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Growth and Development of the *axr1* Mutants of *Arabidopsis*

Cynthia Lincoln, James H. Britton, and Mark Estelle[1]

Department of Biology, Indiana University, Bloomington, Indiana 47405

We have recovered eight new auxin-resistant lines of *Arabidopsis* that carry mutations in the *AXR1* gene. These eight lines, together with the 12 lines described in a previous report, define at least five different *axr1* alleles. All of the mutant lines have a similar phenotype. Defects include decreases in plant height, root gravitropism, hypocotyl elongation, and fertility. Mutant line *axr1-3* is less resistant to auxin than the other mutant lines and has less severe morphological abnormalities. This correlation suggests that the morphological defects are a consequence of a defect in auxin action. To determine whether the altered morphology of mutant plants is associated with changes in cell size or tissue organization, tissue sections were examined using scanning electron microscopy. No clear differences in cell size were observed between wild-type and mutant tissues. However, the vascular bundles of mutant stems were found to be less well differentiated than those in wild-type stems. The auxin sensitivity of rosette-stage plants was determined by spraying plants with auxin solutions. Mutant rosettes were found to be significantly less sensitive to exogenously applied auxin than wild-type rosettes, indicating that the *AXR1* gene functions in aerial portions of the plant. Our studies suggest that the *AXR1* gene is required for auxin action in most, if not all, tissues of the plant and plays an important role in plant development. Linkage studies indicate that the gene is located on chromosome 1 approximately 2 centiMorgans from the closest restriction fragment length polymorphism.

INTRODUCTION

In higher plants, a complex hormonal system appears to play a crucial role in controlling growth and development. One important group of plant hormones, the auxins, has been shown to influence a wide variety of growth processes. Evidence from physiological studies indicates that the major naturally occurring auxin, IAA, affects such diverse processes as cell expansion during shoot elongation (Jacobs and Ray, 1976), tropic responses (Shaw et al., 1973; McClure and Guilfoyle, 1989), apical dominance (Phillips, 1975), and differentiation of vascular elements (Aloni, 1987). The proliferation of cultured plant cells or tissues also requires auxin supplied either exogenously (Krikorian et al., 1987) or endogenously in tumors (Gelvin, 1990). The diversity of growth processes influenced by auxin combined with the requirement for auxin in tissue culture suggests that this particular plant hormone is essential for cell growth and plant viability.

Although physiological studies have clearly illustrated how auxins can influence plant growth and differentiation, the molecular mechanism of auxin action is still not understood. A powerful approach to identify hormone receptors and components of signal transduction pathways is the isolation of mutants altered in hormone response. The genetic approach has been used effectively to study hormone action in animal (Rabindran et al., 1987) and fungal systems (Hartwell, 1980), and promises to be equally useful in the analysis of plant growth hormones (King, 1988). For example, biochemical studies have shown that the auxin-resistant mutant of tomato, *diageotropica*, is deficient in an auxin-binding protein that may function as a receptor (Hicks et al., 1989). In *Arabidopsis thaliana*, a number of hormone-resistant mutants have been isolated, including mutants insensitive to exogenous ABA, GA, and ethylene (Finkelstein et al., 1987). Identification of the biochemical defects in these mutants as well as isolation and characterization of the hormone resistance genes should greatly increase our understanding of hormone action in plants.

We have screened for mutants of *Arabidopsis* that are resistant to exogenous application of auxin. In a previous report, we described the isolation and preliminary characterization of a recessive mutation called *axr1* (Estelle and Somerville, 1987). Plants homozygous for *axr1* mutations exhibit reduced sensitivity to auxin in addition to a number of morphological abnormalities. In this report, we extend our genetic studies and describe the morphological and physiological characterization of two *axr1* mutants. Several independent screens for auxin resistance have resulted in

[1] To whom correspondence should be addressed.

Table 1. Recovery of *axr1* Mutants[a]

M2 population	Mutagen	Selection	Mutants recovered
A[b]	EMS	2,4-D	*axr1-1*
			axr1-2
			axr1-3
			axr1-4
			axr1-5
			axr1-6
B[b]	EMS	2,4-D	*axr1-7*
			axr1-8
			axr1-9
			axr1-11
			axr1-12
			axr1-15
C[c]	EMS	2,4-D	*axr1-16*
			axr1-17
			axr1-18
			axr1-19
			axr1-20
			axr1-21
C[c]	EMS	IAA	*axr1-22*
D[c]	γ	2,4-D	*axr1-23*

[a] A total of 470,000 seeds from four distinct M2 populations was screened for mutants that were able to elongate roots on either 5 μM 2,4-D or 50 μM IAA.
[b] Estelle and Somerville (1987).
[c] This study.

the isolation of 20 *axr1* mutant lines. These lines fall into two classes that differ in the severity of their phenotype. We demonstrate that the *axr1* mutation confers auxin resistance to both the root and rosette and also disrupts root gravitropism and hypocotyl elongation. This unusual phenotype indicates that the *AXR1* gene functions in most plant tissues and is required for auxin-regulated growth processes. Finally, we have used restriction fragment length polymorphisms (RFLPs) to map the *AXR1* gene to chromosome 1.

