The *axr2-1* Mutation of *Arabidopsis thaliana* Is a Gain-of-Function Mutation That Disrupts an Early Step in Auxin Response

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ABSTRACT

The dominant *axr2-1* mutation of *Arabidopsis thaliana* confers resistance to the plant hormones auxin, ethylene, and abscisic acid. In addition, *axr2-1* has pleiotropic effects on plant morphology which include gravitropic defects in roots, hypocotyls and inflorescences of *axr2-1* plants. Two genetic screens were conducted to isolate new mutations at the *AXR2* locus. First, *axr2-1* pollen was γ-irradiated, crossed onto wild-type plants, and the M_1 progeny screened for loss of the *axr2-1* phenotype. Large deletions of the *axr2-1* region on chromosome *3* resulted; however, none of these deletions appeared to be heritable. In the second, M_2 seed obtained from *axr2-1 gl-1* plants was screened for reversion of the *axr2-1* phenotype. One revertant line, *axr2-r3*, has a distinctive phenotype caused by a second mutation at the *axr2* locus. To learn more about the nature of the *axr2-1* mutation, the effects of varying the ratio of wild-type to mutant copies of the *AXR2* gene were examined by comparing plants of the following genotypes: +/+, +/+/+, *axr2-1/axr2-1*, *axr2-1*/+ and *axr2-1*/+/+. Additionally, accumulation of transcripts from the auxin-inducible *SAUR-AC1* gene was examined to determine the response of wild-type and mutant plants to auxin. Wild-type seedlings and mature plants accumulate transcripts with auxin treatment. In contrast, *axr2-1* tissue does not accumulate *SAUR-AC1* transcripts in response to auxin. Taken together, these results indicate that *axr2-1* is a neomorphic or hypermorphic mutation that disrupts an early step in an auxin response pathway.

THE five major plant hormones ethylene, abscisic acid (ABA), cytokinin, gibberellic acid (GA), and auxin act to regulate a wide variety of developmental processes (KING 1988). Auxin is particularly interesting because it appears to play a role in very diverse aspects of plant development including tropic responses (FELDMAN 1985), organ elongation (BARKLEY and EVANS 1970; EVANS 1974, 1984), apical dominance (TAMAS 1988), and vascular tissue differentiation (ALONI 1987). Auxin exerts its effects at the cellular level by controlling cell elongation, cell division, and cell differentiation. Despite the central role of auxin in plant development, little is known about the molecular mechanism of auxin action. It is assumed that auxin interacts with a cellular receptor(s), thus initiating a cellular response, possibly via a series of signal transduction steps. In an attempt to identify auxin receptors, a number of laboratories have isolated auxin-binding proteins. However, the physiological role of these proteins is not known at present (PALME 1992).

Auxin-regulated genes have been identified in soybean, pea, tobacco and Arabidopsis (HOBBIE and ESTELLE 1994). Athough the function of the proteins encoded by these genes remains unknown, recent studies suggest that they perform an important role in auxin-regulated elongation. Transcripts of the SAUR, or *s*mall *a*uxin *u*p *R*NA, gene family accumulate in excised soybean hypocotyls within minutes of auxin application, and are transcriptionally regulated (MCCLURE *et al.* 1989). Additionally, in gravistimulated soybean hypocotyls, SAUR transcripts were observed specifically on the lower side of the hypocotyls prior to the onset of elongation. These experiments establish a correlation between elongating regions and SAUR expression (MCCLURE and GUILFOYLE 1989). An Arabidopsis SAUR homolog has been isolated and designated *SAUR-AC1* (GIL *et al.* 1994). The gene is 78% identical to the soybean SAUR consensus between amino acid 38 and 87 [as numbered in Figure 2 of GIL *et al.* (1994)] and produces a transcript approximately 500 nucleotides in length.

A molecular genetic approach has been used to study auxin action. Mutations which confer auxin-resistance have been identified in tomato (KELLY and BRADFORD 1986), tobacco (BLONSTEIN *et al.* 1991; BITOUN *et al.* 1990; MULLER *et al.* 1985), and Arabidopsis (ESTELLE and SOMERVILLE 1987; MAHER and MARTINDALE 1980; LINCOLN *et al.* 1990; PICKETT *et al.* 1990; WILSON *et al.* 1990; LEYSER *et al.* 1993). The isolation and characterization of the genes identified by these mutations should provide important information about the mechanism of auxin action and the role of auxin during plant growth and development.

The dominant *axr2-1* mutation is one of several auxin-resistance mutations isolated in Arabidopsis. This mutation was originally recovered in a screen for seed-

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lings able to elongate roots on medium containing the naturally occurring auxin, indole-3-acetic acid (IAA). Subsequently, the *axr2-1* mutation was shown to confer resistance to the synthetic auxins naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) as well as the plant hormones ethylene and ABA (WILSON *et al.* 1990). Although the *axr2-1* mutation confers resistance to several plant hormones, most aspects of the *axr2-1* phenotype can be explained by a defect in auxin action. An analysis of cell structure in hypocotyls and stems of *axr2-1* plants showed that mutant plants are deficient in cell elongation in these organs (TIMPTE *et al.* 1992). Extensive physiological studies suggest that both shoot elongation and gravitropism are dependent on auxin-regulated cell growth (FELDMAN 1985). In contrast, there is little evidence to suggest that either ABA or ethylene play an important role in these processes. Mutants in Arabidopsis which are either ABA deficient or ABA-insensitive do not have defects in elongation or gravitropism and are not resistant to auxin (KOORNNEEF *et al.* 1984; A. K. WILSON and M. ESTELLE, unpublished). Similarly, ethylene-insensitive mutations of Arabidopsis do not affect plant morphology (BLEECKER *et al.* 1988; GUZMAN and ECKER 1990).

Previous studies showed that the *axr2-1* mutation is dominant (WILSON *et al.* 1990). In this paper, we report the use of γ-irradiation to generate plants which are hemizygous for the wild-type *AXR2* gene. We also report the isolation of three intragenic revertants of the *axr2-1* mutation. Two of the revertants are wild type in appearance and may restore the wild-type *AXR2* gene function. The third revertant, called *axr2-1-r3*, has a novel phenotype and represents a new mutation in the locus. A detailed comparison of homozygous and heterozygous *axr2-1* plants was performed, including the construction of triplod plants to examine further the effects of mutant to wild-type gene copy number. Additionally, we have examined the accumulation of *SAUR-AC1* transcripts as a molecular assay for auxin response in wild-type and mutant plants. The auxin-induced accumulation of *SAUR-AC1* transcripts was compared in several tissues of wild-type and *axr2* mutant plants. We find that *axr2-1* plants are severely deficient in auxin-regulated expression of the *SAUR-AC1* gene, while *axr2-1-r3* plants have only a slight reduction in *SAUR-AC1* expression. Since the *SAUR* genes respond rapidly to auxin, these results indicate that the *axr2-1* mutation disrupts an early step in an auxin response pathway.

