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The *axr4* auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation

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Summary

To understand the molecular mechanism of auxin action, mutants of *Arabidopsis thaliana* with altered responses to auxin have been identified and characterized. Here the isolation of two auxin-resistant mutants that define a new locus involved in auxin response, named *AXR4*, is reported. The *axr4* mutations are recessive and map near the *ch1* mutation on chromosome 1. Mutant plants are specifically resistant to auxin and defective in root gravitropism. Double mutants between *axr4* and the recessive auxin-resistant mutants *axr1-3* and *aux1-7* were characterized to ascertain possible genetic interactions between the mutations. The roots of the *axr4 axr1-3* double mutant plants are less sensitive to auxin, respond more slowly to gravity, and form fewer lateral roots than either parental single mutant. These results suggest that the two mutations have additive or even synergistic effects. The AXR1 and AXR4 gene products may therefore act in separate pathways of auxin response or perhaps perform partially redundant functions in a single pathway. The *axr4 aux1-7* double mutant has the same sensitivity to auxin as the *aux1-7* mutant but forms far fewer lateral roots than either parental single mutant. The *aux1-7* mutation thus appears to be epistatic to *axr4* with respect to auxin-resistant root elongation, whereas in lateral root formation, the effects of the two mutations are additive. The complexity of the genetic interactions indicated by these results may reflect differences in the mechanism of auxin action during root elongation and the formation of lateral roots. The AXR4 gene product, along with those of the AXR1 and AUX1 genes, is important for normal auxin sensitivity, gravitropic response in roots and lateral root formation.

Introduction

The plant hormone auxin plays a key role in many aspects of plant growth and development. Auxin has been implicated in tropisms (Kaufman and Song, 1987), vascular differentiation (Aloni, 1987), control of lateral branching (Cline, 1994), cell expansion (Cleland, 1987), and cell division (Evans, 1984). Roots are a particularly attractive system in which to study auxin action because of their morphological simplicity and well-characterized auxin responses. Indirect evidence from a variety of studies suggests that auxin has a central role in cell division and elongation in the growing root. In many plants, very low concentrations of auxin stimulate root elongation, while higher concentrations invariably inhibit elongation (Burström, 1969; Evans *et al.*, 1994). Root gravitropism is thought to involve the effects of auxin on cell elongation. According to the Cholodney–Went model, differential growth of the upper and lower sides of a gravistimulated root is caused by an asymmetric auxin concentration across the root (Pickard, 1985). Experiments involving applied auxin also suggest a key role for this hormone in initiation of lateral roots (Street, 1968; Webster and Radin, 1972). Exogenous auxin generally promotes the formation of morphologically normal lateral roots, apparently by stimulating cell division in the pericycle (Webster and Radin, 1972). In an attempt to understand the molecular basis for these growth responses, we are isolating and studying mutants of *Arabidopsis* that have altered root responses to exogenous auxin (reviewed in Hobbie and Estelle, 1994).

At present, five loci involved in auxin response have been mutationally defined in *Arabidopsis*: *aux1* (Maher and Martindale, 1980; Pickett *et al.*, 1990), *dwf* (Mirza and Maher, 1987), *axr1* (Lincoln *et al.*, 1990), *axr2* (Wilson *et al.*, 1990), and *axr3* (Leyser *et al.*, unpublished). All of these auxin-resistant mutants have defects in root gravitropism, confirming the importance of auxin in this process. For example, *axr1* roots are somewhat delayed in gravitropic response whereas *aux1* roots have a more severely reduced gravitropic response (Lincoln *et al.*, 1990; Okada and Shimura, 1992). Since these two mutants are equally auxin resistant, the difference in gravitropism probably reflects different roles for the two gene products. The AXR1 protein appears to be required for auxin response in both the root and aerial parts of the plant. The AUX1 protein may act specifically in the hormonal regulation of root gravitropic response.

In addition to auxin resistance, the *aux1*, *axr1*, *axr2*, and *axr3* mutants also have altered responses to at least two other plant hormones. This cross-resistance may reflect physiological interactions among these compounds. For

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Table 1. Genetic analysis of *axr4* mutants

Cross	Generation	Auxin response[a]		
		Sens.	Resist.	χ^2
AXR4/AXR4 × *axr4-1/axr4-1*	F_1	147	0	
	F_2	3084	638	122.5; $P<0.01$[b]
AXR4/AXR4 × *axr4-2/axr4-2*	F_1	31	0	
	F_2	508	186	1.2; $P>0.1$[b]
axr4-1/axr4-1 × *axr4-2/axr4-2*				
Cross 1	F_1	3[c]	87	
	F_2	0	171	
Cross 2	F_1	0	61	
axr4-2/axr4-2 × *axr1-3/axr1-3*	F_1	9	0	
axr4-1/axr4-1 × *aux1-7/aux1-7*	F_1	124	0	

[a]Seedlings were scored for auxin resistance as described in Experimental procedures.
[b]χ^2 was calculated for an expected 3:1 ratio.
[c]These three seedlings were probably scored as auxin-sensitive because of the presence of unrelated deleterious mutations segregating in the *axr4-2* background.

example, ethylene inhibits longitudinal transport of auxin in stems (Burg and Burg, 1967; Morgan and Gausman, 1966) and lateral auxin transport in roots (Lee *et al.*, 1990), possibly resulting in accumulation of auxin to high levels in certain cells. Inhibition of root elongation by ethylene could thus be a secondary consequence of its effects on auxin transport. Resistance to the accumulated auxin would explain the ethylene resistance of these four mutants.