RESULTS

Isolation of *axr1* Mutants

In a previous report (Estelle and Somerville, 1987), we described the isolation and preliminary characterization of 12 auxin-resistant mutant lines of *Arabidopsis*. Genetic analysis of the 12 mutant lines indicated that 2,4-D resistance is the result of recessive mutations in a single gene that we have called the *AXR1* gene. An additional 170,000 M2 seeds from two independently mutagenized populations (M2 populations C and D, Table 1) were screened for seedlings resistant to either 50 μM IAA or 5 μM 2,4-D. Eight new auxin-resistant lines with a morphological phenotype similar to the original *axr1* mutant lines were recovered. Seven of the new resistant lines were recovered from an ethyl methanesulfonate (EMS)-mutagenized population, and one line was recovered from a γ-mutagenized population. The results of all of our screens are summarized in Table 1.

Genetic Analysis of *axr1* Mutants

To determine the genetic basis for auxin resistance in the new mutant lines (recovered from M2 populations C and D, Table 1), each line was crossed to a wild-type plant and auxin resistance was scored in the F1 and F2 generations by germinating seeds on agar medium containing 1 μM 2,4-D. Segregation data for some of the mutant lines are presented in Table 2. For each line, auxin resistance segregated three sensitive to one resistant, indicating that auxin resistance segregates as a recessive trait. Similar results were obtained for the other four *axr1* mutant lines. To confirm that the new auxin-resistant lines carried an *axr1* mutation, each mutant line was crossed to plants homozygous for the *axr1-3* mutation. The results of this experiment are displayed in Table 3. All of the F1 progeny from these crosses were resistant to 1 μM 2,4-D, indicating that each new mutant line is homozygous for a mutation that does not complement the *axr1* mutation. Thus, auxin resistance in these lines is due to a mutation at the *AXR1* gene. Similar results were obtained for all of the mutants listed in Table 1.

axr1 Mutants Have an Altered Morphology

In addition to auxin resistance, the *axr1* mutations produce a characteristic morphological phenotype that includes defects in leaf, inflorescence, and flower morphology. In

Table 2. Genetic Segregation of 2,4-D Resistance in *axr1* Lines

Cross	Number of Plants		
	Resistant	Sensitive	χ^{2} [a]
axr1-19 × wild-type F1	0	23	
F2	186	493	2.07[b]
axr1-21 × wild-type F1	0	51	
F2	82	281	1:12[b]
axr1-22 × wild-type F1	0	22	
F2	56	216	2.83[b]
axr1-23 × wild-type F1	0	33	
F2	117	383	0.683[b]

[a] χ^{2} was calculated based on an expected ratio of three sensitive to one resistant.
[b] $P > 0.05$.

Table 3. Complementation Analysis of *axr1* Lines

Cross	Number of Plants	
	Resistant	Sensitive
axr1-12 × *axr1-3*	33	0
axr1-19 × *axr1-3*	21	0
axr1-20 × *axr1-3*	13	0
axr1-22 × *axr1-3*	24	0
axr1-23 × *axr1-3*	39	0

all mutant lines, this altered plant morphology cosegregates with auxin resistance. The initial characterization of *axr1* plant morphology suggested that all 12 mutant lines (M2 population A and B, Table 1) were similarly altered with respect to their developmental phenotype (Estelle and Somerville, 1987). However, repeated back-crossing of several of these mutant lines to wild-type plants, combined with a more detailed analysis of plant morphology, revealed that one mutant line differed from all the others. This mutant line, *axr1-3*, is characterized by less severe morphological alterations. The other 19 mutant lines define a second, more severely affected class of mutants. The morphologies of *axr1-3* and *axr1-12*, one of the severely affected mutants, were characterized in detail.

Morphological differences between *axr1* and wild-type plants can be seen in rosettes, inflorescences, and flowers. These differences are illustrated in Figures 1 and 2 and Table 4. Rosette leaves of *axr1* plants are irregular in shape and tend to curl downward (Figure 1). The margins of mutant leaves are slightly toothed and the petioles are shorter than wild-type leaves. These leaf differences are seen in both *axr1-3* and *axr1-12* plants, but are much more pronounced in *axr1-12* mutants. Other differences between mutant and wild-type plants can be seen in the inflorescences (Figure 2 and Table 4). At maturity, the height of wild-type plants is approximately 50 cm. This is only slightly reduced in *axr1-3* plants. However, the height of *axr1-12* plants is less than half that of wild-type plants. Although the total number of inflorescences arising from the rosette does not differ greatly between *axr1* and wild-type plants, the number of lateral branches is dramatically increased. The total number of lateral branches observed on *axr1-3* inflorescences is twice the number seen on wild-type plants. The inflorescences of *axr1-12* mutants are even more highly branched, giving *axr1-12* plants a much bushier appearance (Figure 2). The overall growth habit of *axr1-12* plants indicates a reduction in apical dominance that is not as evident in *axr1-3* plants.

Plants homozygous for the *axr1-12* mutation exhibit greatly reduced fertility relative to both *axr1-3* and wild-type plants and produce significantly less pollen (Table 4). However, *axr1-12* plants can be successfully hand pollinated with either wild-type or *axr1-12* pollen, indicating that pistil development is normal in the mutant and that *axr1-12* pollen is functional. Examination of developing *axr1-12* flowers revealed that very little pollen is deposited on the stigma because the filaments of mutant plants fail to elongate (data not shown). This defect, in combination with the reduced pollen yield, probably accounts for the reduction in self-fertilization in *axr1-12* plants.