MATERIALS AND METHODS

Plant material: Arabidopsis plants were grown at 23° on a commercially available peat-lite mixture with continuous illumination, and fertilized as described (LINCOLN *et al.* 1990). For certain experiments, plants were grown under sterile conditions in Petri plates. Seeds were surface sterilized for 20 min in 30% v/v bleach and 0.01% Triton X-100 and then placed on Petri plates containing the nutrient salts, 8 g/liter agar and 10 g/liter sucrose (minimal medium). Hormones were added to the medium after autoclaving. Sterile plants were grown at 22–24°. All plants were Columbia ecotype unless stated otherwise. Heterozygous *axr2-1* diploids were obtained by crossing *axr2-1/axr2-1* to +/+. Triploid plants were made by crossing diploid *axr2-1* or wild-type plants to wild-type tetraploids (gift of CAREN CHANG, California Institute of Technology) and the resulting F_1 seeds were used in the studies described.

For the *SAUR-AC1* analysis, approximately 2000 seeds (20 mg) were used for each experimental point, and were dispersed in 0.3% agar drops on three plates of minimal media. Seedlings were grown for 7 days in the dark at 22°. The roots and cotyledons were excised, and the hypocotyls cut into 2–3-mm pieces. Samples of *axr2-1* seedlings included both hypocotyl and roots, wild-type seedlings were treated similarly for comparison. Tissue (1 g) was incubated at 30° for a total of 4 hr in 50 ml KPSC [10 mM $KHPO_4$, pH 6, 2% sucrose (w/v) and 50 μg/ml chloramphenicol] with medium changes after 1 and 2 h. Auxin treatment was initiated by transferring tissue into KPSC containing 50 μM 2,4-D or the indicated concentration for 1 h at 30°. Tissue was collected and frozen at −70°. Light-grown seedlings were grown as described above except in constant light for 7 days, then treated as above. Rosette leaves were collected from 3-week-old plants, chopped into 2–4-mm pieces and incubated in KPSC as above. Inflorescences were collected from 1-month-old plants, stripped of cauline leaves and siliques, cut into 4–7-mm pieces and incubated in KPSC followed by auxin. Mature roots were harvested from month-old plants grown in sand by excising the rosettes, inverting the pot and immersing the root mass in water and rinsing until clean. Roots were then cut and incubated in KPSC and treated with auxin.

Pollen mutagenesis: To mutagenize Arabidopsis pollen, whole flowers from *axr2-1/axr2-1* (ecotype Columbia) were picked and placed in a small Petri dish. The entire dish was placed in the irradiator (J. L. Shephard, Glendale, CA) containing ^{137}Cs for the appropriate time period. Flowers from +/+ (ecotype Niederzenz) were emasculated, and the mutagenized pollen was placed on the stigma surface. *In vitro* germination tests indicated that pollen was viable for at least 24 h when stored at 4° after irradiation (J. TURNER and M. ESTELLE, unpublished).

***axr2-1 gl-1* M_2 mutagenesis and revertant screen:** Approximately 37,500 *axr2-1 gl-1* seeds were soaked for 16 hr in 250 ml of 0.2% (v/v) ethyl methanesulfonate (EMS), then washed with 12 changes of water over 4 hr. This M_1 seed was sown at a density of approximately 1 plant/cm^2. M_1 plants were allowed to self-fertilize, and the resulting M_2 seed was collected in 33 individual lots, with seed from 1000 M_1 plants/lot. Thus, 33 distinct *axr2-1 gl-1* M_2 populations were prepared. Twenty-two different M_2 populations were screened by plating seeds onto minimal plates containing 5×10^{-7} M 2,4-D and identifying seedlings with auxin-sensitive roots. After all auxin sensitive plants were removed from the selective medium, the plates were placed in the dark for 3 days to screen for plants with long hypocotyls. M_2 seeds were plated at a density of 500–1000 seeds per plate, and 10,000–20,000 seeds were screened from each individual M_2 population.

Morphometric analysis: Plants used for morphometric analysis were grown under uniform conditions in continuous light. Rosettes were analyzed when the plants were 3 weeks old. Inflorescences were analyzed when plants were 7 weeks old. Average floral internode distance was calculated by measuring the distance from the first to the last silique on the main inflorescence and dividing this distance by the total number of siliques on the inflorescence. Five plants of each genotype were analyzed to calculate internode distance. For every other

character examined, a mean value was obtained by measuring 10 different diploid plants or 7 different triploid plants.

Determination of auxin sensitivity: Auxin sensitivity was measured as described in WILSON *et al.* (1990). The inhibition of root growth on auxin relative to growth on minimal medium was determined. These data were plotted and the auxin concentration which produced 50% inhibition of root growth was estimated by interpolation from the graphs.

RNA preparation: RNA was prepared using guanidine isothiocyanate as previously described (PUISSANT and HOUDEBINE 1990; NEWMAN *et al.* 1993) with the following modifications. Frozen tissue (<1 g) was ground with mortar and pestle in liquid nitrogen. The powder was extracted with 5 ml of 4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7, 1% *N*-lauroylsarcosine and 0.1 M mercaptoethanol and incubated on ice for 20 min. After the addition of 0.5 ml sodium acetate, pH 4, 5 ml phenol and 1 ml chloroform were added and the sample shaken for 2 min. The aqueous phase was collected and RNA precipitated with 8 ml isopropanol. The resulting pellet was dispersed in 0.5 ml of 4 M LiCl, collected by centrifugation, and resuspended in 0.5 ml of 10 mM Tris, pH 8, 0.5 mM EDTA and 0.5% sodium dodecyl sulfate (SDS). After phenol:chloroform (1:1) extraction, followed by chloroform extraction, RNA was precipitated by the addition of 50 µl of 3 M sodium acetate, pH 5, and 1 ml ethanol. The resulting pellet was solubilized in 100 µl sterile water. The ratio of absorbance at 260 and 280 nm was approximately 2. Typical yield for etiolated seedlings was 60–80 µg of total RNA, for rosettes 500–1000 µg.

RNA blot analysis: Total RNA (20–50 µg) was denatured with formaldehyde and separated in a 1% agarose gel containing formaldehyde (SAMBROOK *et al.* 1989). The use of total RNA allowed normalization by comparison of ribosomal RNA levels, since an actin gene initially used for normalization was found to be affected by auxin treatment. RNA was transferred to nylon membrane (Hybond-N, Amersham Corp.) by capillary action in 20 × SSC and baked for 2 hr at 80°. Membranes probed with *SAUR-AC1* were prehybridized in 30% formamide, 5 × SSCP, 5 × Denhardt's solution, 0.1% SDS, and 0.02 mg/ml denatured salmon sperm DNA at 52°. Hybridizations were carried out in 30% formamide, 5 × SSCP, 10X Denhardt's, 0.1 mg/ml salmon sperm DNA and 10% dextran sulfate at 52° for 16 h. Filters were washed in 2 × SSC, 1% SDS for 20 min at room temperature, then in 0.5 × SSC, 1% SDS for 20 min at 65°. Membranes probed with pRE12 were prehybridized in the same solution as above except in 50% formamide, and hybridized in 50% formamide, 5 × SSCP, 1 × Denhardt's solution, 0.1% SDS and 0.02 mg/ml denatured salmon sperm DNA at 42° for 16 h. The *SAUR-AC1* probe was as described by GIL *et al.* (1994) and covered the entire coding region from +56 to +356 (relative to the transcription start site at +1). It was labeled with ^{32}P using the random prime method (FEINBERG and VOGELSTEIN 1983). For normalizations, plasmid pRE12 (DELSENY *et al.* 1983), carrying the 18S rRNA gene, was prepared by cleaving with *Bam*HI and *Xho*I, excising the 1.7-kb fragment corresponding to the 18S rDNA fragment, and labeling as above. Autoradiograms were made on Fugi RX medical x-ray film. Densitometry of resulting autoradiograms was conducted with a Molecular Dynamics Computing densitometer. Equal loading of RNA was checked by staining with ethidium bromide. Additionally, all blots were quantitated by probing with the 18s rDNA probe and normalized.