This paper describes the isolation and characterization of mutants at a new locus required for auxin response, which we have designated *AXR4*. The *axr4* mutants are specifically resistant to auxin, the first such mutants described, and are defective in root gravitropism. We also present a genetic analysis of the interactions between the *axr4* mutations and two other recessive mutations that affect auxin response, *axr1* and *aux1*. These analyses indicate that all three of these loci are important for normal lateral root initiation and that they do not define a single linear pathway of auxin response.

Results

Isolation of the axr4 *mutants*

To identify new genes involved in auxin response, we screened 8100 T-DNA transformed lines of ecotype Wassilewskija (Feldmann, 1991) in pools of 100 families for seedlings able to elongate roots on agar containing 1×10^{-7} M 2,4-dichlorophenoxyacetic acid (2,4-D) (for details see Experimental procedures). Growth of wild-type seedling roots was almost completely inhibited on this medium. Auxin-resistant seedlings from one pool have been most extensively characterized and shown to define a new locus involved in auxin response, called *AXR4*. The T-DNA-induced mutant is called *axr4-1*.

We identified a second mutant with a similar phenotype in a screen of gamma-ray-mutagenized M_2 seed for auxin-resistant seedlings. This mutant was named *axr4-2*.

Genetic characterization

To determine the genetic basis for auxin resistance in *axr4-1* plants, mutant plants were crossed to wild-type and the progeny analyzed. The F_1 plants resulting from this cross were all auxin-sensitive, and in the F_2, auxin-resistant seedlings segregated at a ratio of 4.8:1 (Table 1). Thus, the *axr4-1* mutation is recessive and segregates in a manner most consistent with a single Mendelian gene. The deviation from a 3:1 ratio may result from reduced viability of gametes carrying the *axr4-1* mutation. The *axr4-2* mutation also behaved as a Mendelian recessive allele, but no evidence for reduced transmission was observed (Table 1).

Because *axr4-1* and *axr4-2* plants have similar phenotypes, these two mutants were tested for allelism. F_1 progeny from two independent crosses between *axr4-1* and *axr4-2* were auxin-resistant (Table 1). In addition, 171 F_2 progeny from four individual auxin-resistant F_1 plants were tested and found to be auxin-resistant, demonstrating that *axr4-1* and *axr4-2* are allelic.

To determine if the *axr4-1* and *axr4-2* mutants are alleles of previously isolated auxin-resistant mutants, they were crossed to *axr1-3* (Lincoln *et al.*, 1990) and *aux1-7* (Maher and Martindale, 1980; Pickett *et al.*, 1990) plants. All the F_1 progeny from these crosses were auxin-sensitive (Table 1), indicating that the *axr4* mutations are not alleles of either *aux1* or *axr1*.

The *AXR4* locus was mapped to the lower arm of chromosome 1, approximately 20 cM from the trichome mutation *dis2* (Figure 1; Table 2a). In crosses between *axr4-1* or *axr4-2* and a Landsberg mapping line containing the leaf color mutation *ch1-1*, only one *axr4-2 ch1-1* double mutant seedling was identified out of 766 total F_2 seedlings scored, demonstrating close linkage between *axr4* and *ch1*. To test whether *axr4* lies between *ch1* and the linked dwarf mutation *le* or on the proximal side of *ch1*, *axr4-2* was crossed to a double mutant *ch1-1 le* line. The one *axr4-2 ch1-1* double mutant identified in the F_2 population was wild-type at the *le* locus. Likewise, the one *axr4-2 le* double mutant identified proved to be wild-type at *ch1*. As the recombination events that combined *axr4* with either *ch1* or *le* separated *ch1* and *le* from each other, *axr4* almost certainly lies between *ch1* and *le*. To define the map location of *axr4* more precisely, an *axr4-2 ch1-1* double mutant was crossed to wild-type Landsberg (*erecta*) and recombinant seedlings identified in the F_2 (Table 2b). The results of this analysis place *axr4* close to the *ch1* locus (2.6 cM ± 1.5 cM), at about 61.0 cM on the genetic map of visible markers (Koornneef, 1994). No other known hormone-resistant mutations map to this location. This result, combined with the complementation data, demonstrates that AXR4 is a new locus.

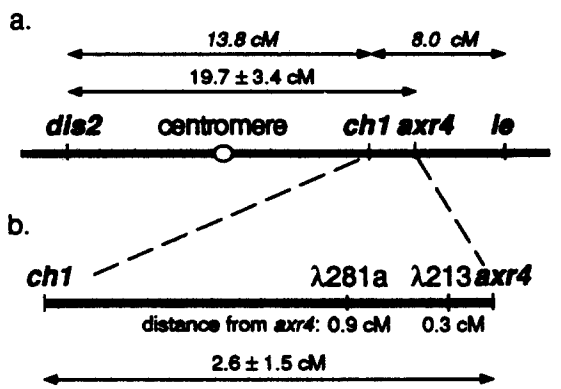

Figure 1. Map Location of *axr4* on chromosome 1.
(a) Visible markers. Distances shown in italics are those given in Koornneef (1994). Distances shown in plain type are derived from the data in Table 2 and were calculated by the method of maximum likelihood using the LINKAGE-1 program (Suiter *et al.*, 1983). The position of the centromere is shown according to Richards (personal communication).
(b) RFLP markers. axr4$^-$ch1$^+$ recombinants (Col/Col at *axr4*, Ler/Col or Col/Col at *ch1*) were scored for the two RFLP markers shown. Nine out of 28 were Col/Ler at λ281a and 4/41 were Col/Ler at λ213. These fractions were multiplied by 2.6 cM, the distance between *axr4* and *ch1*, to give the estimated distances shown.

