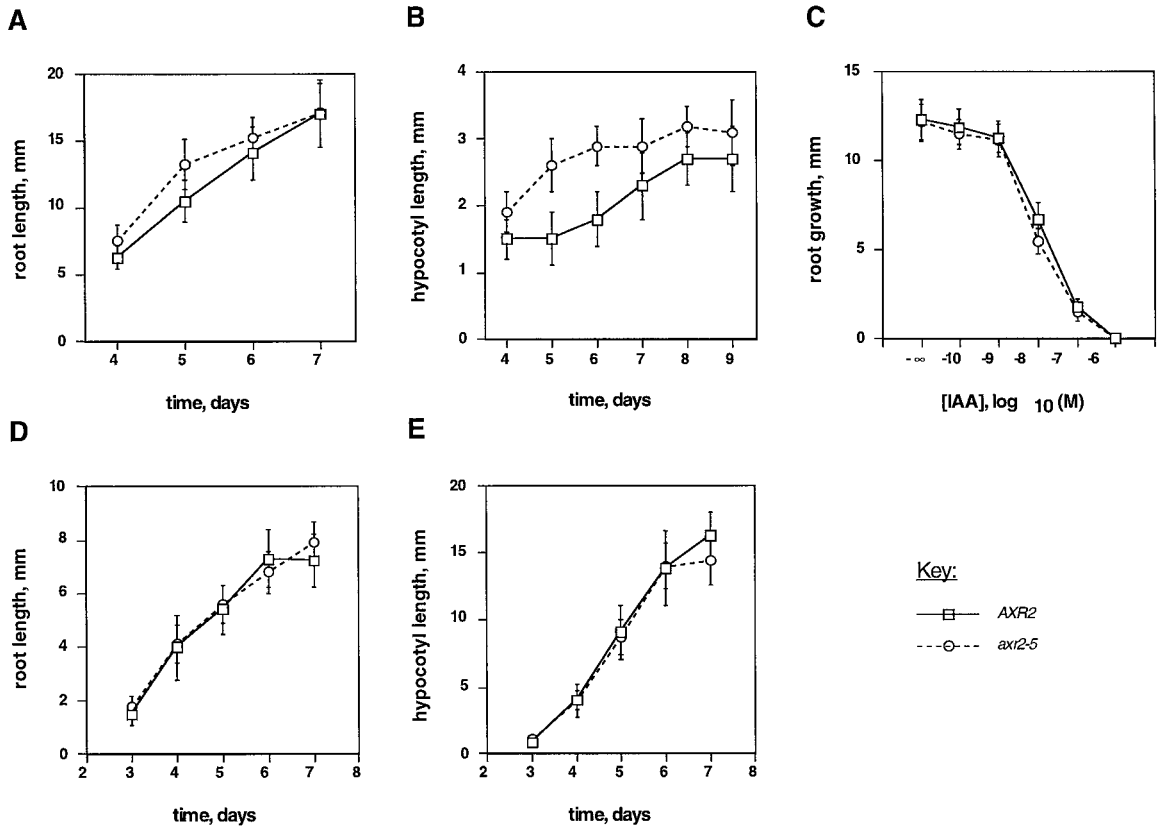

Figure 3. Growth phenotypes of the *axr2-5* T-DNA insertion mutant. A, Kinetics of root elongation in light-grown seedlings; B, kinetics of hypocotyl elongation in light-grown seedlings; C, root growth in the presence of varying amounts of IAA; D, kinetics of root elongation of dark-grown seedlings;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

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* F_1 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 F_1 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* F_1 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.

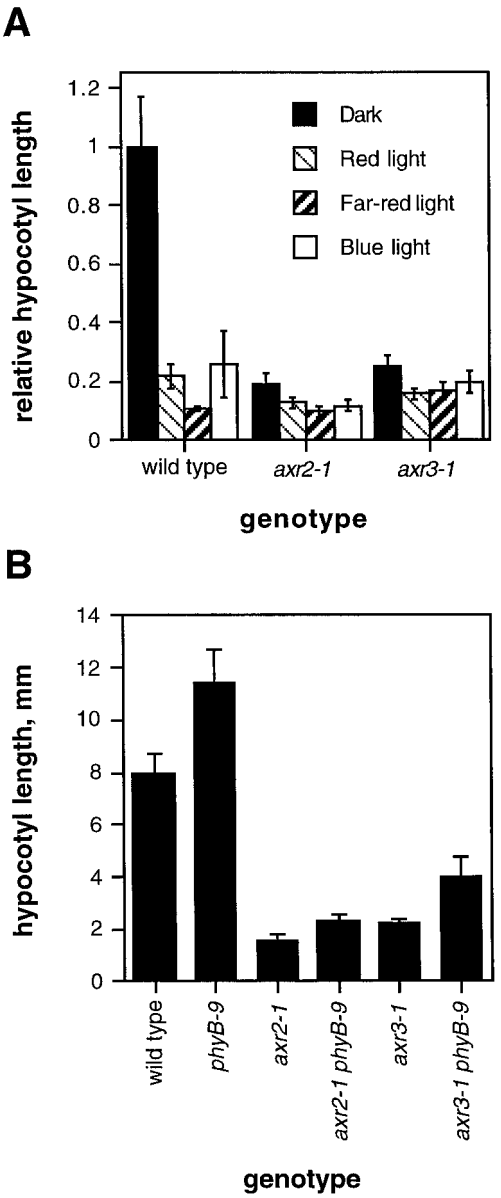

Figure 4. Effects of *axr2-1* and *axr3-1* mutations on light responses. A, Hypocotyl lengths of wild-type, *axr2-1*, and *axr3-1* seedlings grown for 5 d under different light conditions, normalized to the wild-type hypocotyl length in the dark ± SD. B, Hypocotyl lengths of wild-type, *axr2-1*, *axr3-1*, *phyB-9*, *axr2-1 phyB-9*, and *axr3-1 phyB-9* seedlings grown for 7 d in red light ± SD.