Differences between mutant and wild-type plants can also be observed in dark-grown seedlings (Table 4). Etiolated seedlings of both *axr1-3* and *axr1-12* mutant lines have shorter hypocotyls than those of wild-type seedlings. The difference in hypocotyl length is greatest between wild-type and *axr1-12* seedlings. The hypocotyls of etiolated *axr1-12* seedlings are approximately half the length of wild-type hypocotyls. The hypocotyl length of light-grown seedlings is the same in both mutant and wild-type seedlings (data not shown).

Leaf and Stem Ultrastructure of Mutant and Wild-Type Plants

It is possible that the altered morphology of *axr1* plants is due to differences in cell size or tissue organization. To compare wild-type and mutant tissues, we examined stem and leaf sections with the scanning electron microscope. Representative micrographs are shown in Figure 3. Scanning electron micrographs (SEMs) of cross-sections through the leaf blade of wild-type and *axr1-12* rosette leaves did not reveal any major structural differences (Figures 3a and 3d). Cell size and the relative numbers of

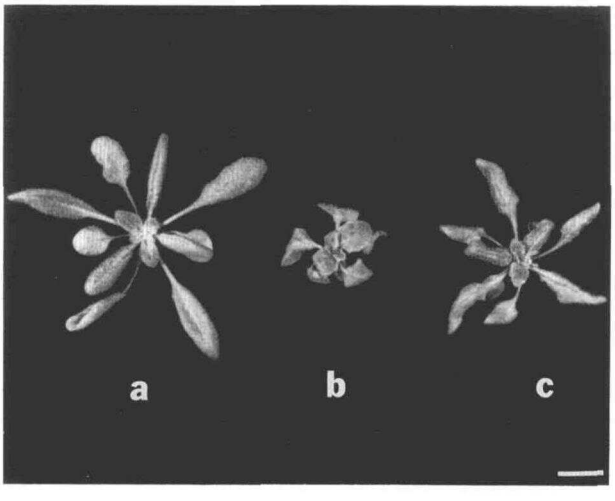

Figure 1. Phenotype of Wild-Type and Mutant Rosettes.

Rosettes were photographed when the plants were 3 weeks old.
(a) Wild type.
(b) *axr1-12/axr1-12*.
(c) *axr1-3/axr1-3*.
Bar = 1 cm.

Figure 2. Comparison of Mature Wild-Type and Mutant Plants.

Wild-type and mutant plants were photographed when 7 weeks old.
(a) Wild type.
(b) *axr1-12/axr-12*.
(c) *axr1-3/axr1-3*.
Bar = 3 cm.

palisade and spongy mesophyll cells are approximately the same in mutant and wild-type leaves. The size and frequency of intercellular spaces within the mesophyll cell layer are also similar. SEMs of cross-sections of the first internode of mature primary inflorescences from mutant and wild-type plants are displayed in Figures 3b and 3e. Comparison of similar cross-sections obtained from each of several different *axr1-12* and wild-type plants reveals subtle differences in the organization of vascular tissue. Vascular bundles in *axr1-12* tissue are less uniform and do not extend as far into the pith as wild-type vascular bundles. In general, the vascular bundles in mutant stem tissue appear to be less differentiated. Longitudinal sections through mutant and wild-type stem tissue were also prepared to examine the relative lengths of the pith parenchyma cells (Figures 3c and 3f). In general, the length of these cells was similar in *axr1-12* and wild-type stem tissue. Stem and leaf sections of *axr1-3* were also examined and found to be similar to *axr1-12* sections.

Root Gravitropism Is Affected in *axr1* Mutants

To determine the effect of the *axr1* mutation on root gravitropism, we measured the response of wild-type and *axr1* roots to gravity on agar plates. The results of this experiment are shown in Figure 4. Roots of *axr1* seedlings exhibit a slower response to a gravity stimulus than the roots of wild-type seedlings. Within 5 hr of gravistimulation, wild-type roots achieved an angle of curvature of 80°. In contrast, the roots of both *axr1-3* and *axr1-12* seedlings had curved only 60° toward the gravity stimulus after 9 hr. There was no difference between the response of *axr1-3* and *axr1-12* roots. The difference in root gravitropic response did not appear to be due to a reduction in root growth rate. In fact, Figure 5 shows that the roots of both *axr1-3* and *axr1-12* seedlings elongated significantly faster than the roots of wild-type seedlings.

axr1 Mutations Confer Auxin Resistance to Both Roots and Leaves

The results in Figure 6 show that growth of the roots of *axr1-3* and *axr1-12* seedlings is less inhibited than wild-type roots over a range of 2,4-D concentrations. Seedlings homozygous for the *axr1-12* mutation are resistant to twofold to threefold higher concentrations of 2,4-D than *axr1-3* seedlings. A similar root growth inhibition assay