Using the lines generated in the above cross with recombination breakpoints between *ch1-1* and *axr4-2*, *AXR4* was mapped using RFLP markers. This analysis indicates that the locus lies 0.3 cM distal to λ213 and 0.9 cM distal to λ281a (Chang *et al.*, 1988; Figure 1).

To determine if the *axr4-1* mutation is due to insertion of a T-DNA into the locus, the F_2 progeny from crosses between *axr4-1* and a wild-type plant were scored for auxin resistance and kanamycin resistance (which is conferred by the T-DNA). Kanamycin resistance segregated as a single dominant gene (data not shown). All 523 of the auxin-resistant progeny tested were also kanamycin resistant, indicating that the T-DNA is closely linked to the *axr4* mutation (≤4.4 ± 2.2 cM).

Physiological characterization

To characterize the response of the *axr4* mutants to auxin, the effects of exogenous hormone on root growth were determined. The results show that both alleles are equally resistant to 2,4-D and indole-3-acetic acid (IAA) (Figure 2a and b). Root growth of *axr4* seedlings is inhibited by a concentration of these auxins about five fold higher than the concentration which inhibits wild-type root elongation. Because other auxin-resistant mutants are resistant to additional plant hormones, the effects of these compounds

Table 2. Mapping of *AXR4* relative to visible markers

(a) Cross	Auxin^R F_2 scored	No. homozygous for 2nd mutation	Calculated map distance[a]
axr4-2/axr4-2 × *dis2-1/dis2-1*[b]	199	7 dis2	19.7 cM ± 3.4 cM
axr4-1/axr4-1 × *ch1-1/ch1-1*[c]	363	0 ch1	
axr4-2/axr4-2 × *ch1-1/ch1-1*[c]	403	1 ch1	(see b)
axr4-2/axr4-2 × *ch1-1 le/ch1-1 le*	779	1 ch1	

(b) Cross	Total F_2	axr4$^+$ ch1$^+$	axr4$^-$ ch1$^-$	axr4$^-$ ch1$^+$	axr4$^+$ ch1$^-$	Calculated map distance[a]
axr4-2 ch1-1 /axr4-2 ch1-1 × *AXR4 CH1/AXR4 CH1*	4307	3270	930	51	56	2.6 cM ± 1.5 cM

[a]Map distances were calculated by the method of maximum likelihood using the LINKAGE-1 program (Suiter *et al.*, 1983).
[b]The full genotype of the line used was *ga4-1 dis2-1 cer5-1 er* (Ler-O ecotype).
[c]The full genotype of the line used was *gl2-1 ch1-1 ap1-1 er* (Ler-O ecotype).