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 *shy2-1* 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

Figure 5. Appearance of wild-type, *axr2-1*, *axr3-1*, and *det1-1* mutant seedlings after 23 d growth in the dark.

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

Phenotypes are from the following references: For *axr2-1:* Wilson et al. (1990); Timpte et al. (1992); Timpte et al. (1994); this work. For *axr3-1:* Leyser et al. (1996). For *shy2-2:* Reed et al. (1998); Tian and Reed (1999). w.t., Wild type.

Phenotype	Mutant Genotype		
	axr2-1	*axr3-1*	*shy2-2*
Roots			
Agravitropism	Extreme	Extreme	Slight
Growth rate	Normal	Reduced	Reduced
Auxin sensitivity	Reduced	Very reduced	Normal
Lateral roots	More than w.t.		Fewer than w.t.
Adventitious roots	Fewer than w.t.	More than w.t.	Fewer than w.t.
Root hairs	Fewer than w.t.	Fewer than w.t.	Normal
Shoots			
Leaves	Wavy	Upcurled	Upcurled
Inflorescence stems	Agravitropic	Upright	Upright
Dark growth			
Hypocotyl	Short	Short	Short
Leaves	Yes	Yes	Yes

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.

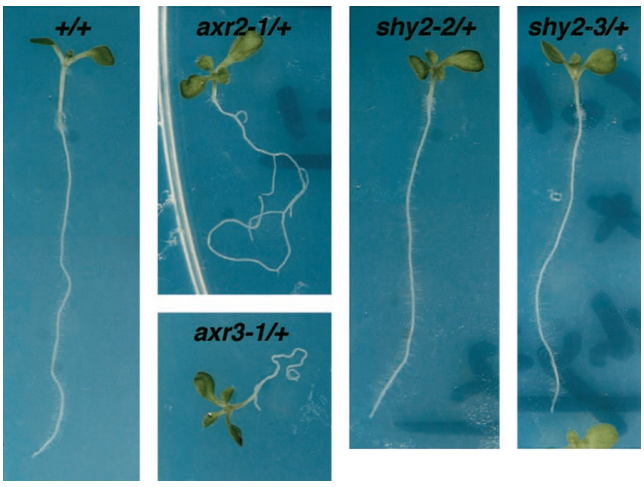

Figure 6. Appearance of Columbia/Landsberg *erecta* hybrid plants heterozygous for *axr2-1*, *axr3-1*, *shy2-2*, or *shy2-3*. Seedlings were grown on vertically oriented MS/agar/2% (w/v) Suc plates for 7 d. As shown in the photograph, *axr3-1/+* seedlings frequently grew in orientations other than upright.

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 II. *Phenotypes of dark-grown Columbia/Landsberg erecta F_1 hybrids heterozygous for gain-of-function mutations*

Seedlings were grown for 23 d in the dark on MS/agar/2% (w/v) Suc medium.

Genotype		Hypocotyl Length	Fraction of Seedlings with Unfolded Cotyledons	Fraction of Seedlings with Leaf Primordia
	n	*mm*		
Wild type	11	20.4 ± 1.0	0.09	0.09
axr2-1/+	21	5.4 ± 0.2	1.00	0.86
axr3-1/+	9	5.2 ± 0.6	1.00	1.00
shy2-2/+	17	8.6 ± 0.5	0.29	0.76
shy2-3/+	16	12.9 ± 1.6	0.38	0.50

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 F_1 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.

axr2 Mutations Reveal Domains Important for AXR2/IAA7 Protein Function

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 SCF^{TIR1} reduce auxin response (Ruegger et al., 1998; Gray et al., 1999). Thus, it is possible that SCF^{TIR1} functions to ubiquitinate the Aux/IAA proteins, resulting in their degradation. Recent studies also suggest that SCF^{TIR1} 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 8 d in liquid MS (1× MS salts [Gibco/BRL, Grand Island, NY], 1× Gamborg's vitamin mix [Sigma, St. Louis])/2% (w/v) Suc. Total RNA was isolated from seedlings grown under these conditions using Trizol reagent (Gibco/BRL), and poly(A^+) RNA was isolated using oligo$(dT)_{25}$ Dynabeads according to manufacturer's instructions (Dynal, Lake Success, NY). mRNA isolated from 50 μg of total RNA was run on formaldehyde gels and blotted to nylon membranes (Hybond N, Amersham, Piscataway, NJ). ^{32}P-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 *Bgl*II 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 oligodeoxyribonucleotide [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. IAAdIV30mer 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 IAAdIV30mer 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 gene-specific 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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