S1 endonuclease assay: A single-stranded S1 probe was prepared as described by GIL *et al.* (1994) and covered the region from −441 to +141 at the 5′ end of *SAUR-AC1*. Total RNA (50 µg) was annealed to the probe and treated with nuclease S1 as described (SAMBROOK *et al.* 1989). The protected fragment was separated by 6% denaturing gel electrophoresis and autoradiographed.

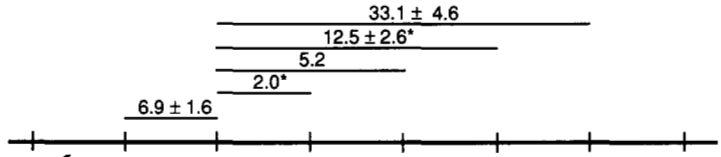
Other molecular techniques: DNA was isolated from leaf tissue using the procedure of DELLAPORTA *et al.* (1983). For a standard DNA blot to analyze restriction fragment length polymorphism (RFLP) genotypes, 3 µg of Arabidopsis DNA were used per lane. Restricted DNA was separated on agarose gels, transferred to Hybond-N (Amersham) membranes and probed with labeled RFLP clones using standard procedures (SAMBROOK *et al.* 1989). Lambda DNA containing RFLP markers was isolated by the procedure of DAVIS *et al.* (1986). The RFLPs used were described by CHANG *et al.* (1988) and were kindly provided by E. MEYEROWITZ. Hybridization probes were prepared with ^{32}P using the random priming method (FEINBERG and VOGELSTEIN 1983).

RESULTS

Genetic location of the *axr2-1* mutation: Previous studies (WILSON *et al.* 1990) showed that the *axr2-1* mutation is located on chromosome three between the visible marker *gl1* and the RFLP marker 105 (CHANG *et al.* 1988) (Figure 1). To localize the gene more precisely in this interval, we isolated additional recombinants between *axr2-1* and either RFLP 105 or *gl1* and scored them for the RFLP markers 6220 (NAM *et al.* 1988) and 255 (CHANG *et al.* 1988). Twelve of 13 recombinants in the 105 to *axr2-1* interval were recombinant at 6220 indicating that this marker is only slightly closer to *axr2-1* than 105 (not shown). Of the 68 recombinants in the *axr2-1* to *gl1* interval, only 11 were recombinant at the 255 locus. Since the distance between *axr2-1* and *gl1* is approximately 12.5 cM (WILSON *et al.* 1990), the estimated distance between *axr2-1* and RFLP 255 is 12.5 × 11/68, or 2 cM.

Mutagenesis of *axr2-1* pollen: To identify recessive loss-of-function mutations at the *axr2-1* locus, we attempted to recover revertants of the dominant mutant by mutagenizing *axr2-1 gl1* pollen with γ-irradiation. M_1 plants were screened for the loss of the *axr2-1* phenotype. Two types of analysis were performed to characterize the putative revertants. First, the selfed M_2 progeny were examined for the presence of any novel phenotype which might be due to a recessive mutation in the *AXR2* gene. Second, the M_2 progeny were analyzed for the segregation of RFLPs near the *AXR2* gene to investigate the nature of the mutation associated with reversion and its inheritance.

Two doses of γ-irradiation were employed. When pollen was treated with 50 krad of irradiation, eleven *axr2-1* revertants were isolated out of 1025 M_1 plants screened. The roots, leaves and inflorescences of the M_1 revertants were wild type in appearance (data not shown). Similarly, all of the M_2 progeny from each M_1 plant had a wild-type phenotype. Plants with the *gl1* phenotype were not present in the M_2 families, although RFLP analysis demonstrated that the M_1 plants were produced by outcrossing. None of the M_2 families segregated the Col RFLP alleles of 105 or 255 (Figure 1). Taken together



Class	Number	243	105	axr2	255	abi-3	gl-1	249	460
A	1	N/C	N/-		N/-			N/-	N/-
B	4	N/-	N/-		N/-			N/-	N/C
C	1	N/-	N/-		N/-			N/C	N/C
D	2	N/C	N/-		N/-			N/-	N/C
E	3	N/C	N/-		N/-			N/C	N/C
F	1	N/C	N/C		N/-			N/C	N/C

FIGURE 1.—Characterization of revertants generated by pollen mutagenesis. All map distances are in centimorgans. Numbers in parentheses are from CHANG *et al.* (1988). Map distances marked with an asterisk (*) are from WILSON *et al.* (1990). Genomic DNA was isolated from M_2 progeny derived from M_1 revertants, restricted with the appropriate enzymes and run on 1% agarose gels. The DNA was transferred and probed with RFLPs 243, 105, 255, 249 and 460. The relative position of these markers on chromosome *3* is shown on the bold horizontal line with the map distance in centimorgans indicated above (map not drawn to scale). The revertants were grouped into six classes (A through F) based on their genotype. N represents the Niederzenz allele and C the Columbia allele of each RFLP. Each revertant was scored as either heterozygous (N/C) or homozygous (N/N) at each locus. The number of revertants in each class is indicated. All the revertants were generated using 50 krad of irradiation except the single revertant in class F which was generated with 5 krad. In the case of the class F line, *axr2-1-d1* DNA from the original M_1 plant was used for the analysis.

with the loss of the *gl1* marker, these results indicate that reversion of the *axr2-1* mutation is associated with loss of a region of chromosome *3* which spans at least 19.5 cM. Since these mutations removed a large number of loci in addition to *AXR2*, the 50-krad mutants were not analyzed further.

In an attempt to isolate revertants with smaller deletions, the dose of γ-irradiation was reduced to 5 krad. One putative *axr2-1* revertant was isolated out of 1026 M_1 plants screened. This revertant, *axr2-1-d1*, was similar to wild type in appearance, and no novel phenotype was observed among the self progeny of this plant. Both Col and Nd-O alleles were present for all the RFLPs examined in the original M_1 *axr2-1-d1* plant, except for RFLP 255 (Figure 1). In the M_2 generation, DNA from plants homozygous for *axr2-1-d1* should not hybridize to the RFLP 255 probe, as that region of the genome is deleted in *axr2-1-d1*. If the deletion were passed to the next generation with 100% efficiency through both the male and female gametes, 25% of the progeny should be homozygous for the deletion. All 46 M_2 plants examined had the Nd-O allele of RFLP 255, indicating that none was homozygous for the *axr2-1-d1* deletion (data not shown). This result suggests that the *axr2-1-d1* mutation results in lethality either in the gametophyte, during embryogenesis, or at the seedling stage of development. To determine if the *axr2-1-d1* mutation was transmitted, the original revertant plant was crossed to a plant homozygous for *gl1*. If the *axr2-1-d1* chromosome was transmitted with 100% frequency, 50% of the F_1 progeny from this cross should display the *gl1* phenotype. If the deletion chromosome is not transmitted, *gl1* plants will only be observed when the *gl1* allele recombines away from the *axr2-1-d1*. In this case, the frequency of *gl1* plants in the F_1 would be 12% or slightly less, depending on the size of the deletion and possible effects of the deletion on recombination. When *axr2-1-d1* was the female parent, 11% (2/18) of the progeny were *gl1*, while when *axr2-1-d1* was the male parent 30% (17/57) of the progeny were *gl1*. These results suggest that the deletion can be transmitted through the male gametophyte, albeit at a reduced frequency. The F_1 population derived from the cross with *axr2-1-d1* as a female parent is too small to draw definite conclusions. However the results suggest that the deletion is not transmitted through the female gametophyte. These plants were not analyzed further and all lines carrying the *axr2-1-d1* mutation were lost subsequent to this study.