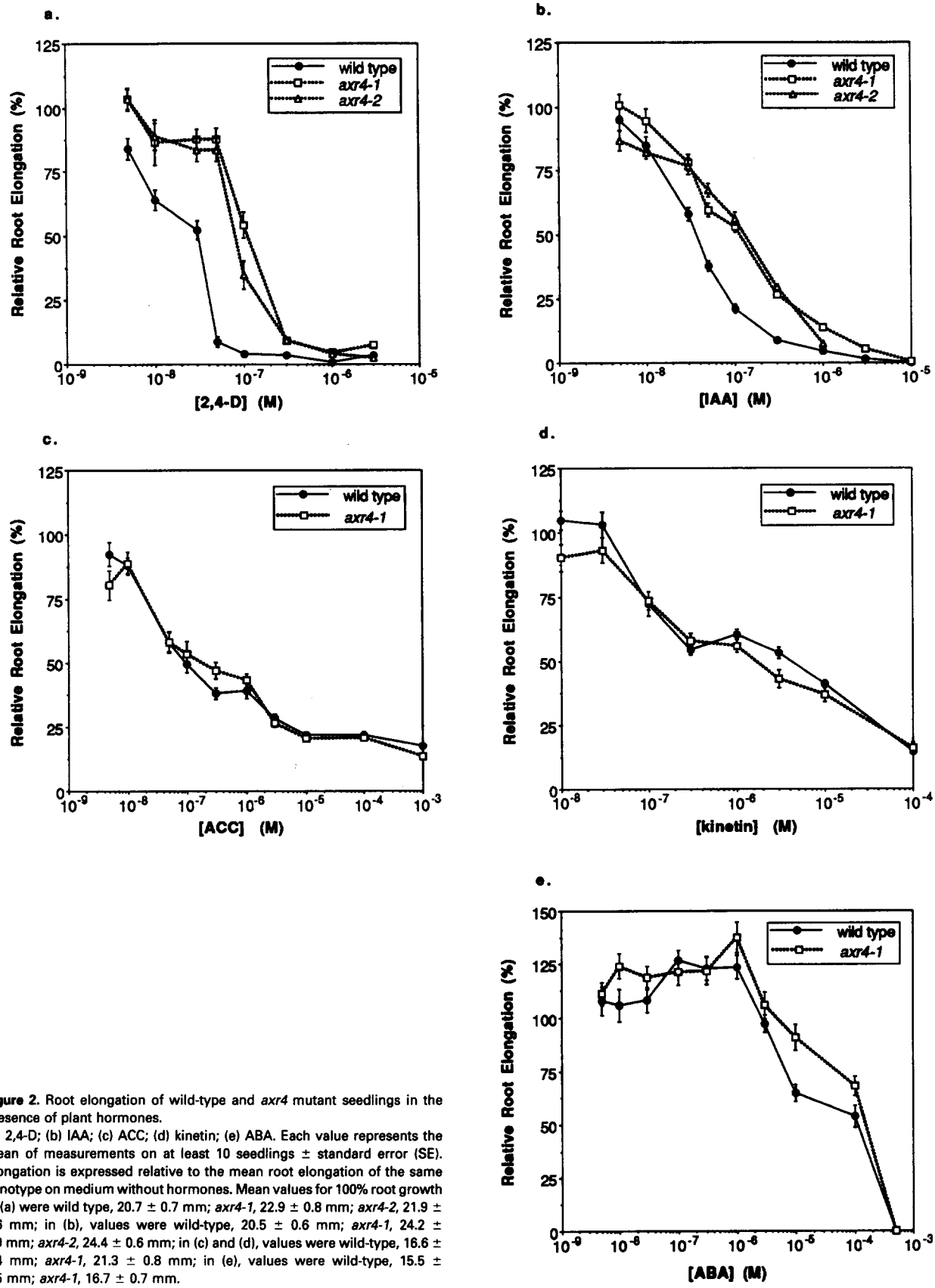

Figure 2. Root elongation of wild-type and *axr4* mutant seedlings in the presence of plant hormones.
(a) 2,4-D; (b) IAA; (c) ACC; (d) kinetin; (e) ABA. Each value represents the mean of measurements on at least 10 seedlings ± standard error (SE). Elongation is expressed relative to the mean root elongation of the same genotype on medium without hormones. Mean values for 100% root growth in (a) were wild type, 20.7 ± 0.7 mm; *axr4-1*, 22.9 ± 0.8 mm; *axr4-2*, 21.9 ± 0.6 mm; in (b), values were wild-type, 20.5 ± 0.6 mm; *axr4-1*, 24.2 ± 0.9 mm; *axr4-2*, 24.4 ± 0.6 mm; in (c) and (d), values were wild-type, 16.6 ± 0.4 mm; *axr4-1*, 21.3 ± 0.8 mm; in (e), values were wild-type, 15.5 ± 0.5 mm; *axr4-1*, 16.7 ± 0.7 mm.

on root growth of the *axr4* mutants were also determined. *axr4-1* has approximately the same sensitivity as wild-type to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Figure 2c), the cytokinins kinetin (Figure 2d) and benzyladenine (data not shown), and the amino acid analog α-methyltryptophan (data not shown). Similar results were obtained for the *axr4-2* allele (data not shown). *axr4* plants occasionally were more resistant than wild-type to ACC and cytokinin at certain concentrations (e.g. in Figure 2, 3×10^{-7} M ACC and 3×10^{-6} M kinetin). In four of five experiments *axr4* showed slight and variable resistance to abscisic acid (ABA) (e.g. Figure 2e). Thus, auxin was the only plant hormone tested to which the *axr4* mutants were substantially and consistently resistant.

Morphology

One striking aspect of the *axr4* mutant phenotype is defective root gravitropism. To assess this defect quantitatively, vertically grown seedlings were reoriented by 90° and the time course of root tip curvature was measured. Plants of both alleles were consistently delayed in their response to gravity relative to wild-type (Figure 3a), although the majority of roots did eventually orient vertically downward (by 24 h, not shown). This defect does not appear to result from a defect in root elongation, as *axr4* roots grew faster than wild-type roots on hormone-free medium (Figure 3b).

Roots of *axr4* plants appeared similar to those of wild-type with respect to root hair number and morphology. There was a reduction in the number of lateral roots formed by *axr4* plants (Figure 3c).

The aerial portion of *axr4* mutant plants is largely wild-type in appearance. Morphometric analysis confirmed that there were no significant and consistent differences from wild-type in rosette weight, time of bolting, number of inflorescences, height of main inflorescence, number of lateral branches, or number and spacing of siliques (data not shown). The rosette leaves of *axr4* plants are somewhat curled along their long axis, making the leaves appear narrower than wild-type (Figure 4). To see if the mutation also affects the auxin sensitivity of the rosette leaves, 14-

