Mutations in the AXR3 gene of Arabidopsis result in altered auxin response including ectopic expression from the SAUR-AC1 promoter

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Summary
A new auxin response gene in Arabidopsis called AXR3 has been identified. This gene is defined by two semi-dominant mutations which affect many auxin-regulated developmental processes. Auxin has been shown to maintain apical dominance, inhibit root elongation, stimulate adventitious rooting, mediate root gravitropism, and stimulate transcription from the SAUR-AC1 promoter. Mutant axr3 plants show enhanced apical dominance, reduced root elongation, increased adventitious rooting, no root gravitropism, and ectopic expression from the SAUR-AC1 promoter. These phenotypes suggest an increased auxin response in the mutants. In support of this hypothesis, many of the phenotypes are partially restored to wild-type by exogenous cytokinin, a treatment that could restore a more wild-type auxin to cytokinin ratio.

Introduction
The plant hormone auxin has been implicated in the regulation of almost every aspect of plant growth and development (King, 1998). For example, auxin is thought to inhibit the growth of axillary meristems (apical dominance) (Tamas, 1988), mediate gravitropism (Feldman, 1985), promote stem elongation (Yang et al., 1993), inhibit root elongation (Evans, 1984), and promote adventitious root formation (Katsumi et al., 1969). How auxin regulates such diverse processes is unknown.

Two rapid auxin responses have been well characterized. Auxin is thought to induce proton extrusion through the plasma membrane by stimulating a proton pumping ATPase (Rayle and Cleland, 1992). The resulting acidification of the apoplast loosens the cell wall, and allows cell elongation. Auxin also induces the transcription of specific genes (Theologis, 1986). For example, transcription of the SAUR genes, originally identified in soybean, is rapidly and specifically induced by auxin (McClure et al., 1989). SAUR gene transcription can be detected as soon as 2.5 min after auxin application (McClure and Guilfoyle, 1987). While the function of the SAUR gene products is unknown, their expression in intact plants correlates with rapid cell elongation (Gee et al., 1991).

Despite intensive study of these auxin responses, little is known about how the auxin signal is perceived and transduced. Biochemical studies have identified a number of auxin-binding proteins, but the evidence that any of these proteins act as auxin receptors is inconclusive (Palme, 1992). Genetics provides an alternative approach to identify proteins involved in auxin action. The isolation and characterization of mutants defective in auxin responses can identify genes involved in auxin perception or signal transduction. A number of such mutants have been identified, primarily by screening for plants resistant to the growth inhibitory effects of auxin (Bitoun et al., 1990; Bonstein et al., 1991; Estelle and Somerville, 1987; Hobbie and Estelle, 1995; Kelly and Bradford, 1986; Lincoln et al., 1990; Maher and Martindale, 1980; Muller et al., 1985; Pickett et al., 1990; Wilson et al., 1990). These mutants frequently have pleiotropic phenotypes with defects in a variety of auxin-regulated processes. In addition, many such mutations confer resistance to other hormone classes (Bitoun et al., 1990; Pickett et al., 1990; Timpte et al., 1995; Wilson et al., 1990). Two models have been proposed to explain this cross-resistance. The same protein could be required for response to several different hormones. Alternatively, cross-resistance could reflect the close interactions between the various hormone classes. Some interactions are well characterized. For example, in many instances, it is the ratio of auxin to cytokinin that regulates developmental events, and not the absolute levels of either hormone (Klee and Estelle, 1991).

In this paper we describe the identification of a new auxin-response locus in Arabidopsis called AXR3. Like previously described auxin-resistant mutants, the axr3 phenotype is highly pleiotropic with defects in many auxin-related processes, and cross-resistance to several hormone classes. However, unlike previous auxin-resistant mutants, many aspects of the axr3 phenotype are indicative of auxin hypersensitivity rather than auxin-resistance.
Table 1. The F₂ segregation of the axr3 and axr1 phenotypes in crosses between axr3 and axr1 homozygotes

<table>
<thead>
<tr>
<th>Cross</th>
<th>F₂ phenotype</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Axr3</td>
<td>Axr1</td>
<td>Wild-type</td>
<td>Total</td>
<td>Map distance (cM)</td>
</tr>
<tr>
<td>axr3-1×axr1-3</td>
<td>4665</td>
<td>1492</td>
<td>44</td>
<td>6201</td>
<td>1.43±1.27</td>
</tr>
<tr>
<td>axr3-3×axr1-3</td>
<td>926</td>
<td>314</td>
<td>10</td>
<td>1250</td>
<td>1.59±2.83</td>
</tr>
</tbody>
</table>

Map distances were estimated using the linkage 1 program and are based on the arbitrary assumption that axr3 is epistatic to axr1.

