Growth and Development of the axr1 Mutants of Arabidopsis

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We have recovered eight new auxin-resistant lines of Arabidopsis that carry mutations in the AXR7 gene. These eight lines, together with the 12 lines described in a previous report, define at least five different axr7 alleles. All of the mutant lines have a similar phenotype. Defects include decreases in plant height, root gravitropism, hypocotyl elongation, and fertility. Mutant line axr7-3 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 AXR7 gene functions in aerial portions of the plant. Our studies suggest that the AXR7 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...
Table 1. Recovery of axr1 Mutants

<table>
<thead>
<tr>
<th>M2 population</th>
<th>Mutagen</th>
<th>Selection</th>
<th>Mutants recovered</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>EMS</td>
<td>2,4-D</td>
<td>axr1-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>axr1-2</td>
</tr>
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<td></td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>axr1-6</td>
</tr>
<tr>
<td>B</td>
<td>EMS</td>
<td>2,4-D</td>
<td>axr1-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>axr1-8</td>
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<td>axr1-9</td>
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<td></td>
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<td>axr1-11</td>
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<td></td>
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<td>axr1-12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>axr1-15</td>
</tr>
<tr>
<td>C</td>
<td>EMS</td>
<td>2,4-D</td>
<td>axr1-16</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>axr1-17</td>
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<td>axr1-18</td>
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<td></td>
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<td>axr1-21</td>
</tr>
<tr>
<td>C</td>
<td>EMS</td>
<td>IAA</td>
<td>axr1-22</td>
</tr>
<tr>
<td>D</td>
<td>γ</td>
<td>2,4-D</td>
<td>axr1-23</td>
</tr>
</tbody>
</table>

* 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.

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
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 axr7 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 axr7-3 and axr7-72 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 infloresences 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

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Table 3. Complementation Analysis of axrl Lines

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of Plants</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td>Sensitive</td>
<td></td>
</tr>
<tr>
<td>axrl-12 × axrl-3</td>
<td>33</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>axrl-19 × axrl-3</td>
<td>21</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>axrl-20 × axrl-1-3</td>
<td>13</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>axrl-22 × axrl-3</td>
<td>24</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>axrl-23 × axrl-3</td>
<td>39</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

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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.
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

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**Table 4. Morphology of Wild-Type and Mutant Plants**

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>axr1-3</th>
<th>axr1-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (cm)</td>
<td>50.9 ± 1.0</td>
<td>38.4 ± 1.3</td>
<td>19.2 ± 1.8</td>
</tr>
<tr>
<td>No. of inflorescences</td>
<td>5.75 ± 0.25</td>
<td>6.25 ± 1.4</td>
<td>6.6 ± 1.6</td>
</tr>
<tr>
<td>No. of lateral branches</td>
<td>46.2 ± 4.5</td>
<td>96.7 ± 21.4</td>
<td>124.0 ± 24.4</td>
</tr>
<tr>
<td>Distance between siliques (cm)</td>
<td>0.65 ± 0.04</td>
<td>0.30 ± 0.01</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>No. of siliques</td>
<td>673.75 ± 68.0</td>
<td>526.5 ± 56.0</td>
<td>2.4 ± 1.3</td>
</tr>
<tr>
<td>No. of pollen grains/flower</td>
<td>2035.0 ± 500.0</td>
<td>2700.0 ± 337.0</td>
<td>680.0 ± 32.0</td>
</tr>
<tr>
<td>Hypocotyl length in etiolated seedlings (cm)</td>
<td>1.40 ± 0.04</td>
<td>1.20 ± 0.05</td>
<td>0.77 ± 0.03</td>
</tr>
</tbody>
</table>
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
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
The levels of measurement for inhibition of root growth by E. coli (axr1-72). Levels of inhibition of root growth by 2,4-D are expressed relative to growth on nonsupplemented medium. Each value represents the mean of measurements 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 axr1 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 ialaM 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, diageetropica, 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 Eckers, 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**