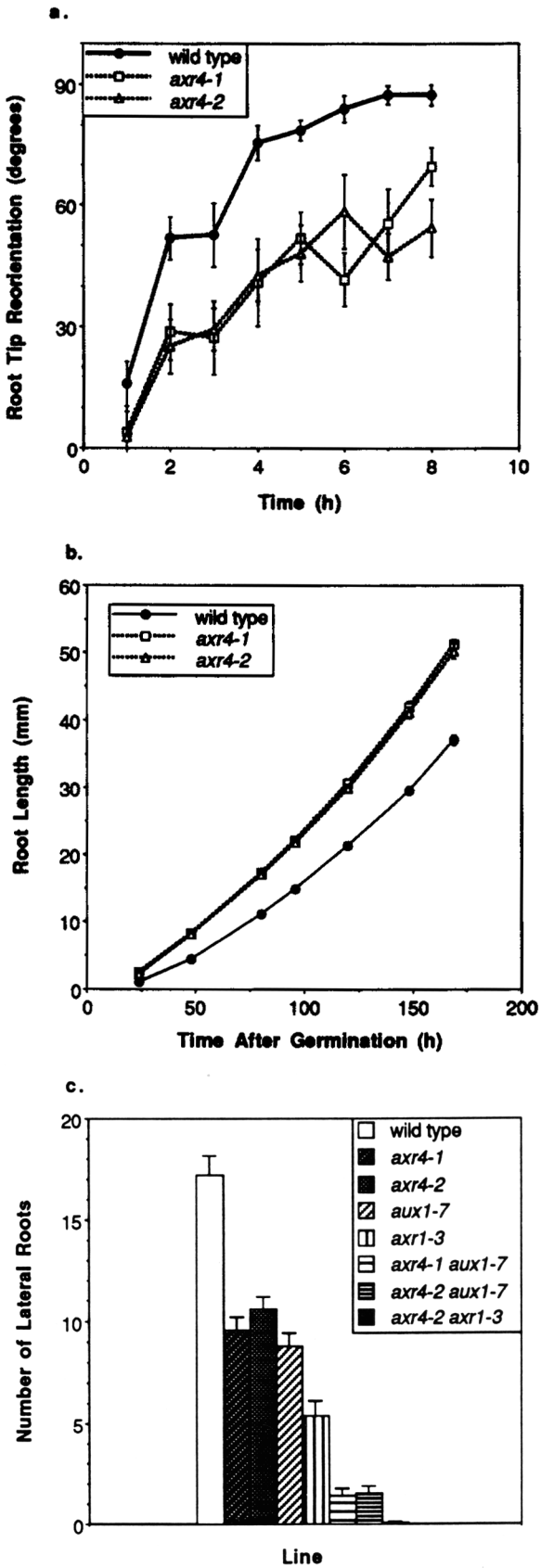

Figure 3. Root phenotype of wild-type and *axr4* mutant seedlings.
(a) Root reorientation in response to gravity. Seedlings on vertical plates were turned 90° to a horizontal position and the orientation of the root tips measured at the indicated times thereafter; 90° represents complete reorientation downward. Values shown represent the mean ± SE of at least 10 seedlings.
(b) Time course of root growth. Roots of seedlings on vertical plates of hormone-free medium were measured daily; only seedlings whose radicles emerged at approxmately the same time were used. The values shown represent the mean ± SE of at least 12 seedlings.
(c) Lateral root formation. Seedlings were grown for 14 days on vertically oriented plates containing media without hormones. At the end of this period lateral roots were counted using a dissecting microscope. Values shown are means of at least 13 seedlings ± SE.

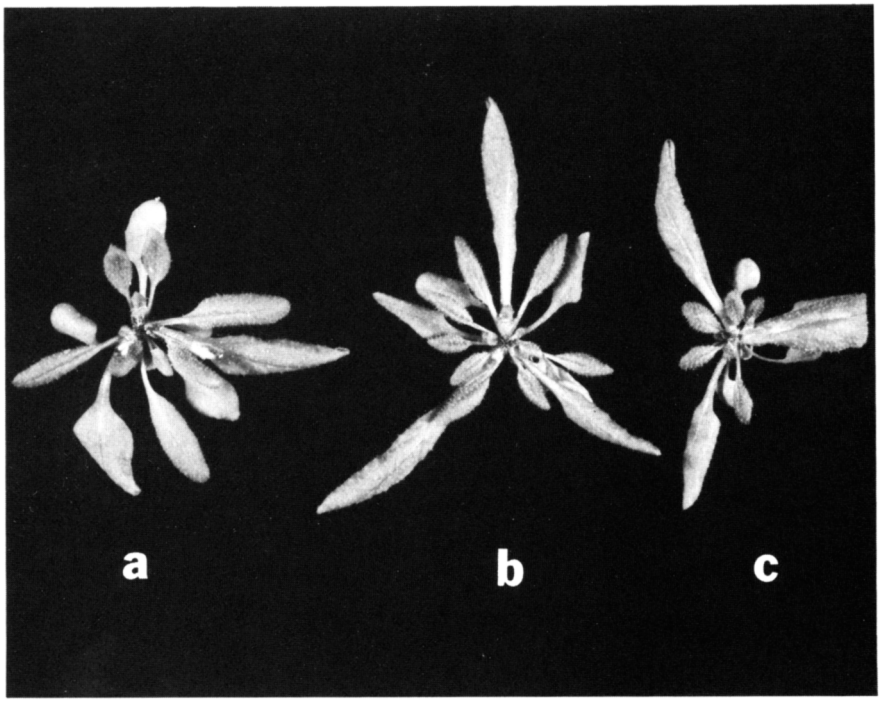

Figure 4. Rosettes of wild-type Columbia (a), *axr4-1* (b), and *axr4-2* (c) plants, grown for 29 days with continuous light.

day, 16-day, or 19-day-old plants (three separate experiments) were sprayed with 2,4-D for 3 successive days and weighed 1 week later. Growth of both *axr4* and wild-type plants was found to be equally inhibited by 2,4-D in these experiments (data not shown). However, this assay might not detect a small change in auxin sensitivity.