Table 4. Morphology of Wild-Type and Mutant Plants

	Wild Type	*axr1-3*	*axr1-12*
Height (cm)	50.9 ± 1.0	38.4 ± 1.3	19.2 ± 1.8
No. of inflorescences	5.75 ± 0.25	6.25 ± 1.4	6.6 ± 1.6
No. of lateral branches	46.2 ± 4.5	96.7 ± 21.4	124.0 ± 24.4
Distance between siliques (cm)	0.65 ± 0.04	0.30 ± 0.01	0.32 ± 0.02
No. of siliques	673.75 ± 68.0	526.5 ± 56.0	2.4 ± 1.3
No. of pollen grains/flower	2035.0 ± 500.0	2700.0 ± 337.0	680.0 ± 32.0
Hypocotyl length in etiolated seedlings (cm)	1.40 ± 0.04	1.20 ± 0.05	0.77 ± 0.03

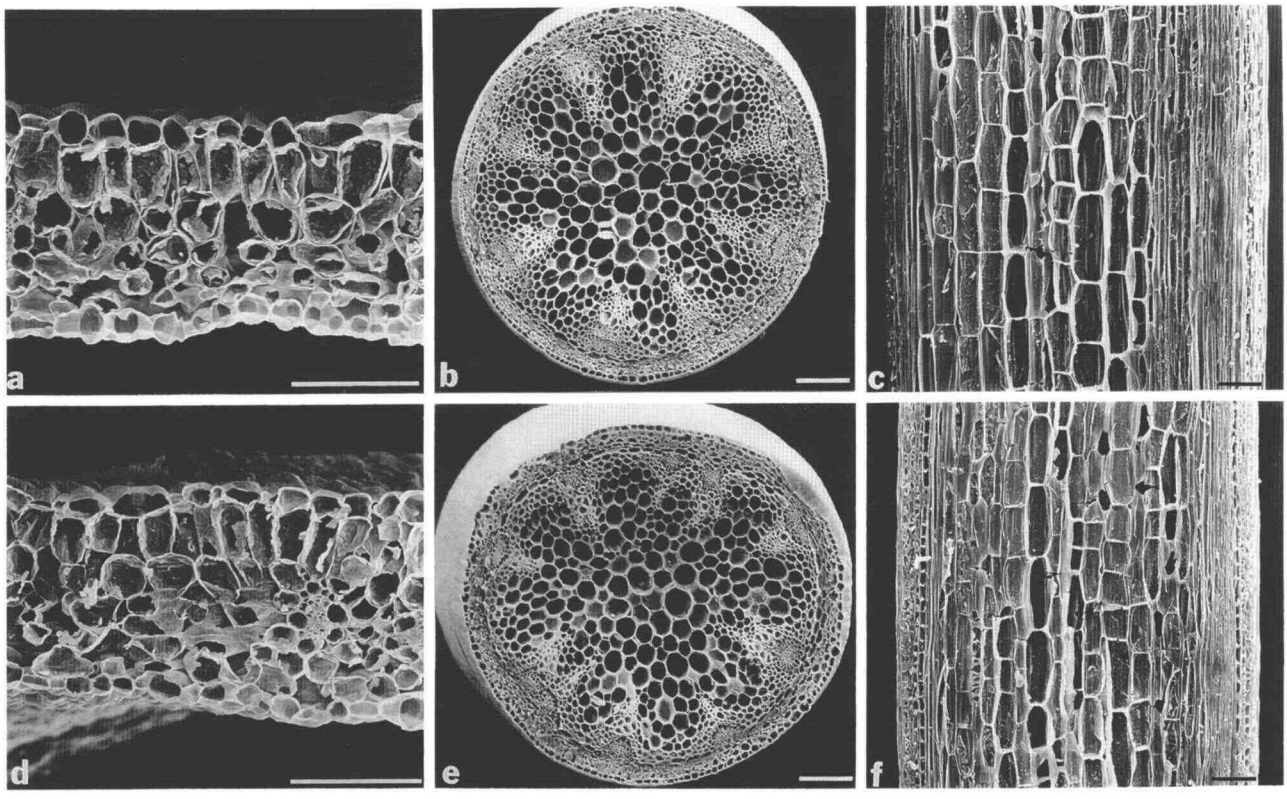

Figure 3. Scanning Electron Micrographs of Sections Prepared from Wild-Type and *axr1-12* Plants.

(a) Cross-section of wild-type rosette leaf.
(b) Cross-section of wild-type stem.
(c) Longitudinal section of wild-type stem.
(d) Cross-section of *axr1-12* rosette leaf.
(e) Cross-section of *axr1-12* stem.
(f) Longitudinal section of *axr1-12* stem.
Bar = 100 μm.

using IAA was performed, and the results indicate that *axr1-12* seedlings are also more resistant to IAA than *axr1-3* seedlings (data not shown).

The rosette leaves of *axr1-3* and *axr1-12* plants are also less sensitive to 2,4-D than wild-type leaves, as shown in Table 5. In general, 2,4-D inhibited leaf growth but in *axr1-3* rosettes, leaf growth was actually stimulated by treatment with 10 μM 2,4-D. The same concentration of 2,4-D only slightly inhibited *axr1-12* rosette growth. A comparison of the percent growth inhibition of wild-type, *axr1-3*, and *axr1-12* rosettes treated with 100 μM 2,4-D reveals that *axr1-12* plants exhibit the highest level of resistance.