M_2 screen for revertants of the *axr2-1* mutation: As a second method of isolating recessive loss-of-function alleles of the *AXR2* gene, seedlings were screened for loss of the *axr2-1* phenotype as described. Over 220,000 M_2 seedlings derived from approximately 22,000 M_1 plants were screened. Three putative revertants were identified in this screen which were *gl1/gl1* indicating that they are probably not contaminants. Because these new mutations were isolated in the *axr2-1* background, they were given the designations *axr2-1-r1*, *axr2-1-r2*, and *axr2-1-r3*. Both *axr2-1-r1* and *axr2-1-r2* were completely wild type in appearance. The *axr2-1-r3* revertant has a distinctive phenotype which is described below.

To determine whether reversion of the *axr2-1* phenotype was due to second-site suppression or to a mutation in the *axr2-1* gene, the *axr2-1-r1* and *axr2-1-r2*

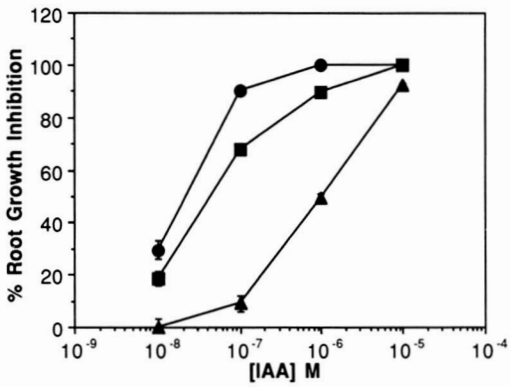


FIGURE 2.—Auxin sensitivity of *axr2-1-r3* seedlings. Sensitivity to IAA was determined in +/+ (circles), *axr2-1/axr2-1* (triangles), and *axr2-1-r3/axr2-1-r3* (squares). Each value is the mean of measurement of at least 10 seedlings. Error bars indicate the standard error.

lines were crossed to wild-type plants. No *axr2-1* plants appeared in the F_2 population ($n = 50$ and 60 for *axr2-1-r1* and *axr2-1-r2* respectively) from either cross indicating that reversion was due to a mutation in the *axr2-1* locus or in a gene closely linked to the *axr2-1* gene. Moreover, when *axr2-1-r3* plants were crossed to wild type, all 85 F_1 plants had the *axr2-1-r3* phenotype. In the F_2, 69 plants examined had the *axr2-1-r3* phenotype and 29 had the wild-type phenotype, indicating that the *axr2-1-r3* mutation segregates as a single dominant mutation ($\chi^2 = 1.1$, $P > 0.05$). In total, no *axr2-1* plants were recovered from 323 F_2 plants examined, indicating that reversion of the *axr2-1* phenotype is probably due to a second mutation at the *axr2-1* locus. When 50 F_1 progeny of a cross between *axr2-1/axr2-1* and *axr2-1-r3/axr2-1-r3* were examined, all the F_1 plants had the *axr2-1* phenotype indicating that the *axr2-1* mutation is dominant to *axr2-1-r3*. Furthermore, the two phenotypes segregated three *axr2-1* to one *axr2-1-r3* in the F_2 (data not shown).

The *axr2-1-r3* line: Auxin sensitivity of the revertant line was determined by measuring IAA-inhibition of root growth (Figure 2). Revertant plants are more sensitive to IAA than *axr2-1* plants but less sensitive than the wild type. In contrast, *axr2-1-r3* and wild-type plants have a similar response to 2,4-D (data not shown).

The appearance of *axr2-1-r3* plants is intermediate between *axr2-1* and wild type (Figure 3 and Table 1). Etiolated hypocotyls of revertant plants are slightly shorter than wild-type hypocotyls and revertant stems are about two-thirds the height of wild-type stems. Similarly, revertant rosette weight is intermediate between *axr2-1* and wild-type rosette weight. Unlike *axr2-1* plants, gravitropism of the revertant primary inflorescence is normal. However, the inflorescence branches on revertant plants grow away from the primary inflorescence at an unusual angle. The branch angle on revertant plants is much closer to perpendicular and causes them to have a broader profile than wild-type plants. Finally, roots of revertant plants are similar to wild type in appearance with abundant root hairs and a normal gravitropic response.

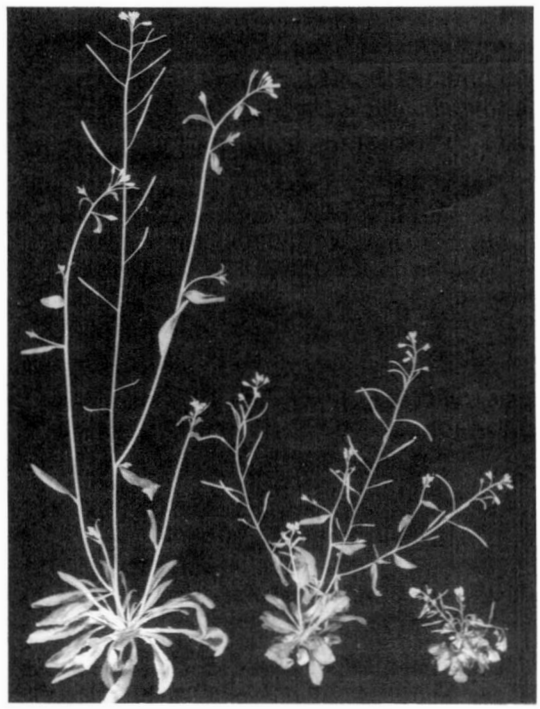


FIGURE 3.—Morphology of mature *axr2-1-r3* plants. Plants were grown as described in MATERIALS AND METHODS and photographed when 6 weeks old. +/+ (left), *axr2-1-r3/axr2-1-r3* (center) and *axr2-1/axr2-1* (right).

TABLE 1

Comparison of wild-type, axr2–1 and axr2–1-r3 morphology

Character	Wild type	$\frac{axr2-1}{axr2-1}$	$\frac{axr2-1-r3}{axr2-1-r3}$
Light-grown hypocotyl length (cm) [a]	0.21 ± 0.01	0.14 ± 0.01	0.20 ± 0.01
Dark-grown hypocotyl length (cm) [a]	1.46 ± 0.02	0.29 ± 0.01	1.26 ± 0.01
Rosette weight (gm)	0.26 ± 0.03	0.08 ± 0.01	0.14 ± 0.01
Inflorescence length (cm)	38.72 ± 1.90	ND [b]	18.30 ± 1.82
Branch angle (° from vertical)	32.6 ± 0.53	ND	77.1 ± 1.8

[a] Hypocotyls were measured 7 days after sowing.
[b] Not done.