Double mutant analysis

To characterize the epistatic relationships between *axr4* and other mutations that cause auxin resistance, the *axr4* mutants were crossed to *aux1-7* and *axr1-3* plants and double mutants identified among the F_2 progeny. The genotypes of the double mutants were confirmed by analysis of F_3 progeny and by backcrossing to the parental lines and showing that all the F_1 plants were also auxin-resistant. The auxin sensitivity, gravitropic response, and morphology of double mutant plants were characterized.

axr4 axr1 *double mutants*

Roots of the *axr4–2 axr1-3* double mutant plants differed from those of the parental single mutants in auxin resistance, gravitropism, and lateral root formation. The double mutant plants were strikingly more resistant to 2,4-D than the parental single mutants in a root growth assay (Figure 5a). The concentration of 2,4-D which caused a 50% inhibition of root growth was 2.5×10^{-6} M, compared to 4×10^{-8} M for wild-type, 1.5×10^{-7} M for *axr4* and 4×10^{-7} M for *axr1-3*. The root gravitropic response of this double mutant was also consistently slower than that of either of the single mutants (Figure 5b). Lateral root formation, which was somewhat reduced in the parental single mutants, was dramatically reduced to an average of less than one lateral root per 14-day-old seedling in the *axr4-2 axr1-3* double mutants (Figure 3c). In similar experiments, single mutant plants with the severe *axr1-12* allele (Lincoln *et al.*, 1990) also developed an average of less than one lateral root per seedling by 14 days after germination (unpublished data; Lincoln, 1992). The aerial morphology of the *axr4-2 axr1-3* double mutant plants was similar to that of *axr1-3* plants, although occasionally the rosette leaves showed downward lengthwise curling similar to that of *axr4* plants (not shown). Double mutants between both *axr4* alleles and *axr1-12* have been identified and in preliminary studies the level of auxin resistance of the *axr4 axr1-12* plants appears very similar to that of the *axr4-2 axr1-3* plants. However, *axr1-12* plants have reduced fertility and not enough seed has yet been obtained from the *axr4 axr1-12* double mutants for detailed physiological analysis.

axr4 aux1 *double mutants*

The *axr4 aux1-7* double mutants had the same level of 2,4-D resistance as the *aux1-7* mutant alone. The concentration of 2,4-D that caused 50% inhibition of root elongation for both *aux1-7* and *axr4 aux1-7* was about 4×10^{-7} M (Figure 5a). The roots of *aux1-7* and *axr4 aux1-7* plants were severely reduced in gravtropism, such that gravitropism

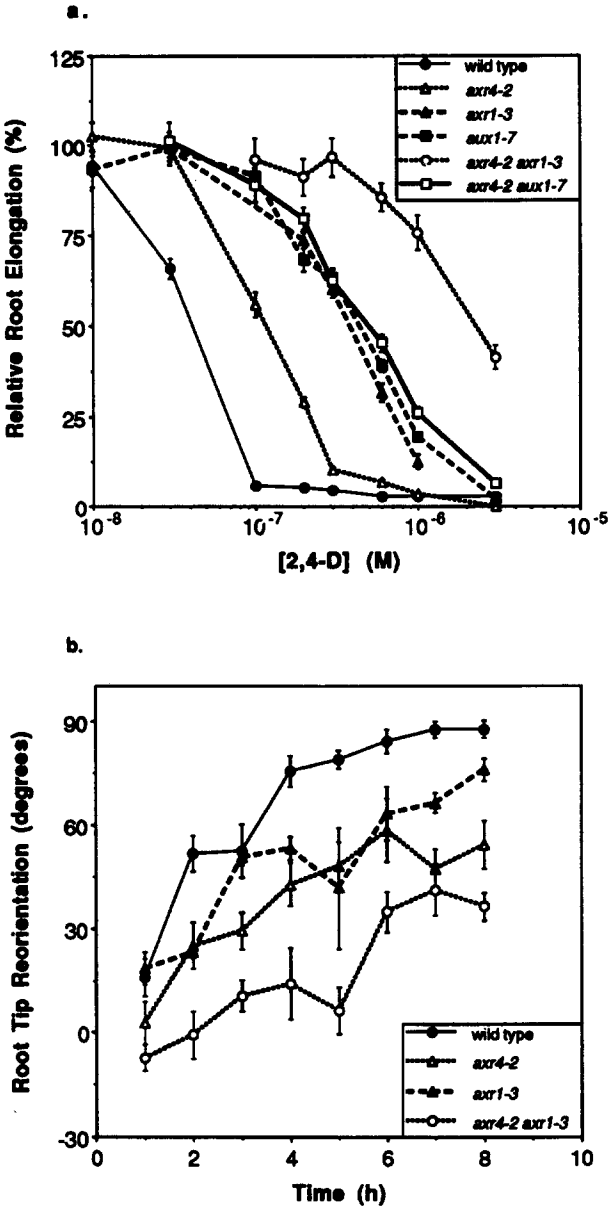

Figure 5. Phenotypes of double mutant seedlings.
(a) Root elongation of wild-type and mutant seedlings on medium containing 2,4-D. Experiment and data analysis as described for Figure 2; values represent the mean of at least 12 seedlings ± SE. Mean values for 100% root growth were wild type, 17.7 ± 0.4 mm; *axr4-2*, 20.1 ± 0.6 mm; *axr1-3*, 27.8 ± 0.4 mm; *aux1-7*, 20.4 ± 0.7 mm; *axr4-2 axr1-3*, 24.3 ± 0.9 mm; *axr4-2 aux1-7*, 20.3 ± 0.7 mm.
(b) Root reorientation in response to gravity. Experiment and data analysis as described for Figure 3(a).

assays of the type shown in Figures 3(a) and 5(b) were not informative. The *aux1-7* mutation alone caused a reduction in the number of lateral roots (Figure 3c). The *axr4 aux1-7* double mutants had greatly reduced lateral root formation in14-day-old seedlings (Figure 3c). The aerial phenotype of the *axr4-2 aux1-7* double mutant was variable but generally similar to wild-type. Occasionally these plants displayed longitudinal leaf curling that was exaggerated relative to *axr4* plants, although no such curling was noted in *aux1* plants (not shown).