Map Position of *axr1*

The chromosomal location of *axr1* was established by determining the degree of linkage between *axr1* and RFLP markers on several different chromosomes. This analysis, shown in Table 6, indicates that the mutation lies on chromosome 1, approximately 2.6 centiMorgans from RFLP 488 (Chang et al., 1988).

DISCUSSION

We have identified eight new auxin-resistant lines of *Arabidopsis* that carry recessive mutations of the *AXR1* gene. Among the 20 *axr1* mutant lines that have been recovered, the majority exhibit dramatic alterations in plant morphology, including a significant reduction in apical dominance and fertility. However, the phenotype of one of the mutants, *axr1-3*, is characterized by less severe morphological abnormalities. Four independent M2 populations, three generated by EMS treatment and one by γ irradiation, have been screened. Each screen has resulted in the

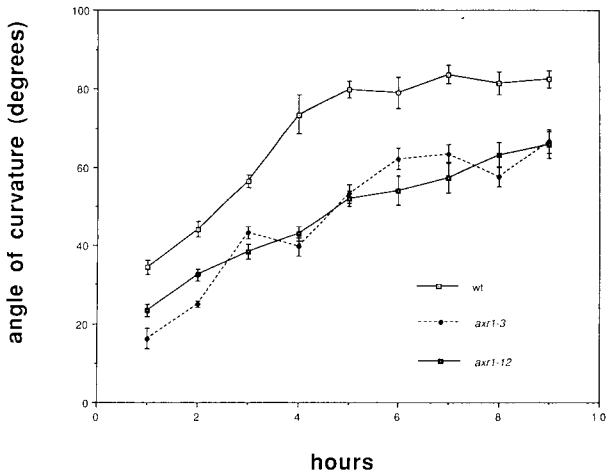

Figure 4. Gravitropic Response of Wild-Type (wt) and Mutant (*axr1-3*, *axr1-12*) Seedlings.

Seedlings grown on agar medium were rotated 90° at time 0. The angle of curvature from the horizontal was measured at the times indicated. Each point represents the mean of 10 measurements. Error bars indicate the standard error.

recovery of at least one new *axr1* allele. Because *axr1-3* mutants exhibit a unique phenotype, we believe that this line represents one additional *axr1* allele. Therefore, we have recovered at least five *axr1* alleles.

In our early studies of the *axr1* mutants, we showed that the roots of mutant seedlings are resistant to auxin inhibition of growth (Estelle and Somerville, 1987). In addition, we demonstrated that higher concentrations of auxin are required to promote callus growth from mutant stem segments than from wild-type segments (Lincoln and Estelle, 1990), indicating that stimulation of growth by auxin is also affected in the mutant. In this study, we show that the rosettes of mutant plants are auxin resistant. Therefore, auxin resistance is expressed in most parts of *axr1* plants. The results of these morphological and physiological analyses of *axr1* mutants indicate that the wild-type *AXR1* gene product is required for normal auxin response throughout the plant. Other components of auxin response may be restricted to specific tissues. Plants homozygous for recessive mutations of the *AUX1* gene are similar to *axr1* mutants in their level of resistance to exogenous auxin (Maher and Martindale, 1980; Pickett et al., 1990). However, *aux1* mutants are morphologically normal except for a severe defect in root gravitropism (Maher and Martindale, 1980). Unlike the *AXR1* gene product, the *AUX1* gene may encode a function specifically required for hormonal regulation of root gravitropism.

Plants homozygous for the *axr1-12* allele display more severe alterations in morphology than *axr1-3* mutants. *axr1-12* mutants also exhibit greater auxin resistance in both seedling roots and rosette leaves than plants that are homozygous for the *axr1-3* allele. Thus, there is a good correlation between level of resistance and severity of the morphological abnormalities, suggesting that some of the morphological differences in *axr1* mutants may be directly due to a reduction in auxin response. However, it is important to note that many aspects of the mutant phenotype may not be directly related to the auxin defect. For example, auxin is known to induce ethylene biosynthesis in most plant tissues (Yang and Hoffman, 1984). In *axr1* plants, induction of ethylene biosynthesis by exogenous auxin is reduced compared with wild type (Lincoln and Estelle, 1990). Thus, it is possible that some features of the *axr1* phenotype, such as the altered leaf morphology, are due to a reduction in ethylene biosynthesis. In addition, several recent studies have shown that the auxin-resistant mutants of *Arabidopsis*, *aux1* and *axr2*, display cross resistance to several plant hormones (Pickett et al., 1990; Wilson et al., 1990). We are currently determining the response of the *axr1* mutants to hormones other than auxin.