Table 2. Morphometric analysis of axr3 plants

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>axr3-1</th>
<th>axr3-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>25.8±0.3 cm</td>
<td>8.7±0.8 cm</td>
<td>18.7±0.6 cm</td>
</tr>
<tr>
<td>Internode length</td>
<td>2.0±0.2 cm</td>
<td>1.8±0.1 cm</td>
<td>2.2±0.2 cm</td>
</tr>
<tr>
<td>Pith cell length</td>
<td>149±8.65 μm</td>
<td>141±6.3 μm</td>
<td>168±13.6 μm</td>
</tr>
<tr>
<td>Primary inflorescences</td>
<td>3.4±0.3</td>
<td>1.1±0.1</td>
<td>1.2±0.8</td>
</tr>
<tr>
<td>Branches from main inflorescence</td>
<td>4.2±0.3</td>
<td>0.06±0.06</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>Light-grown hypocotyl length</td>
<td>1.9±0.1 mm</td>
<td>2.0±0.1 mm</td>
<td>1.9±0.1 mm</td>
</tr>
<tr>
<td>Dark-grown hypocotyl length</td>
<td>11.5±0.8 mm</td>
<td>3.8±0.1 mm</td>
<td>4.4±0.1 mm</td>
</tr>
<tr>
<td>Light-grown hypocotyl epidermal cell length</td>
<td>89±2.9 μm</td>
<td>79±3.8 μm</td>
<td>85±7.6 μm</td>
</tr>
<tr>
<td>Dark-grown hypocotyl epidermal cell length</td>
<td>460±18 μm</td>
<td>129±5.0 μm</td>
<td>173±11 μm</td>
</tr>
</tbody>
</table>

Measurements were taken from samples of at least 10 plants grown as described in Experimental procedures. For cell length measurements the sample size was at least 10 cells from at least three different plants, except for wild-type hypocotyl epidermal cells where n = 5. Pith cell lengths were measured on scanning electron micrographs of longitudinal stem sections. Hypocotyls and hypocotyl epidermal cell lengths were measured 5 days after planting.

Results

Mutant isolation

In order to isolate mutants in hormone response we have screened Arabidopsis M₂ populations (Columbia ecotype) for individuals able to elongate roots on concentrations of hormone that inhibit growth of wild-type roots (Lincoln et al., 1990; Pickett et al., 1990). Two mutant lines of strikingly similar phenotype were isolated in these screens. The first was isolated from an EMS-mutagenized population screened for resistance to the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC). Because subsequent analysis demonstrated that this mutant is also resistant to auxin, the mutant was named auxin-resistant 3 (axr3-1). The second mutant line was isolated from a DEB mutagenized population screened for resistance to the synthetic auxin 2,4-D. Back-crossing mutant plants to wild-type showed that both phenotypes resulted from single semi-dominant Mendelian mutations (data not shown).

To determine the map positions of the genes defined by these mutations, axr3-1 plants (ecotype Columbia, Col-0) were crossed to wild-type plants of the Niederzenn ecotype (Nd-0) and the F₂ was scored for segregation of AFLPs between the two ecotypes (Chang et al., 1988). This analysis showed that AXR3 is on chromosome 1 close to the AXR1 gene (data not shown). We then crossed both axr3-1 plants and the second auxin-resistant line described above to plants homozygous for the axr1-3 mutation. The F₂ phenotypes from these crosses were scored by examining root growth on 2,4-D supplemented medium. Three phenotypes were observed, Axr3, Axr1, and wild-type. The segregation ratios of these phenotypes show that both mutations are approximately 1.5 map units from the AXR1 locus (Table 1). Their similar phenotype, map position and semi-dominance strongly suggest that these mutations are allelic. This second line was named axr3-3.