Discussion

The *axr4-1* and *axr4-2* mutants define a new locus involved in auxin response. The *axr4* mutant phenotype includes curled rosette leaves, delayed root gravitropic response, reduced lateral root formation, and auxin-resistant root elongation. The defects in root gravitropism and lateral root formation in *axr4* and other auxin resistant mutants are consistent with the importance of auxin in these processes (Pickard, 1985; Street, 1968). The altered leaf morphology presumably reflects the involvement of auxin in cell expansion (Cleland, 1987) or in vascular differentiation (Aloni, 1987). More severe changes in leaf morphology have been observed in the more strongly auxin-resistant *axr1* and *axr2* mutants. The somewhat subtle effects of the *axr4* mutation on rosette leaves could result from the lower level of auxin resistance in these mutants compared with other mutants, or could reflect tissue-specific expression patterns of the *AXR4* gene. In sum, the *axr4* phenotype is likely the result of a mutation that alters auxin sensitivity.

These mutants are the first described in *Arabidopsis* that are specifically resistant to auxin. This specificity may be the consequence of the relatively low level of auxin resistance in *axr4* plants, only 1/2 to 1/3 the level of resistance of the *aux1* and *axr1* mutants. Indirect effects of the *axr4* mutations on responses to other hormones may therefore be minor and not easily detectable. Alternatively, the product of the *AXR4* gene may act in an auxin-specific response pathway. We cannot distinguish between these possibilities.

We have analyzed the genetic relationships between *AXR4*, *AXR1*, and *AUX1* by characterizing double mutant plants. The *axr4-1*, *axr4-2* and *axr1-12* mutations are all the most severe alleles known for these loci (Lincoln *et al.*, 1990) and so may represent complete loss-of-function mutations. *aux1-7* is a severe allele of this locus (Pickett, unpublished) and is probably a near-null allele. Although *axr1-3* is a weak allele of the *AXR1* gene (Lincoln *et al.*, 1990), the similar levels of auxin resistance seen in *axr4 axr1-3* and *axr4 axr1-12* double mutants indicate that the leakiness of the *axr1-3* mutation does not invalidate our interpretations.

Roots of the *axr4 axr1* double mutant are more resistant to auxin, respond more slowly to gravity, and form fewer lateral roots than those of either parental single mutant. The severity of the defects in the double mutant suggests that *axr4* and *axr1* may interact synergistically, and that the AXR4 and AXR1 proteins may perform overlapping or partially redundant functions in a single pathway.

Mutations in one of the two genes would still allow partial function of the pathway; mutations in both genes would completely block the pathway and result in much more severe effects. It is also possible that *AXR4* and *AXR1* act in separate pathways with largely independent roles in auxin response.

axr4 aux1 double mutant plants form fewer lateral roots than either parental single mutant, but are only as resistant to auxin as *aux1* plants. The *axr4 aux1* double mutants are therefore significantly less resistant to auxin than are the *axr4 axr1* double mutants. The phenotype of the *axr4 aux1* double mutant presents a paradox. The two mutations appear to have additive effects on lateral root formation, which suggests that the two gene products act in separate pathways. However, *aux1* is epistatic to *axr4* in its effects on auxin-sensitive root elongation, suggesting that *AXR4* and *AUX1* act in the same pathway. These results may reflect differences in the mechanism of auxin action during root elongation and formation of lateral roots. A precise explanation awaits a molecular characterization of the two genes.

Our results support the proposed role of auxin in lateral root development. Exogenously applied auxin has been shown to increase lateral root formation (Street, 1968; Webster and Radin, 1972). The data presented here provide genetic evidence that auxin has an important role in controlling this process *in vivo*. Lateral root formation was somewhat decreased relative to wild-type in the *aux1*, *axr1*, and *axr4* mutants, dramatically reduced in the *axr4 aux1* double mutants, and almost eliminated in the *axr4 axr1* double mutant. The auxin-insensitive *dgt* mutant of tomato (Kelly and Bradford, 1986) also lacks lateral roots (Zobel, 1973). It is interesting to note that the roots of the *axr4 axr1* double mutant still respond weakly to gravity despite their low level of sensitivity to auxin and absence of lateral roots. This suggests that lateral root formation requires a higher degree of auxin sensitivity than does root gravitropism.

Root elongation in the absence of exogenous hormones is greater in *axr4* and other auxin-resistant mutant seedlings than in wild-type seedlings (Evans *et al.*, 1994; Lincoln *et al.*, 1990; this work). This strong correlation between reduced sensitivity to auxin and increased elongation suggests that the endogenous auxin levels may be supra-optimal in wild-type *Arabidopsis* roots, thus inhibiting root elongation. However, the finding that very low concentrations of auxin stimulate elongation of wild-type roots (Evans *et al.*, 1994) is not consistent with this model. This apparent contradiction may arise from differences in metabolism or processing of exogenous vs. endogenous auxin. The roots of all single and double mutants characterized in the current work elongated at approximately the same rate in a 7 day time course (unpublished data). Therefore, this aspect of the mutant phenotype was not found to be additive in the *axr4 axr1-3* double mutant or in the *axr4 aux1-7* double mutants. Other factors may be limiting for elongation under the growth conditions used.

The complete co-segregation of auxin resistance and kanamycin resistance in *axr4-1* F_2 seedlings indicates that the *axr4-1* mutation appears to be tagged with a T-DNA insertion. Analysis of the T-DNA insert shows that multiple rearranged copies of the T-DNA are present at the single insertion site. Perhaps because of the complex nature of the T-DNA insert, both plasmid rescue and genomic library screening attempts to retrieve a junction fragment of T-DNA and plant DNA have thus far been unsuccessful. Eventual molecular characterization of *AXR4* will help to clarify the mechanism of auxin action.