Effects of gene copy number on the *axr2-1* phenotype: There are several possible explanations for the dominant nature of the *axr2-1* mutation (MULLER 1932). For example, if one copy of the wild-type *AXR2* gene is not sufficient to produce a wild-type plant, *axr2-1* could be a loss-of-function mutation. Alternatively, *axr2-1* may be a gain-of-function mutation and act by increasing wild-type gene function (hypermorphic), by generating a novel function (neomorphic), or by producing a product which has no function in itself but acts to antagonize action of the wild-type gene product (antimorphic or dominant-negative).

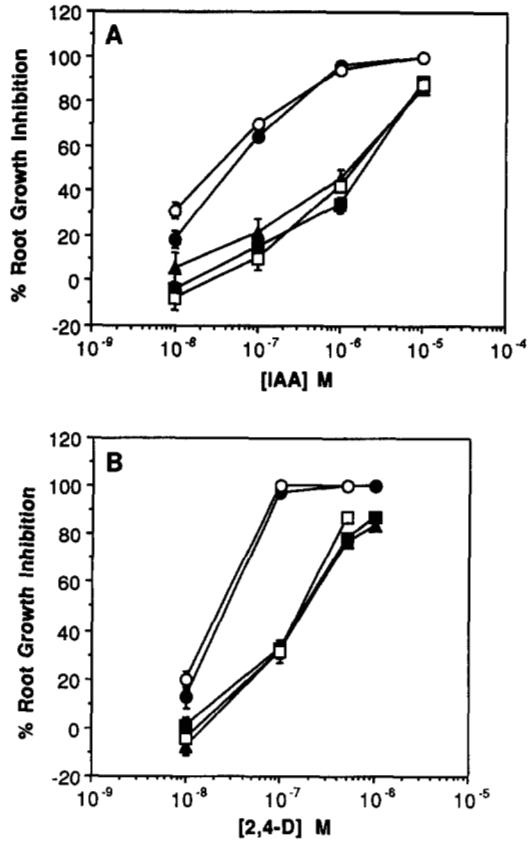


FIGURE 4.—Root growth inhibition on auxin. Inhibition of root growth by auxin is expressed relative to growth on non-supplemented medium. Filled circles represent +/+, filled triangles represent *axr2-1/axr2-1*, filled squares represent *axr2-1*/+, open squares represent *axr2-1*/+/+, and open circles represent +/+/+. (A) Growth inhibition in response to IAA. (B) Growth inhibition in response to 2,4-D.

To distinguish among these possibilities we have examined the effects of altering wild-type gene copy number on the *axr2-1* phenotype. Because well characterized deletion and duplication stocks are not currently available in Arabidopsis, it is not possible to alter *AXR2* gene copy number specifically. As an alternative, we constructed diploid and triploid lines which differ in genotype at the *AXR2* locus. However, this approach has a major limitation. Since triploid plants have larger cells than diploid plants, the cellular concentration of any particular gene product may not significantly increase as ploidy level increases. Consequently, it is not possible to determine the effects of increasing wild-type gene number simply by examining triploid plants. Nonetheless, it is possible to alter the ratio of mutant to wild-type gene copy number by constructing different diploid and triploid lines. Thus plants with the following ratios of mutant to wild-type *AXR2* genes were examined and compared: 0:2 (+/+), 0:3 (+/+/+), 1:2 (*axr2-1*/+/+), 1:1 (*axr2-1*/+), and 2:0 (*axr2-1/axr2-1*). Because *axr2-1* tetraploid plants are not available, it was not possible to generate *axr2-1/axr2-1*/+ plants.

TABLE 2

Auxin level required for 50% inhibition of root growth

Genotype	IAA (μM)	2,4-D (μM)
+/+	0.03 ± 0.02	0.02 ± 0.01
axr2–1/+	1.40 ± 0.70	0.21 ± 0.01
axr2–1/axr2–1	1.30 ± 0.50	0.20 ± 0.10
+/+/+	0.03; 0.02	0.04; 0.02
axr2/+/+	2.00; 1.20	0.12; 0.20

The auxin concentration required for 50% root growth inhibition was determined by interpolation from graphed data similar to that shown in Figure 4. For the three diploid genotypes, the mean and standard error from at least three experiments are presented. Two experiments were performed with each triploid genotype and both values are shown.

Auxin sensitivity in roots: The response of +/+, +/+/+, *axr2-1*/+/+, *axr2-1*/+ and *axr2-1/axr2-1* seedling roots to increasing doses of two different auxins, IAA and 2,4-D, was examined and compared (Figure 4, A and B, and Table 2). Plants with one or more *axr2-1* genes were approximately 40-fold less sensitive to IAA and approximately 10-fold less sensitive to 2,4-D. Roots of wild-type diploid and triploid plants also had a similar sensitivity to auxin, indicating that the comparison between *axr2-1*/+/+, *axr2-1*/+ and *axr2-1/axr2-1* plants is valid. The auxin sensitivity of plants carrying a copy of *axr2-1* are similar, suggesting that an increase in the ratio of wild-type to mutant gene copies does not alter the effects of the *axr2-1* mutation with respect to auxin sensitivity.

Plant morphology: To determine if varying the ratio of wild-type to *axr2-1* genes causes a change in expression of *axr2-1* phenotype, we compared plant morphology of each of the five genotypes described above (Figure 5 and Table 3). In the light, the hypocotyls of wild-type (+/+ and +/+/+) seedlings are slightly longer than mutant (*axr2-1/axr2-1*, *axr2-1*/+ and *axr2-1*/+/+) hypocotyls (Table 3). However, there is no difference among the three mutant genotypes. The difference in length between mutant and wild-type hypocotyls is more pronounced in dark-grown seedlings. Wild-type hypocotyls are approximately five times longer than dark-grown mutant hypocotyls. However, there is no apparent difference in length between the mutant hypocotyls. Therefore, increasing the ratio of wild-type to *axr2-1* gene number has no effect on hypocotyl length in both light- and dark-grown plants.

Mature triploid plants are larger than diploid plants (Figure 5). Therefore, to allow comparison between diploid and triploid plants, each character examined is also expressed relative to the appropriate wild-type genotype (Table 3). A comparison of *axr2-1*/+ and *axr2-1/axr2-1* plants indicates that *axr2-1* is not completely dominant with respect to rosette weight. Rosettes from homozygous mutant plants were approximately 50% the weight of either heterozygous plants or wild-type plants. The more extreme phenotype of homozygous mutant