Morphological phenotype

The axr3 mutations are highly pleiotropic (Table 2, Figures 1–3). For all aspects of the morphological phenotype, axr3-1 plants are more severely affected than axr3-3 plants. The mutant plants are generally less vigorous than wild-type. axr3 leaves are small and curled, with epinastic petioles, and high levels of anthocyanins (Figure 1). For most mutant plants, a single unbranched inflorescence is produced compared with an average of 3.4 branched inflorescences in wild-type plants (Figure 1, Table 2). The axr3 inflorescence is shorter than wild-type due to reduced internode number, rather than reduced internode length or cell length (Figure 1, Table 2). The decreased internode number may be a reflection of the less vigorous growth of the mutant plants. The flowers of axr3 plants are wild-type in appearance but there is reduced seed set compared with wild-type plants. Hypocotyl elongation in the dark is much
Auxin sensitivity of the axr3 mutants

Root growth inhibition assays were carried out to compare the sensitivity of wild-type and axr3 roots to the naturally occurring auxin IAA. The results of these assays are shown in Figure 4. Root growth inhibition is expressed as a percentage of growth on no hormone, allowing direct comparisons of wild-type and axr3 roots despite the slow rate of elongation of axr3 roots. However, axr3 roots are absolutely longer, and elongate more rapidly than wild-type roots at concentrations above $10^{-7}$ M auxin. The results show that axr3 roots are approximately 500-fold less sensitive to auxin than wild-type roots (Figure 4, Table 3). This level of resistance is much greater than that of previously described auxin-resistant mutants (Table 3) (Lincoln et al., 1990; Wilson et al., 1991). The roots of axr3-3 plants are more resistant than axr3-1 roots at indole-3-acetic acid (IAA) concentrations below $10^{-5}$ M but at higher concentrations axr3-1 roots are more resistant. For both axr3-3 and axr3-1 plants, root hairs develop on roots grown on high auxin levels. Root hairs are induced at concentrations above $10^{-7}$ M on axr3-3 roots, but $10^{-6}$ M auxin is required for root hair development on axr3-1 roots. axr3 root elongation is also highly resistant to the synthetic auxins 2,4-D and 1-napthaleneacetic acid (1-NAA) (data not shown).

Sensitivity of the axr3 mutants to other hormone classes

In addition to auxin-resistance, the axr3 mutations confer resistance to the ethylene precursor ACC (Figure 4b). In this case, axr3-1 roots were more resistant than axr3-3 roots at all concentrations of ACC tested.

When the response to the synthetic cytokinin benzyladenine (BA) was examined, an unusual result was observed (Figure 4c). Elongation of axr3 roots is stimulated by concentrations of BA which are severely inhibitory to wild-type root growth. Stimulation was stronger in axr3-1 plants than in axr3-3 plants.

Root growth inhibition by abscisic acid (ABA) and the tryptophan analogue alpha-methyl-tryptophan was not significantly affected by the axr3 mutations (data not shown).

Cytokinin and axr3 morphology

In light of the interesting effect of cytokinin on axr3 root elongation, we tested the effect of cytokinin on other morphological phenotypes conferred by axr3. Growth of axr3 plants on medium supplemented with $10^{-6}$ M BA was found to abolish adventitious rooting completely (data not shown). In addition, hypocotyl elongation in dark-grown axr3 plants was found to be slightly but significantly stimulated by $10^{-6}$ M BA, whilst wild-type hypocotyl elonga-
tion was markedly inhibited under these conditions (Figure 5).

In contrast, cytokinin had no effect on axr3 gravitropism or root hair formation (data not shown).