Although there is experimental evidence to suggest that auxin controls gravitropic curvature, the precise role of auxin in root gravitropic response is still unclear (Feldman, 1985). The Cholodny-Went theory postulates that the differential growth rates observed on the upper and lower portions of a gravistimulated root are due to an asymmetric distribution of auxin (Feldman, 1985). Roots of *axr1* seedlings respond much more slowly to a gravity stimulus than roots of wild-type seedlings. Upon perception of a gravity stimulus, the roots of *axr1* mutants may be unable to establish the correct distribution of auxin because of a

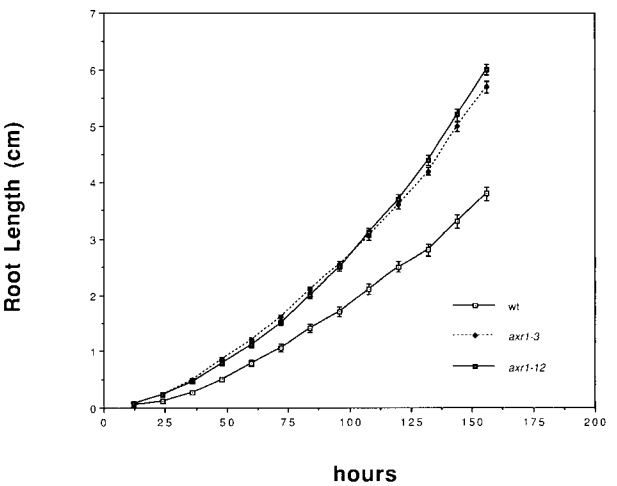

Figure 5. Elongation of Wild-Type (wt) and Mutant (*axr1-3*, *axr1-12*) Seedling Roots.

Each point represents the mean of at least 10 measurements. Error bars indicate the standard error.

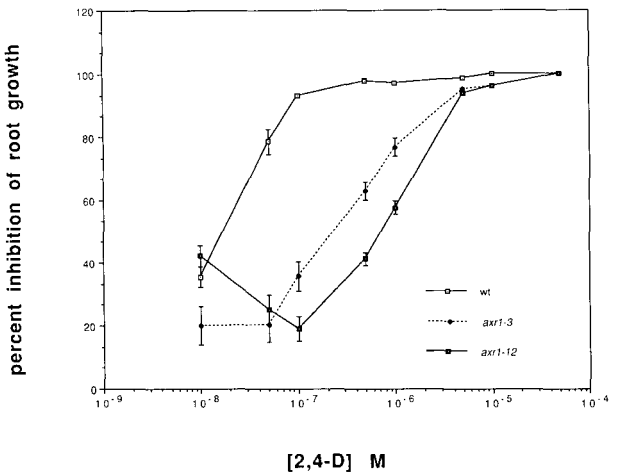

Figure 6. Dose-Response Curve for Wild-Type (wt) and Mutant (*axr1-3*, *axr1-12*) Seedlings on 2,4-D.

Inhibition of root growth by 2,4-D is expressed relative to growth on nonsupplemented medium. Each value represents the mean of measurement for 10 seedlings. Error bars indicate the standard error. The levels of 2,4-D producing 50% root growth inhibition are 1.5×10^{-8} M for wild type, 2.5×10^{-7} M for *axr1-3*, and 7.5×10^{-7} M for *axr1-12*.

disruption in auxin transport. Alternatively, auxin may be transported normally in gravistimulated *axr1* roots, but differential growth may not occur because of a reduction in auxin sensitivity. All auxin-resistant mutants of *Arabidopsis*, including *aux1* mutants (Maher and Martindale, 1980) and the dominant *axr2* mutant (Wilson et al., 1990), display defects in root gravitropism, further supporting the theory that auxin functions in regulating root gravitropism.

Auxin appears to play a major role in vascular tissue differentiation (Aloni, 1987). The generation of transgenic petunia plants that constitutively express the *Agrobacterium tumefaciens iaaM* gene has made it possible to examine the effects of large alterations in endogenous auxin levels on plant development (Klee et al., 1987). Such transgenic petunia plants contain approximately 10-fold higher levels of IAA and produce more secondary vascular tissue than normal petunia plants. In contrast, the secondary vascular tissue in the auxin-resistant mutant of tomato, *diageotropica*, is poorly developed (Zobel, 1974; Kelly and Bradford, 1986). Our results indicate that the development of vascular tissue is also affected in *axr1* plants. SEMs show that the vascular bundles in mutant stems are more peripheral and less regular than those in wild-type plants. These observations are consistent with a role for auxin in vascular development.

The SEMs of longitudinal sections of stem tissue indicate that cell elongation occurs normally in mutant plants. This result suggests that the short stature of *axr1* plants is due to a reduction in cell number. Two tissues that typically undergo rapid growth, the etiolated hypocotyl and the root, are also abnormal in *axr1* mutants. Elongation of the hypocotyl of etiolated *axr1* seedlings is reduced, whereas elongation of roots of mutant seedlings is increased. Further characterization is required to determine how growth is specifically affected in these two tissues in *axr1* mutants.

The phenotype of the *axr1* mutants is most easily explained by an alteration in a protein required for auxin perception or action. The extremely pleiotropic nature of the mutant phenotype suggests that this protein is required for auxin response and normal growth in most tissues of the plant. The isolation and characterization of the *AXR1* gene will enable us to begin to determine the cellular function of the *AXR1* gene product. Linkage analysis shows that RFLP 488 (Chang et al., 1988) lies approximately 2.6 centiMorgans from the *AXR1* gene. We intend to use this RFLP as a starting point for a chromosomal walk to the gene. The recent construction of a yeast artificial chromosome library using *Arabidopsis* DNA will greatly facilitate such a chromosomal walk (Guzmán and Ecker, 1988; Somerville, 1989). The isolation and molecular characterization of the *AXR1* gene as well as other hormone-resistance genes will provide new insight into the hormonal regulation of plant development.