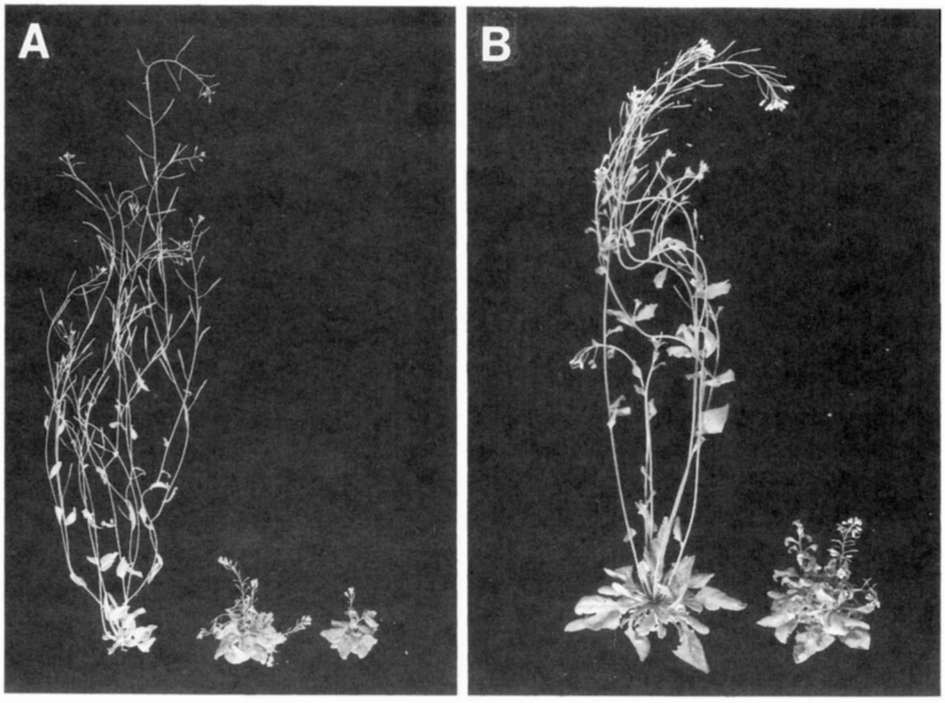


FIGURE 5.—Morphology of mature wild-type and mutant plants. Plants were grown as described in MATERIALS AND METHODS and photographed when 7 weeks old. (A) +/+ (left), +/*axr2-1* (center), and *axr2-1*/*axr2-1* (right). (B) +/+/+ (left) and +/+/*axr2-1* (right).

TABLE 3

Morphometric analysis of axr2 mutants

Morphology	Genotype				
	+/+	*axr2–1*/+	*axr2–1*/*axr2–1*	+/+/+	*axr2–1*/+/+
Rosette weight (g)	0.19 ± 0.06	0.20 ± 0.03	0.1 ± 0.02	0.54 ± 0.11	0.37 ± 0.13
Height of primary inflorescence (cm)	43.1 ± 5.8	7.9 ± 0.8	6.1 ± 1.1	60.8 ± 10.5	13.0 ± 1.1
Floral internode distance (cm)	0.99 ± 0.12	0.20 ± 0.01	0.20 ± 0.02	0.79 ± 0.09	0.19 ± 0.01
Hypocotyl length (cm)					
Light	0.18 ± 0.03	0.11 ± 0.02	0.14 ± 0.02	0.28 ± 0.04	0.12 ± 0.02
Dark	1.46 ± 0.12	0.30 ± 0.03	0.25 ± 0.03	1.32 ± 0.28	0.30 ± 0.03

plants could be due to the presence of two copies of the mutant gene or the lack of wild-type genes. In contrast, any differences observed between *axr2-1*/+ and *axr2-1*/+/+ plants should be due to an increase in the ratio of wild-type to mutant genes (from 0.5 to 0.66). Increasing the ratio of wild-type to mutant genes results in only a minor increase in inflorescence height and internode distance (relative to the appropriate wild-type control). Thus increasing the wild-type gene copy number from 2 to 3 does not result in a significant amelioration of the mutant phenotype.

The *axr2-1* mutation also affects root hair development and root gravitropism. Both *axr2-1*/+ and *axr2-1*/*axr2-1* seedlings lack root hairs except at the root-hypocotyl junction (data not shown). Seedlings of *axr2-1*/+/+ have a few root hairs along the length of the root. However, these are irregularly positioned, non-uniform in length, and are much fewer in number than wild type. Thus, increasing the ratio of wild-type to mutant *AXR2* genes seems to have a minor effect on the root hair phenotype. When wild-type plants are grown on vertically oriented plates, the roots are gravitropic, growing downward. In contrast, roots of mutant seedlings are agravitropic and do not necessarily orient downward. This growth defect appears to be slightly more severe in *axr2-1*/*axr2-1* than in *axr2-1*/+ seedlings, but increasing the ratio of wild-type to mutant gene copies, as in *axr2-1*/+/+ plants, does not correct the gravitropic defect (data not shown).

Expression of *SAUR-AC1* in *axr2-1* seedlings: Recent studies have shown that auxin induces the rapid accumulation of the *SAUR-AC1* transcript in etiolated wild-type Arabidopsis seedlings (GIL *et al.* 1994). To investigate the rapid response of wild-type and mutant plants to auxin, accumulation of *SAUR-AC1* mRNA was examined. In the absence of auxin, neither wild-type nor *axr2-1* etiolated seedlings accumulated *SAUR-AC1* RNA (Figure 6A). In the presence of 2,4-D, wild-type seedlings accumulated significant levels of *SAUR-AC1* transcript. In contrast, transcript was not detected in RNA samples prepared from auxin-treated *axr2-1* etiolated seedlings. Similar results have been obtained by GIL *et al.* (1994). In addition, an S1 nuclease assay was performed, since this assay proved to be several fold

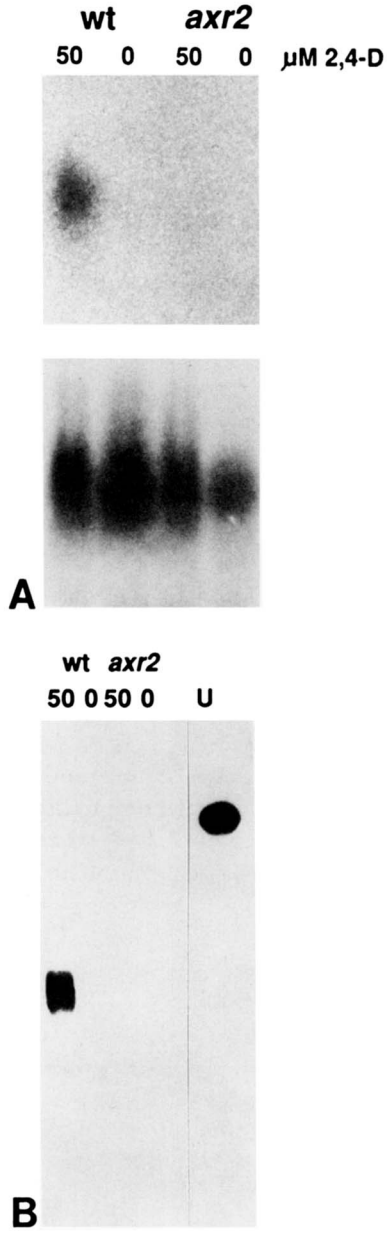


FIGURE 6.—Analysis of *SAUR-AC1* expression in *axr2-1* seedlings. Etiolated wild-type and *axr2-1* seedlings were depleted of auxin as described in MATERIALS AND METHODS and treated with 0 or 50 μM 2,4-D for 1 h. (A) Northern blot analysis of total RNA (20 μg) fractionated in a 1% agarose/formaldehyde gel and probed with *SAUR-AC1*. Lower panel is the same blot stripped and reprobed with rDNA. (B) Total RNA (50 μg) was annealed to the *SAUR-AC1* probed and treated with S1 nuclease, followed by separation in 6% polyacrylamide gel. U designates the uncut probe.

more sensitive for the detection of *SAUR-AC1* RNA from wild-type plants (our unpublished results). In the S1 analysis, neither auxin-depleted wild-type nor *axr2-1* etiolated seedlings accumulated *SAUR-AC1* transcripts (Figure 6B). *SAUR-AC1* RNA was readily detected in wild-type seedlings after incubation with 2,4-D, but was not detectable in etiolated *axr2-1* seedlings following auxin treatment.