**Callus growth from axr3 explants**

To assay callus induction and proliferation, 10 cotyledons from 5-day-old plants were excised and incubated for 4 weeks on agar-solidified MS medium supplemented with a range of 2,4-D concentrations. After 4 weeks the tissue was weighed and any organ differentiation was noted. The results are shown in Figure 6. Callus induction occurs over a similar range of auxin concentrations in both wild-type and mutant explants. However, callus growth in the mutants is much more vigorous than wild-type. This is a particularly striking result since axr3 mutant plants are much less vigorous than wild-type. In addition, root formation is more frequent on axr3 explants and occurs at higher concentrations of 2,4-D than on wild-type explants. These results indicate that with respect to cell growth in culture, the axr3 mutants display normal sensitivity to auxin, but the magnitude of the response is increased relative to wild-type.

**Transcription of the SAUR-AC1 gene in axr3 plants.**

To characterize a more rapid auxin-response in the mutants, we have examined expression of the SAUR-AC1 gene. In soybeans, transcription of the SAUR genes is specifically and rapidly induced by auxin (McClure and Guilfoyle, 1987). The SAUR-AC1 gene of Arabidopsis has also been shown to be under auxin control (Gil et al., 1994; Timpte et al., 1995). The expression of this gene in 2-week-old axr3 plants was examined on RNA gel blots. The results of one such experiment are shown in Figure 7. No consistent differences were observed in the accumulation of SAUR-AC1 transcript in the mutant plants compared with wild-type plants. Similar levels of transcript were detected in untreated wild-type and mutant plants. For all genotypes, SAUR-AC1 transcript levels were much reduced by soaking the tissue for 5 h in buffer without auxin. Treatment of the tissue with 2,4-D at a concentration of \(5 \times 10^{-7}\) M or higher for 1 h resulted in the accumulation of detectable SAUR-AC1 transcript. A maximum level of transcript was observed following treatment with between \(5 \times 10^{-6}\) and \(5 \times 10^{-5}\) M 2,4-D. Higher concentrations of 2,4-D resulted in reduced transcript levels.

To analyse the tissue specificity of the SAUR-AC1 expression, axr3-1 plants were crossed to transgenic plants containing a SAUR-AC1 promoter–Gus fusion linked to an NPT-II gene conferring kanamycin-resistance (Gil and Green, 1996). Plants true breeding for both the axr3 phenotype and kanamycin-resistance were selected from the progeny of this cross. Gus activity, and hence transcription from the SAUR-AC1 promoter, was detected in whole, 8-day-old plants by incubation with the chromogenic Gus substrate X-Gluc. The spatial distribution of Gus expression was assayed in untreated plants and in plants grown on medium supplemented with either \(10^{-6}\) M auxin (1-NAA) or \(10^{-6}\) M cytokinin (BA). Typical plants from these experiments are shown in Figure 8. In wild-type plants, Gus activity was detected at the hypocotyl base, in the cotyledons, and in the leaves (Figure 8a). In axr3-1 plants, a similar distribution of Gus activity was detected, but in addition there was Gus activity in the root vasculature (Figure 8b). This was never observed in untreated wild-type plants but could be induced by treatment with exogenous auxin. In addition, treatment of axr3-1 plants with exogenous cytokinin was found to eliminate the ectopic root vascular expression.

Interestingly, other than in the root, the tissue specificity of expression was not affected by either hormone treatment. The intensity of staining was increased by auxin, and to a lesser extent by cytokinin, particularly at the hypocotyl base.

**Discussion**

We have identified a new auxin-response gene in Arabidopsis called AXR3. Semi-dominant mutations in this gene affect a variety of auxin-regulated developmental processes, consistent with a primary defect in auxin-response. Auxin has been shown to promote apical dominance (Tamas, 1988), inhibit root elongation (Evans, 1984), stimulate adventitious rooting (Katsumi et al., 1989), mediate gravitropism (Feldman, 1985), inhibit hypocotyl elongation in dark-grown plants (Carrington and Esnard, 1988; Sargent et al., 1974), promote callus growth and stimulate transcription from the Arabidopsis SAUR-AC1 promoter (Gil et al., 1994; Timpte et al., 1995). axr3 plants display enhanced apical dominance, reduced root elongation, increased

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**Figure 2.** Root growth in axr3 mutants. (a) The photograph shows the phenotype of the root systems of 10-day-old plants, from left to right wild-type, homozygous axr3-1, and homozygous axr3-3. The graphs show (b) the mean length of wild-type and axr3 roots over time and (c) the mean number of adventitious roots initiated from the hypocotyl of wild-type and axr3 plants over time. The error bars represent the standard errors of the means. The sample size for each data point was at least 10 plants.