Table 5. Effect of 2,4-D on Rosette Growth of Wild-Type and Mutant Plants

	0 μM 2,4-D		10 μM 2,4-D		100 μM 2,4-D	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
Wild type	59.5 ± 2.9	67.8 ± 2.8	46.0 ± 2.5 (23%)	44.5 ± 2.1 (34%)	19.6 ± 0.74 (67%)	21.4 ± 1.0 (68%)
axr1-3	45.2 ± 3.1	54.7 ± 3.0	50.3 ± 2.8	59.7 ± 2.7	26.3 ± 1.5 (42%)	26.5 ± 1.5 (52%)
axr1-12	49.6 ± 2.5	55.5 ± 2.9	47.1 ± 2.3 (5%)	47.3 ± 3.1 (15%)	33.8 ± 2.0 (32%)	38.0 ± 1.8 (32%)

Values are fresh weight in milligrams ± SE. Percentages in parentheses are percent inhibition of growth.

Table 6. Linkage Analysis between the *AXR1* Gene and RFLP Markers on Chromosome 1

Markers	Recombination Frequency (%)	Number Scored[a]	χ^2 Associated[b]	P
RFLP 219	12.4 ± 2.0	136	123.3	<0.05[c]
RFLP 253	47.8 ± 6.3	63	5.6	0.231
RFLP 488	2.6 ± 1.0	99	174.0	<0.05
phyA1	7.1 ± 2.0	102	135.0	<0.05

[a] Refers to number of F3 families scored.
[b] χ^2 associated is the total χ^2 adjusted for deviations of each individual marker from Mendelian segregation.
[c] P value < 0.05 indicates deviation from nonlinkage (i.e., linkage).

METHODS

Plant Materials and Growth Conditions

The isolation and initial characterization of some of the auxin-resistant mutant lines used in this study were originally described by Estelle and Somerville (1987). The mutant lines *axr1-3* and *axr1-12* correspond to the lines previously designated as ME-3 and ME-12, respectively. All mutant lines described in this report were derived from the Columbia ecotype. Plants were grown under constant fluorescent illumination (80 μE m^{-2} sec^{-1} to 150 μE m^{-2} sec^{-1}) at 21°C to 23°C. The soilless, Peat-Lite mixture Metro-Mix (W. R. Grace & Co.) was normally used for growing plants in 11-cm or 13-cm clay pots. A mineral nutrient solution containing 5 mM KNO_3, 2.5 mM KPO_4 (adjusted to pH 5.5), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 50 μM Fe-EDTA, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM Na_2MoO_4, 10 μM NaCl, and 0.01 μM $CoCl_2$ was supplied to the plants during the first 3 weeks of growth. Sterile plants were grown on an agar medium in sterile Petri plates. Seeds were first surface sterilized by agitation in a 30% v/v bleach (5.25% sodium hypochlorite) and 0.01% Triton X-100 solution for 15 min to 20 min, followed by several rinses in sterile water. The sterilized seeds were dispersed on the surface of a medium consisting of the nutrient solution described above supplemented with 7 g/L agar and 10 g/L sucrose. Hormones were added to the media after autoclaving. Sterile plants were grown in an incubator at 20°C to 21°C with a 16-hr photoperiod at a light intensity of 50 μE m^{-2} sec^{-1} to 60 μE m^{-2} sec^{-1}. To enhance germination, seeds in pots or in Petri plates were first placed in the dark at 4°C for 3 days to 4 days.

Mutagenesis

For chemical mutagenesis, approximately 25,000 seeds were soaked for 16 hr in 100 mL of 0.3% (v/v) EMS, then washed in several volumes of water over a period of 4 hr. This M1 seed was sown at a density of approximately 1/cm^2. At maturity, the resulting plants were bulk harvested to produce M2 seed. To mutagenize with γ irradiation, 20,000 seeds were incubated in water for 3 hr. A dose of 50 krad was administered to the imbibed seed in an irradiator (J.L. Shephard, Glendale, CA) containing ^{137}Cs. The seeds were sown and the M2 population was harvested as described above.

Morphological Characterization

All measurements were made on 8-week-old to 9-week-old plants grown under the light and temperature conditions described above. Plant height was determined by measuring the length of the longest inflorescence. The average distance between siliques, or internode length, was obtained by measuring the length of an inflorescence and dividing this length by the number of siliques present. This measurement was repeated for several separate inflorescences on each plant. The development of the stamens and the pistil in flowers of each genotype was examined using the procedure described by Estelle and Somerville (1987). Pollen yield was also determined as previously described (Estelle and Somerville, 1987). Each value for the number of pollen grains per flower represents the mean of four samples (five flowers per sample). To examine hypocotyl elongation in wild-type and mutant seedlings, seeds were distributed on minimal medium. The plates were then placed either in the light or dark at 20°C to 21°C for 5 days. Hypocotyl measurements of etiolated or light-grown seedlings were made by placing the seedlings under a dissecting microscope and determining hypocotyl length with a ruler. Twenty hypocotyls of each genotype were measured.

