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Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology

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Summary. Mutant lines of *Arabidopsis thaliana* resistant to the artificial auxin 2,4-dichloro phenoxyacetic acid (2,4-D) were isolated by screening for growth of seedlings in the presence of toxic levels of 2,4-D. Genetic analysis of these resistant lines indicated that 2,4-D resistance is due to a recessive mutation at a locus we have designated *Axr-1*. Mutant seedlings were resistant to approximately 50-fold higher concentrations of 2,4-D than wild-type and were also resistant to 8-fold higher concentrations of indole-3-acetic acid (IAA) than wild-type. Labelling studies with (^{14}C)2,4-D suggest that resistance was not due to changes in uptake or metabolism of 2,4-D. In addition to auxin resistance the mutants have a distinct morphological phenotype including alterations of the roots, leaves, and flowers. Genetic evidence indicates that both auxin resistance and the morphological changes are due to the same mutation. Because of the pleiotropic morphological effects of these mutations the *Axr-1* gene may code for a function involved in auxin action in all tissues of the plant.

Key words: Auxin resistance – 2,4-Dichloro phenoxyacetic acid – Phytohormone – Plant development – Herbicide-resistance

Introduction

Plant hormones play a central role in normal plant development. However, despite their importance, very little is known about either the sites at which they act or the mechanisms by which they exert their effects. One group of plant hormones, the auxins, are thought to play an essential role in many aspects of plant development. The major naturally occuring auxin, indole-3-acetic acid (IAA), has been detected in a variety of tissues from a number of different plant species (Bearder 1980), and has been implicated as a regulatory molecule in processes as diverse as cell expansion during shoot elongation (Jacobs and Ray 1976) and the differentiation of vascular elements (Shininger 1979). An important unanswered question is whether or not auxin mediates these various developmental processes via one primary site of action or through several distinct mechanisms.

Because the concentration of phytohormones in plant tissues is very low, conventional approaches to metabolic studies and mode of action have been fraught with technical difficulties. However, a genetic approach has been used effectively to study the biosynthesis of gibberellins (Phinney and Gibberellin 1984) and abscissic acid (Moore and Smith 1985) in maize. In addition mutants of *Arabidopsis* have been identified which appear to be altered in their response to these growth hormones (Koorneef et al. 1984, 1985). A comparable genetic analysis of auxin biosynthesis and response is complicated by the fact that a dramatic disruption of these processes by mutation is likely to result in lethality early in the development of the plant. There is no direct evidence to support this view. However, no auxin-deficient mutants have been identified in any species and since auxin is required for growth of tissue in culture, auxin would appear to be essential for viability. In principle, it should be possible to isolate conditionally auxin-deficient mutants. However, the identification of a conditional mutation in the auxin biosynthetic pathway is problematic since it is not clear that exogenous application of auxin will rescue an auxin-deficient plant. The role of auxin is thought to be regulatory and precise spatial patterns of concentration (i.e., gradients) are likely to be critical. Similarly the morphological phenotype of a mutant defective in auxin response is unknown and may not be specific.

As an alternative approach, mutants resistant to the exogenous application of synthetic auxins have been isolated in several tissue culture systems (Chaleff and Parsons 1978; Davidonis et al. 1982; Muller et al. 1985) and in *Arabidopsis* seedlings (Maher and Martindale 1980). Among other possibilities such mutants might be resistant because the level of endogenous hormone is different from that in wild type or because of changes at the primary site of auxin action. Several of the auxin-resistant mutants have defects in some but not all of the processes believed to be regulated by auxin. In tobacco, mutants resistant to 1-Naphthaleneacetic acid isolated from haploid protoplast culture were unable to regenerate roots. However shoots were able to grow and set seed normally after being transplanted to a wild-type root stock (Muller et al. 1985). In *Arabidopsis*, mutations conferring an auxin-resistant phenotype were found to alter the geotropic behavior of the roots but produced no other obvious phenotype (Mirza et al. 1984). These observations suggest that any genes required for auxin action in all tissues have not been identified by mutation. One auxin-resistant mutant of *Arabidopsis* has been

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isolated with a dwarf phenotype. However this mutation appeared to cause lethality when homozygous and only a preliminary report has appeared (Mirza and Maher 1980).

In order to expand the spectrum of auxin response mutants to include those with more dramatic developmental phenotypes we have screened a large population of mutagenized *Arabidopsis* seedlings for resistance to the artificial auxin 2,4-dichloro phenoxyacetic acid (2,4-D). This paper describes the isolation and characterization of an allelic set of mutants that are resistant to auxin and have a pleiotropic morphological phenotype. The gene defined by these mutants appears to be important for normal development and may encode a function required for response to endogenous auxin.