**Figure 3.** The photomicrographs show the root/hypocotyl interface of 1-week-old -axr3-1 (left) and wild-type (right) plants. Note the absence of root hairs and the presence of many lateral root initials in the axr3-1 plant.
adventitious rooting, no root or hypocotyl gravitropism, reduced hypocotyl elongation in dark-grown seedlings, and ectopic expression from the SAUR-AC1 promoter. The highly pleiotropic nature of this phenotype might suggest some general growth defect, but the fact that almost all these phenotypes are consistent with enhanced auxin-responses, and many can be phenocopied by exogenous auxin addition, is compelling evidence for a primary defect in auxin action.

In support of this hypothesis, many axr3 phenotypes can be partially rescued with cytokinin. Developmental events are frequently regulated by the ratio of auxin to cytokinin rather than the absolute levels of either. If the axr3 phenotype results from enhanced auxin action, addition of cytokinin would restore a more wild-type auxin to cytokinin

Table 3. The concentration of IAA giving 50% inhibition of root growth for wild-type, axr3-1, axr1-12 (Lincoln et al., 1990), and axr2-1 (Wilson et al., 1990)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>[IAA] μM giving 50% root growth inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.03±0.00</td>
</tr>
<tr>
<td>axr3-1</td>
<td>16.7±2.9</td>
</tr>
<tr>
<td>axr1-12</td>
<td>0.22±0.03</td>
</tr>
<tr>
<td>axr2-1</td>
<td>1.3±0.5</td>
</tr>
</tbody>
</table>

Values were estimated by interpolation of graphs like those shown in Figure 4. For each genotype, the mean and standard error were calculated from at least three independent experiments.

Figure 4. The graphs show dose-response curves for wild-type and axr3 seedling root inhibition on (a) IAA, (b) ACC and (c) BA. For each genotype, inhibition of root growth is expressed relative to growth on no hormone. Each value represents the mean measurement for at least 10 seedlings. The error bars represent the standard error of the means.
ratio, hence restoring a more wild-type phenotype. It seems unlikely that a cytokinin response is the primary defect in axr3 mutants. This would not be expected to result in the axr3 phenotypes in which there is no evidence for a role for cytokinin, such as loss of gravitropism and ectopic SAUR-AC1 expression. Furthermore, the cytokinin responses shown cannot be considered as cross-resistance, similar to that observed in several other auxin-resistant mutants (Timpte et al., 1995; Wilson et al., 1990). In the case of axr3, we observe concentration-dependent cytokinin responses which do not occur in wild-type plants. For example, both root and dark-grown hypocotyl elongation are stimulated by cytokinin concentrations which are severely inhibitory to wild-type elongation. Similarly, ectopic expression from the SAUR-AC1 promoter is abolished by cytokinin, which in contrast has a slight stimulatory effect on SAUR expression in wild-type plants (Timpte et al., 1995). Interestingly, McClure and Guilfoyle (1987) have shown that in soybean, SAUR expression is unaffected by cytokinin alone, but auxin-induced SAUR transcript accumulation can be reduced by 50% with cytokinin treatment. This supports the hypothesis that in axr3 plants, exogenous cytokinin is acting to alleviate increased auxin activity.

The resistance of axr3 plants to ACC is more difficult to understand. It is possible that this resistance is also a consequence of enhanced auxin action. It could result from adaptation to the ACC/ethylene signal because of overproduction of these compounds caused by increased auxin-induced ACC biosynthesis (Abel et al., 1995). We are currently taking a number of approaches to test this idea. First, we are assaying ethylene biosynthesis by axr3 plants. Secondly, this model predicts that ethylene resistance and possibly some other axr3 phenotypes such as epinasty are an indirect result of ethylene overproduction. Therefore, it should be possible to alleviate these phenotypes using moderate levels of ethylene biosynthesis inhibitors. Experiments to test this are underway.