<table>
<thead>
<tr>
<th></th>
<th>0 µM 2,4-D</th>
<th>10 µM 2,4-D</th>
<th>100 µM 2,4-D</th>
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<tbody>
<tr>
<td></td>
<td>Exp 1</td>
<td>Exp 2</td>
<td>Exp 1</td>
</tr>
<tr>
<td>Wild type</td>
<td>59.5 ± 2.9</td>
<td>67.8 ± 2.8</td>
<td>46.0 ± 2.5 (23%)</td>
</tr>
<tr>
<td>axr1-3</td>
<td>45.2 ± 3.1</td>
<td>54.7 ± 3.0</td>
<td>50.3 ± 2.8 (42%)</td>
</tr>
<tr>
<td>axr1-12</td>
<td>49.6 ± 2.5</td>
<td>55.5 ± 2.9</td>
<td>47.1 ± 2.3 (5%)</td>
</tr>
</tbody>
</table>

Values are fresh weight in milligrams ± SE. Percentages in parentheses are percent inhibition of growth.
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/cm². 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 137Cs. The seeds were sown and the M2 population was harvested as described above.

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 (60 µE m⁻² sec⁻¹ to 150 µE m⁻² sec⁻¹) at 21°C to 23°C. The soilless, Peat-Lite mixture (Grace & Co.) was normally used for growing plants in 11-cm or 13-cm clay pots. A mineral nutrient solution containing 5 mM KNO₃, 2.5 mM KPO₄ (adjusted to pH 5.5), 2 mM MgSO₄, 2 mM Ca(NO₃)₂, 50 µM Fe-EDTA, 70 µM H₂BO₃, 14 µM MnCl₂, 0.5 µM CuSO₄, 1 µM ZnSO₄, 0.2 µM Na₂MoO₄, 10 µM NaCl, and 0.01 µM CoCl₂ 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 for 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.

Table 6. Linkage Analysis between the AXR7 Gene and RFLP Markers on Chromosome 1

<table>
<thead>
<tr>
<th>Markers</th>
<th>Recombination Frequency (%)</th>
<th>Number Scored</th>
<th>χ² Associated</th>
<th>P</th>
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<tr>
<td>RFLP 219</td>
<td>12.4 ± 1.0</td>
<td>136</td>
<td>123.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>RFLP 253</td>
<td>47.8 ± 6.3</td>
<td>63</td>
<td>5.6</td>
<td>0.231</td>
</tr>
<tr>
<td>RFLP 488</td>
<td>2.6 ± 1.0</td>
<td>99</td>
<td>174.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>phyA1</td>
<td>7.1 ± 2.0</td>
<td>102</td>
<td>135.0</td>
<td>&lt;0.05</td>
</tr>
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</table>

*Refers to number of F3 families scored.

χ² associated is the total χ² adjusted for deviations of each individual marker from Mendelian segregation.

P value < 0.05 indicates deviation from nonlinkage (i.e., linkage).

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% (v/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 \( \mu \text{M} \) or 100 \( \mu \text{M} \) 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 \( \times \) 15 mm) also containing minimal medium. A separate Petri plate containing 10 seedlings was prepared for each measurement. Seedlings were oriented 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 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 \( \mu \text{g} \) 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 \( \times \) SSC (1 \( \times \) SSC = 0.15 M NaCl/0.015 M sodium citrate), 5 \( \times \) Denhardt’s solution (1 \( \times \) 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 \( \mu \text{g/mL} \) denatured salmon sperm DNA. Hybridization was carried out at 42°C for 48 hr in the same solution used for prehybridization except 1 \( \times \) Denhardt’s solution was used. Filters were washed twice at room temperature in 2 \( \times \) SSC and 0.2% SDS for 15 min per wash before a final 10-min to 15-min wash in 0.1 \( \times \) 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 (Suter et al., 1983). The RFLP clone phyA1 was generously provided by P. Quail (Plant Gene Expression Center, Albany, CA).

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