To determine if *SAUR-AC1* induction and accumulation is delayed in mutant seedlings compared to wild type, etiolated *axr2-1* seedlings were treated with 50 μM 2,4-D for 4 hr. Accumulation of *SAUR-AC1* transcripts was not observed. Similarly, treatment of *axr2-1* tissue with 500 μM 2,4-D for 1 hr did not stimulate *SAUR-AC1* RNA accumulation. In both cases, wild-type seedlings accumulated *SAUR-AC1* RNA. It is possible that *axr2-1* plants are deficient in *SAUR-AC1* expression under dark-grown conditions only. To investigate this possibility, wild-type and *axr2-1* seedlings were grown in the light for seven days, depleted of auxin and treated with 50 μM 2,4-D for 1 hr. Transcripts accumulated in auxin-treated wild-type seedlings, but to a lesser extent than in etiolated seedlings. Again, *SAUR-AC1* RNA was not detectable in light-grown *axr2-1* seedlings (data not shown).

***SAUR-AC1* induction in mature *axr2-1* tissue:** Induction of SAUR genes in mature plant tissues has not been reported. To determine if *SAUR-AC1* is inducible in mature Arabidopsis plants, excised inflorescences, rosettes and roots of mature wild-type and *axr2-1* plants were analyzed. As in etiolated seedlings, auxin-depleted tissue had undetectable amounts of *SAUR-AC1* transcript. Auxin treatment induced the accumulation of *SAUR-AC1* RNA in wild-type inflorescences (Figure 7A). Neither depleted nor auxin-treated *axr2-1* inflorescences accumulated detectable *SAUR-AC1* transcripts. Using the more sensitive S1 nuclease assay, transcript was easily detected in auxin-treated wild-type inflorescences, but was not detected in *axr2-1* (data not shown). A similar analysis was performed on the rosettes and roots from mature plants, and auxin-induced accumulation of *SAUR-AC1* RNA was seen in all wild-type organs. *SAUR-AC1* transcripts were not detected in any mature *axr2-1* tissue following auxin depletion and treatment with 2,4-D (data not shown).

Expression of *SAUR-AC1* in other genotypes: Plants with one or more copies of the *axr2* gene were analyzed for induction of *SAUR-AC1* transcription and compared to wild-type. Light-grown wild-type, *axr2-1/axr2-1*, *axr2-1/+*, and *axr2-1/++* seedlings were depleted of auxin and incubated for 1 hr in the presence of 50 μM 2,4-D. In contrast to the accumulation of *SAUR-AC1* transcripts in wild-type tissue, mutant plants carrying one or more copies of the *axr2-1* gene did not accumulate detectable levels of *SAUR-AC1* RNA (data not shown).

The revertant *axr2-1-r3* was also analyzed for induction of *SAUR-AC1* RNA. In the absence of auxin, *SAUR-AC1* RNA was not detectable in rosette leaves of wild-type, *axr2-1*, and *axr2-1-r3* plants (Figure 7B). However, in the presence of auxin, both wild-type and *axr2-1-r3* tissue accumulated *SAUR-AC1* transcripts. The extent of accumulation of *SAUR-AC1* transcripts in *axr2-1-r3* was consistently lower than that of wild type.

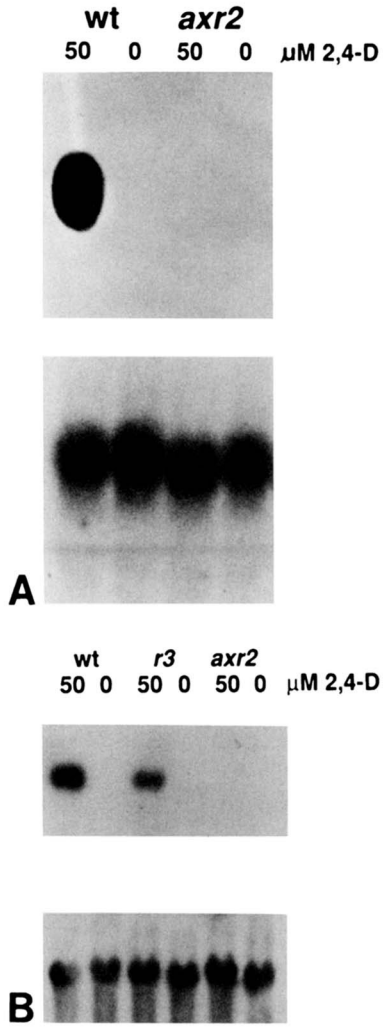


FIGURE 7.—Analysis of *SAUR-AC1* induction in mature wild-type and *axr2* plants. Northern blot analysis of total RNA (20 μg) fractionated in a 1% agarose/formaldehyde gel and probed with *SAUR-AC1*. (A) Mature wild-type and *axr2-1* inflorescences were depleted of auxin as described in MATERIALS AND METHODS and treated with 0 or 50 μM 2,4-D for 1 h. (B) Rosette leaves of wild-type, *axr2-1-r3*, and *axr2* plants were treated as described. Lower panels are blots stripped and reprobed with rDNA.

DISCUSSION

The dominant *axr2-1* mutation confers resistance to auxin, ethylene, and abscisic acid. It was recovered from screens of greater than 500,000 M_2 seedlings. Since loss-of-function mutations typically occur much more frequently than 1 in 500,000 M_2 plants (ESTELLE and SOMERVILLE 1986), the *axr2-1* mutation is probably a gain-of-function mutation. This hypothesis is supported by the results described here. By treating *axr2-1* pollen with γ-irradiation, we generated plants which are heterozygous for deletions which include the *AXR2* locus. These plants are wild type in appearance indicating that a single copy of the *AXR2* gene is sufficient for normal development. Thus we would expect that a loss-of-function mutation at the locus would be recessive.

We adopted two approaches to identify recessive loss-of-function mutations at the *AXR2* locus. The first strategy involved crossing γ-irradiated *axr2-1* pollen onto wild-type plants and screening for loss of the *axr2-1* phenotype among the M_1 progeny. The advantage of this screen is that lethal mutations can be recovered as heterozygotes. We were able to recover revertant M_1 plants at a reasonable frequency. However, analysis of segregating RFLP markers in the M_1 or M_2 plants indicated that each reversion event was associated with the loss of a large segment of chromosome *3*. Since our primary goal was to isolate recessive mutations at the *AXR2* locus we did not analyze these lines further. Nonetheless, it is clear that γ-irradiation of pollen is an effective method for generating large deletions.