Whilst most features of the axr3 phenotype are consistent with increased auxin activity, the resistance of axr3 root elongation to the inhibitory effects of exogenous auxin superficially suggests a reduction in auxin sensitivity or levels. It is possible that axr3 root elongation is already so auxin-inhibited that additional auxin has little further effect.

**Figure 5.** The graph shows the effect of $10^{-8}$ M BA on the length of 5-day-old dark-grown hypocotyls of wild-type and axr3-1 plants.

**Figure 8.** Auxin responses in axr3 explants.
The left-hand graph shows the fresh weight of callus 4 weeks after initiation from 10 cotyledons of wild-type and axr3 seedlings. The right-hand graph shows the percentage of these explants showing root regeneration. Each value represents the mean from three independent experiments and the error bars indicate the standard errors of these means.
This could explain why such a high level of resistance is observed in axr3 roots as compared with those of other auxin-resistant mutants (Table 3). The stimulation of root elongation by cytokinin is certainly consistent with this idea. None the less, this explanation cannot be true in its simplest form, since there are concentrations of auxin where axr3 roots are longer than wild-type.

If the primary defect in axr3 mutants is in auxin action, this could occur at any point in auxin signalling that is common to all the developmental responses affected. For example, the mutations could result in increased auxin levels. Preliminary auxin determinations have shown that free and conjugated IAA levels in axr3-1 plants are not substantially different from wild-type (Turner and Estelle, unpublished). This suggests that overall, auxin levels are not affected by the mutations although they could alter auxin transport or subcellular localization. Auxin over-producing mutants have been isolated in Arabidopsis, such as those at the superroot (sur) locus on chromosome 2 (Boerjan et al., 1995). These mutants have many similarities to axr3 plants, such as short highly branched roots, however, there are also significant differences. For example, no defects in gravitropism are observed in sur plants, and sur explants show auxin autonomous growth in tissue culture. Taken together, these data suggest that the axr3 phenotype does not result from auxin over-production.

An alternative hypothesis is that the axr3 mutations result in increased auxin sensitivity. However, the induction of SAUR-AC1 transcript accumulation and the stimulation of callus growth occurs over the same range of auxin concentrations as in wild-type tissue, clearly demonstrating that the phenotype does not result from a uniform increase in auxin sensitivity. It is possible that only a subset of tissues are affected. However, the mutations affect virtually every part of the plant so it seems unlikely that the total number of cells affected is too small to detect any increase in auxin sensitivity in these assays.

A third possibility is that the mutations could result in a constitutive auxin-response. Contrary to this idea, in axr3 plants the level of SAUR-AC1 transcript can be reduced below the level of detectability by soaking tissue in buffer to deplete endogenous auxins. Transcript levels can be restored by exogenous addition of auxin. This clearly demonstrates that SAUR-AC1 expression is an auxin-dependent response in axr3, suggesting that the mutation does not cause an auxin-responsive phenotype. Similarly, callus initiation from axr3 cotyledons is dependent on exogenous auxin.

Fourthly, it is possible that the mutations affect the amplitude of the auxin-response. This is certainly the case for callus proliferation. The shape of the growth response curve for callus is very similar in wild-type and mutant tissue, but the amplitude of the axr3 curve is much greater than wild-type. The AXR3 gene could be involved in modulating the level of response to auxin. In the axr3 mutants, this modulation is impaired such that the plants over-react to the auxin stimulus. We are investigating this model further by constructing double mutant lines carrying mutations in axr3 and a range of auxin-resistant mutations.

Of particular interest are axr3, axr1 double mutants since axr1 mutations confer a phenotype which is almost the exact opposite of the axr3 phenotype. axr1 plants have a short highly branched shoot system and a long relatively unbranched root system. In the F2 of crosses between axr1 and axr3 plants (Table 3) only three phenotypes were observed at the seedling stage. The absence of a fourth, novel phenotype suggests that either axr1 is epistatic to axr3 or vice versa. Alternatively, it is possible that the double mutant has a wild-type phenotype but if this is the case, then axr3 must be dominant in this respect since the selfed progeny of all wild-type F2s tested showed a 3:1 segregation ratio of wild-type to axr1 plants (data not shown). A third possibility is that the phenotype of the
double mutant is lethal or is not manifest at the seedling stage.