Experimental procedures

Plant material

Arabidopsis plants were grown at 23°C on a commercially available peat-lite mixture such as Metro-Mix360 (Grace-Sierra), with continuous illumination at an intensity of 85–105 μE m^{-2} sec^{-1}. Plants were fertilized initially with nutrient solution as described (Wilson *et al.*, 1990). To score for auxin and kanamycin resistance and to perform root growth and gravitropism assays, seedlings were grown under sterile conditions on Petri plates containing nutrient solution supplemented with 7 g l^{-1} agar and 10 g l^{-1} sucrose (minimal medium). Hormones or kanamycin (obtained from Sigma, St. Louis, Missouri) were added after autoclaving. Seeds were surface sterilized for 15–25 min with 30% (v/v) bleach + 0.01% Triton X-100, washed with sterile water, placed on the plates, and then spread using 3 ml per plate of 0.7% agar in water. Sterile plants were grown at 22°C–24°C with 16 or 24 h light cycle at a light intensity of 20–60 μE m^{-2} sec^{-1}. Lines used for mapping studies were obtained from C. Somerville (Michigan State University) and from the Ohio State University Arabidopsis Biological Resource Center. The *axr4-1* mutant, originally isolated in the Wassilewskija ecotype, was backcrossed repeatedly into the Columbia ecotype (Col) to facilitate direct comparison with the other mutants isolated in this laboratory, all in the Col background. *axr4-1* was backcrossed at least three times before use in the physiological experiments shown (eight backcrosses for most physiological experiments and morphometric analysis). *axr4-2* was backcrossed at least twice before use in experiments.

Mutagenesis

Col ecotype seeds (25 000) were allowed to imbibe water for 12 h and were then exposed to 50 krad of gamma rays from a J.L. Shepherd cesium source. These M_1 seeds were sown in two independent populations at a density of 1 seed cm^{-2}, grown to maturity, and the M_2 seed harvested.

Mutant isolation

The DuPont T-DNA transformed collection of 8100 lines (generated by Dr Ken Feldmann and colleagues in Wassilewskija ecotype; Feldmann, 1991) was screened by placing 5000 seeds derived

from pools of 100 lines (aliquoted and sterilized by Florence Garelick) on minimal medium plates containing 1×10^{-7} M 2,4-D and scoring after 5, 6, and 7 days for seedlings that had elongated roots. Putative resistant mutants were transferred to minimal medium plates and then transplanted to pots. Seventy potential mutant seedlings were identified in the initial screen. Of these, 37 came from two of the pools and appear to represent (based on Southern blot analysis of the T-DNA insertion patterns) one original transformant per pool. Plants from both pools, after back-crossing, segregated auxin-resistant seedlings at ratios consistent with single recessive mutations. However, those derived from one pool showed no co-segregation of the auxin resistance with kanamycin resistance, whereas the other set (*axr4-1*) did. The other putative mutants either did not appear to be auxin-resistant in the next generation or segregated auxin-resistant mutants in the F_2 at aberrant ratios (e.g. 1 resistant: 9 sensitive). These plants have not been further characterized. *axr4-2* was isolated in a screen of 50 000 gamma ray-mutagenized Columbia M2 seed on 1×10^{-7} M 2,4-D.

Genetic characterization

Crosses were done by standard procedures. Auxin resistance was scored on minimal medium plates containing $1\text{–}3 \times 10^{-7}$ M 2,4-D. To test co-segregation of auxin resistance and kanamycin resistance, F_2 seedlings were scored for 2,4-D resistance, transferred to minimal medium plates for 2 days to allow recovery, and then placed on medium containing 50 μg ml^{-1} kanamycin. Kanamycin sensitivity was scored after 2–4 weeks by degree of growth and bleaching relative to controls. DNA isolation, Southern blotting and DNA hybridization were done by standard procedures as described (Wilson *et al.*, 1990) except that in most cases gels were blotted using a Posiblotter apparatus (Stratagene, La Jolla, California) according to the manufacturer's instructions, and hybridizations and washings were carried out in roller bottles in an Appligene hybridization oven (American Synthesis Inc., Pleasanton, California) at 42°C. Restriction digests of 1–5 μg of genomic plant DNA were routinely carried out in a volume of 150 μl containing 1 mM spermidine and 0.05% NP-40 in addition to the appropriate restiction enzyme and buffer. RFLP markers were obtained from the Ohio State University Arabidopsis Biological Resource Center.

Physiological characterization

Root growth assays were performed essentially as described (Wilson *et al.*, 1990). Seeds were placed on minimal medium plates, cold-treated at 4°C for 2–4 days to promote uniform germination, and then the plates placed vertically in a growth chamber. After 5 days, seedlings were transferred to plates containing the hormone and the position of the root tip marked. After 3 days of growth in a vertical orientation on the hormone-containing plates, root elongation was measured using a dissecting scope. Data are presented as the elongation on the hormone relative to the elongation on plates containing minimal medium. Morphometric analysis was performed on plants that had been grown directly from seed in pots with a 24 h light period as described above. Nine or 10 plants were analyzed. Measurement of aerial auxin sensitivity was performed as described by Lincoln *et al.* (1990). Double mutants were constructed using *axr4-1* plants that had been backcrossed four times and *axr4-2* plants that had been backcrossed twice to Columbia wild-type. Columbia ecotype was used as wild-type in all assays.

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