A second strategy for isolating recessive mutations at the *AXR2* locus involved screening an *axr2-1/axr2-1* M_2 population for revertants of the *axr2-1* phenotype; three revertant lines were recovered. Genetic analysis indicates that reversion in each line is probably due to a mutation at the *axr2-1* locus, although the possibility of a suppressing mutation in a closely linked gene cannot be eliminated. Since *axr2-1-r1* and *axr2-1-r2* are identical to wild type in appearance, a second mutation has probably restored wild-type function to the *axr2-1* gene. The third revertant line, *axr2-1-r3,* appears to carry a second mutation at the *AXR2* locus which modifies the *axr2-1* phenotype. This line has a new phenotype which is intermediate between wild type and *axr2-1*. Plants which are *axr2-1-r3/+* exhibit the *axr2-1-r3* phenotype while *axr2-1/axr2-1-r3* plants have an *axr2-1* phenotype. These results support the hypothesis that *axr2-1* is a gain-of-function mutation and suggest that the new mutation modifies gene activity to a state closer to that of the wild-type gene. This second *axr2* allele will be a useful tool in future attempts to understand the function of the wild-type gene product.

The three revertant lines recovered in the M_2 screen probably do not carry hypomorphic mutations at the *AXR2* gene. It is possible that loss of *AXR2* gene function does not result in a mutant phenotype. However, if this were the case, we would expect to recover a much larger number of revertants in a screen of 220,000 seedlings. An alternative explanation is that *AXR2* is an essential gene and recessive mutations result in death of the gametophyte or sporophyte before mutants can be identified. Since auxin is essential for growth of plant cells in culture, and is thought to regulate diverse aspects of plant development, it would not be surprising for the *AXR2* gene to be essential for cell viability.

To further characterize the *axr2-1* mutation we have analyzed diploid and triploid plants with different ratios of wild-type and mutant *AXR2* genes. All of the mutant genotypes examined have approximately the same level of auxin sensitivity indicating that a single *axr2-1* gene

is sufficient to confer the full reduction in auxin sensitivity. This result suggests that the *axr2-1* mutation is either neomorphic or hypermorphic. The mutant and wild-type gene products do not appear to be antagonistic since an increase in the relative level of wild-type gene copy number does not result in increased auxin sensitivity (compare *axr2-1/+* and *axr2-1/+/+*). The morphology of the various mutant genotypes is also very similar. Inflorescence height and internode length (relative to the appropriate wild-type line) increase slightly as the relative level of wild-type gene number increases, but these values are still greatly reduced compared to wild type. Again, there is little evidence for antagonism between mutant and wild-type gene products. Homozygous mutant plants have significantly smaller rosettes than either +/+ or *axr2-1/+* plants. Since the wild-type gene product does not appear to ameliorate the effects of the mutant gene product, the difference between these two genotypes must be due to an increase in the number of mutant genes.

These results do not distinguish between the effects of a neomorphic mutation which generates a novel function and a hypermorphic mutation which, for example, causes an overexpression of wild-type *AXR2* gene product. Plants with the genotype *axr2-1*/deficiency may help to resolve this question. Unfortunately, deficiencies are not presently available. The *axr2-1-d1* mutation, which appeared to carry a small and heritable deficiency in this region, was lost before these experiments could be performed. In addition, the dominant nature of the *axr2-1* mutation precludes the generation of *axr2-1*/deficiency plants directly by pollen mutagenesis.

To learn more about the effects of *axr2-1* on auxin response, we have examined expression of the auxin inducible *SAUR-AC1* gene in wild-type and mutant plants. Transcription of the SAUR genes is thought to be a primary cellular response to auxin (McClure *et al.* 1989). Accumulation of *SAUR-AC1* RNA was examined in several wild-type plant organs, both in the presence and absence of exogenously applied auxin. When depleted of auxin, *SAUR-AC1* RNA is not detectable in any wild-type plant tissue. However, treatment with 2,4-D induces accumulation of *SAUR-AC1* transcripts in hypocotyl, root, rosette and inflorescence tissue in wild-type plants. These results contrast with observations in soybean that *SAUR* transcripts accumulate only in hypocotyl and plumules of seedlings, but are not inducible in mature plant tissues (Gee *et al.* 1991).

In the *axr2-1* mutant, *SAUR-AC1* accumulation is dramatically altered relative to wild-type plants. In contrast to the strong response observed in wild-type plants, *SAUR-AC1* transcripts are not detectable in any *axr2-1* tissue after auxin depletion and subsequent auxin treatment. These results indicate that the *axr2-1* mutation confers auxin resistance in the leaves, inflorescences and roots of mature plants. Treatment with higher concentrations of auxin or treatment for longer time periods did not increase *SAUR-AC1* accumulation. Thus, the *axr2-1* mutation appears to prevent auxin induction of *SAUR-AC1* expression. This defect is consistent with the high levels of auxin resistance of *axr2-1* plants and suggests that auxin resistance is not due to a defect in either auxin uptake or metabolism. Rather, the *axr2-1* mutation appears to disrupt an early step in an auxin response pathway.

A single mutant copy of the *axr2-1* gene is sufficient to abolish *SAUR-AC1* transcript accumulation in *axr2-1/+* and *axr2-1/+/+* plants. These results agree with the morphological data and suggest that additional copies of the wild type gene do not alter the effects of the *axr2-1* mutant gene. Furthermore, the revertant *axr2-1-r3* restores accumulation of *SAUR-AC1* transcripts, but not to wild-type levels. This result is consistent with the increased root growth sensitivity to auxin in *axr2-1-r3* plants, and suggests that this mutation partially restores wild-type gene function.

The results presented here do not define the exact role of AXR2 in auxin response. One formal possibility is that *axr2-1* is a mutation in the *SAUR-AC1* gene. However, this seems unlikely since the SAUR genes are redundant, comprising a gene family in soybean and Arabidopsis (Guilfoyle *et al.* 1993). Additionally, *axr2-1* is also deficient in the accumulation of transcripts of the *IAA1* and *IAA2* genes of *Arabidopsis* (S. Abel and A. Theologis, personal communication). These genes are representative members of another family of auxin-inducible genes (Theologis *et al.* 1985; Abel *et al.* 1994) and further indicate that *axr2-1* disrupts an early step in an auxin response pathway.

The evidence presented here suggests that *axr2-1* is a gain-of-function mutation which dramatically affects the morphology of the plant and severely disrupts an early auxin response. Previous analysis of the *axr2-1* phenotype demonstrated that the *axr2-1* mutation confers a high level of auxin resistance as well as a dramatic reduction in cell elongation in the hypocotyl and inflorescence (Wilson *et al.* 1990; Timpte *et al.* 1992). In soybean, the SAUR genes are transcribed rapidly in response to auxin and are expressed primarily in elongating tissue, therefore SAUR genes are likely to function in auxin regulated cell elongation (McClure *et al.* 1989; Gee *et al.* 1991). Thus, the failure of *axr2-1* cells to accumulate *SAUR-AC1* transcripts may be related to the failure of these cells to elongate and hence, to respond to gravity signals. We speculate that the *axr2-1* phenotype is due to a defect in this auxin regulated cell elongation and that the *axr2-1* mutation acts early in an auxin response pathway. One possibility is that *AXR2* encodes a repressor of auxin-regulated transcription. If this is the case, the gain-of-function *axr2-1* mutation may cause the synthesis of unusually high levels of AXR2, or alternatively, the mutation may prevent auxin-mediated inactivation of the repressor.

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