We are currently conducting a chromosome walk to clone the AXR3 gene. The molecular characterization of this locus may clarify the function of this gene in auxin signalling.

**Experimental procedures**

**Plant materials and growth conditions**

Plants were grown under constant illumination from fluorescent lamps (80–150 μmol m$^{-2}$ sec$^{-1}$) at 21–23°C in clay pots in the soilless medium, Metromix™, supplemented with mineral nutrient...
solution (Wilson et al., 1990). To culture plants in petri dishes under sterile conditions, seeds were surface-sterilized in a solution containing 10% bleach and 0.01% Triton X-100 (octyl-phenoxypolyeth-oxyethanol) for 15 min, washed in 70% ethanol, and then rinsed four times with sterile distilled water. The seeds were dispersed on medium containing 0.8% agar, 1% sucrose and the mineral nutrient solution mentioned above. The plants were grown in an incubator at 20–21°C with a 16 h photoperiod. For darkgrown plants, the plates were wrapped in aluminium foil. For most experiments, plants were grown aseptically for 2 weeks and then transferred to clay pots to complete their development. Homozygous axr3-1 plants grown from seed in clay pots frequently die at the rosette stage due to inadequate penetration of the root system into the Metromix™.

Mutagenesis and screening

Approximately 25 000 seeds were soaked in mutagen for an appropriate period of time and then washed with several volumes of water over a 4 h period. For ethyl methanesulphonate (EMS) 0.3% (v/v) for 16 h was used. For diepoxybutane (DEB) 10 mM for 4 h 30 min was used. This M1 seed was sown at a density of approximately 1 seed per cm², the M1 plants were bulk harvested, and the M2 seed was screened in petri dishes containing the medium described above supplemented with 100 μM ACC for the EMS M2 population or 6.1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) for the DEB M2 population.

Microscopy

Pith cell length measurements were made from scanning electron micrographs. Hypocotyl cell length measurements were made from cleared 5-day-old seedlings viewed under Nomarski optics. Lateral root initiation was examined using phase contrast microscopy. The tissue was prepared and examined as described by Timpte et al. (1992).

Root growth inhibition assay

Root growth inhibition assays were carried out as described in Lincoln et al. (1990) except seeds were allowed to germinate for about 2 days, until the radicle had emerged, before transfer to hormone supplemented medium.

Callus induction

Cotyledons were cut from 5-day-old seedlings and placed on MS medium (Murashige and Skoog, 1962), solidified with 0.8% agar and containing 1% sucrose. The plates were incubated at 20–

SAUR-AC1 induction

SAUR-AC1 induction was carried out as described by Gil et al. (1994). Plants were grown aseptically for 2 weeks as described above. A control sample of untreated plants was collected directly from the petri dishes, frozen in liquid nitrogen and stored at –70°C. For the induction experiments, plants were cut into small pieces and 1 g was soaked in 50 ml of buffer containing 10 mM potassium phosphate pH 6, 2% sucrose (w/v) and 50 μg ml⁻¹ chloramphenicol for 4 h at 30°C with buffer changes after 1 and 2 h. The plants were then incubated for a further h in buffer supplemented with 0, 0.5, 5.0, 50, or 500 μM 2,4-D. The tissue was then frozen in liquid nitrogen and stored at –70°C.

RNA gel blot analysis

RNA was extracted by a method based on that of Puissant and Houdebine (1990). The RNA was subjected to gel electrophoresis as described by Tsang et al. (1993), transferred to Hybond-N nylon membrane (Amerham International plc) and probed with 32p-labelled DNA probe according to the manufacturer’s instructions. Probes were prepared using random oligonucleotide primers (Feinberg and Vogelstein, 1983).

Histochemical localization of β-glucuronidase (Gus)

GUS activity (Jefferson et al., 1987) in the transgenic plants was detected by incubating the plants in 50 mM sodium phosphate pH 7, containing 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide and 0.3 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) at 37°C for 16 h.

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