Leaf and stem tissue used for SEM was obtained from plants of the same age and grown under identical light and temperature conditions. Leaf sections were prepared by cutting transversely across the leaf blade of rosette leaves of each of the different genotypes. Cross-sections of stems were made at the level of the first internode of mature inflorescences. All tissue pieces were fixed in a solution containing 4% (w/v) *para*-formaldehyde, 0.05 M potassium phosphate (pH 7.0), and 0.02% Triton X-100. The samples were then placed under a vacuum for several hours to ensure infiltration of fixative into the tissue. Tissue samples were left in fixative at 4°C overnight, then rinsed several times with 0.05 M potassium phosphate (pH 7.0) and distilled water. After the last rinse, the tissue was dehydrated in a graded ethanol series at room temperature. Tissue samples were critical point dried in liquid carbon dioxide. After mounting individual sections of leaves or stems on SEM stubs, the tissue was sputter coated with gold and palladium (60:40). A Cambridge Stereoscan microscope was used to perform SEM, and photographs were taken using Kodak 4127 film.

Determination of Auxin Sensitivity

Surface-sterilized wild-type and mutant seeds were distributed on the surface of minimal medium. Petri plates were placed in an incubator in a vertical position so that the roots would grow along the agar surface. After 5 days, 10 seedlings per treatment were transferred to new plates supplemented with various concentrations of 2,4-D. All seedlings were positioned so that their root tips were aligned on a line drawn along the diameter of the Petri plate bottom. After 3 days, the amount of new root growth was measured and the percent root growth inhibition was calculated relative to root growth on minimal medium minus hormone. The light and temperature conditions were those described for sterile plants.

To analyze auxin sensitivity in the leaves of wild-type and *axr1* plants, 2-week-old plants were sprayed once daily for 3 consecutive days with 10 μM or 100 μM 2,4-D in water. Each application delivered approximately 1 mL of an aqueous 2,4-D solution to the pot surface. Control plants were sprayed with water only. Both treated and untreated plants were allowed to grow 1 additional week after the last application of 2,4-D. The fresh weight of rosette leaves was then determined for 15 separate plants per treatment. Inhibition of rosette growth was calculated relative to the growth of control plants.

Measurement of Root Gravitropic Response

Seedlings of each genotype were germinated and grown for 4 days on minimal medium. Temperature and light conditions were those described for sterile plants. Plates were oriented vertically so that roots would grow along the surface of the agar. After 4 days, seedlings were transferred to square Petri plates (100 × 15 mm) also containing minimal medium. A separate Petri plate containing 10 seedlings was prepared for each measurement. Seedlings were aligned on the agar medium so that all root tips were perpendicular to a line marked on the Petri plate bottom. As before, the plates were oriented vertically to allow the roots to continue to grow in the same direction as on the germination plate. After 2 days, a gravity stimulus was applied by rotating the plates 90°. At 1-hr intervals, the angle of curvature of the growing root tips was measured with a protractor.

Measurement of Root Growth

Surface-sterilized seedlings were germinated on minimal medium in plates placed vertically in an incubator. The date of germination, as defined by radicle emergence, was recorded for each seed. Only seedlings that had the same approximate time of germination were used for root growth measurements. At least 10 seedlings of each genotype were analyzed. Determination of root length was initiated 12 hr after germination and was repeated at 12-hr intervals for 6 days.

Linkage Analysis

The genetic location of *axr1* was established by determining linkage between the mutant gene and RFLPs identified and mapped by Chang et al. (1988). A homozygous *axr1-3* plant (ecotype Columbia) was crossed to a wild-type plant (ecotype Niederzenz), and the resulting F1 plants were allowed to self in order to generate an F2 population of plants segregating both the *axr1* mutation and RFLPs between the Columbia and Niederzenz ecotypes. Seed was collected from 150 individual F2 plants to establish F3 families. The genotype of each F2 plant was determined by examining F3 families for segregation of the *axr1* phenotype. Using the procedure of Dellaporta et al. (1983), DNA was isolated from a pool of 15 to 20 plants per F3 family. Approximately 3 μg of DNA was restricted with the appropriate restriction enzyme; the resulting fragments were separated in a 1% agarose gel and blotted onto a Hybond-N (Amersham Corp.) membrane (Maniatis et al., 1982). ^{32}P-labeled DNA probes were prepared using the random priming method (Feinberg and Vogelstein, 1983). Filters were prehybridized for 1 hr to 12 hr at 42°C in a solution consisting of 50% formamide, 5 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution = 0.02% PVP/0.02% Ficoll/0.02% BSA), 0.05 M $NaPO_4$ (pH 6.5), 0.1% SDS, and 20 μg/mL denatured salmon sperm DNA. Hybridization was carried out at 42°C for 48 hr in the same solution used for prehybridization except 1 × Denhardt's solution was used. Filters were washed twice at room temperature in 2 × SSC and 0.2% SDS for 15 min per wash before a final 10-min to 15-min wash in 0.1 × SSC and 0.1% SDS at 55°C. The filters were re-used after the probe was removed with boiling 0.1% SDS. The segregation of RFLPs was scored in the F3 families and linkage determined using the LINKAGE-1 program (Suiter et al., 1983). The RFLP clone phyA1 was generously provided by P. Quail (Plant Gene Expression Center, Albany, CA).

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