Materials and methods

Plant materials and growth conditions. All the plants used for these experiments were derived from Columbia wild type. Normally plants were grown in a support medium consisting of equal parts of perlite, vermiculite and spaghnum saturated with a mineral nutrient solution containing 5 mM KNO_3, 2.5 mM KH_2PO_4 (adjusted to pH 5.5), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 50 μM Fe-EDTA (Sigma EDFS), 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4$, 10 μM NaCl, 0.01 μM $CoCl_2$. When plants were grown under sterile conditions seeds were surface sterilized for 20 minutes in 30% v/v bleach and 0.02% Triton X-100 and placed in petri dishes containing the nutrient solution described above supplemented with 8 g/l agar and 20 g/l sucrose. Where appropriate, hormones were added to the medium after autoclaving. All plants were grown at 22° C–24° C with continuous illumination supplied at an intensity of 100–150 $\mu E/m^2$ per second.

Mutagenesis. Approximately 50,000 seeds were soaked for 16 h in 100 ml 0.3% (v/v) ethyl methane sulfonate, then washed in water over a period of 4 h. This M1 seed was sown at a density of approximately $1/cm^{/2}$. At maturity the resulting plants were bulk harvested to produce M2 seed.

Determination of auxin sensitivity. The level of resistance to either IAA or 2,4-D was determined by first incubating surface sterilized seeds on minimal medium for 3 days in continuous illumination and then transferring the seedlings onto plates containing minimal medium plus various concentrations of auxin. The position of the root tip was marked on the surface of the plate so that new root growth could be measured. These plates were incubated for 48 h in continuous illumination with the agar surface oriented vertically so that root growth occurred on the surface of the plate. Growth inhibition was calculated relative to growth on non-supplemented medium.

Analysis of flower development. In order to determine pollen yield, anthers were collected at a stage just prior to dehiscence and placed into light mineral oil. Each sample contained the anthers from 5 flowers. The anthers were ruptured with a small glass rod to allow release of pollen grains and the pollen was counted with a hemocytometer.

The development of the stamens and the pistil was examined by determining the ratio of the length of the longest stamen to that of the pistil in flowers of various ages. Length measurements were done to the nearest 0.1 mm with a magnifying comparator. In *Arabidopsis,* each inflorescence carries a series of flowers of increasing age with the youngest flower at the apical end. For the purposes of this analysis the flower immediately apical to the youngest flower with visible pollen was designated stage 1. The youngest flower with visible pollen was then called a stage 2 flower and so on proceeding basally down the inflorescence.

Uptake of radiolabelled 2,4-D. Surface sterilized seeds were distributed on petri plates containing standard mineral solution supplemented with 1 μM (2-^{14}C)2,4-D at a specific activity of 55 mCi/mMol (Amersham). The plates were incubated under standard growing conditions for 72 h. The seedlings were harvested, washed thoroughly in several changes of distilled water and homogenized in 2–5 volumes of 95% ethanol in a glass homogenizer. Cell debris was removed with a 10 min centrifugation in a Fisher microfuge, the pellet was reextracted with a further 1 ml of 95% ethanol, and the two supernatants were pooled.

Fractionation of 2,4-D metabolites. The metabolites of 2,4-D were fractionated according to the procedures of Davidonis et al. (1982) with minor modifications. The ethanol extract was adjusted to a pH of between 2 and 3 with H_3PO_4 and extracted 4 times with equal volumes of diethyl ether. The ether fractions were saved and the aqueous fraction was then extracted 4 times with equal volumes of water-saturated 1-butanol. The butanol fraction was retained and evaporated to dryness under a N_2 jet. In order to digest 2,4-D-sugar conjugates, the extract was rehydrated in 4 ml 0.1 *M* sodium acetate (pH 5.0), 0.1 mg/ml β-glucosidase (Sigma Type II) and digestion was carried out for 12 h at 23° C. The ether soluble components were removed by extracting four times with diethyl ether. The ether fractions from the first ether extraction and the ether fractions extracted after enzymatic digestion were dried seperately under N_2 and the metabolites were resuspended in a small volume of 95% ethanol. Metabolites were fractionated on silica gel thin-layer plates using a solvent system of a diethyl ether/petroleum ether/formic acid (70:30:1, v/v/v) and visualized by flourography. The radioactivity in each fraction was determined by liquid scintillation counting and the size of each metabolite pool was calculated after correcting for the efficiency of recovery from the TLC plate.

Results

Isolation of 2,4-D resistant mutants

When wild-type *Arabidopsis* seeds are placed on agar solidified minimal media containing 5 μM 2,4-D, the seedlings emerge from the seedcoat, accumulate chlorophyll but do not grow. Elongation of both the radicle and the hypocotyl are completely inhibited and the cotyledons do not expand. In order to screen for plants resistant to 2,4-D, 150,000 M2 seeds were distributed onto plates containing minimal medium supplemented with 5 μM 2,4-D at a density of 5,000 seeds/plate. A total of six plants (ME-1 to ME-6) that showed significant growth on the selective plates were recovered. Since it is possible that these six plants are all descendents from the same M1 plant a further 150,000 M2 seeds derived from an independent mutagenesis were also

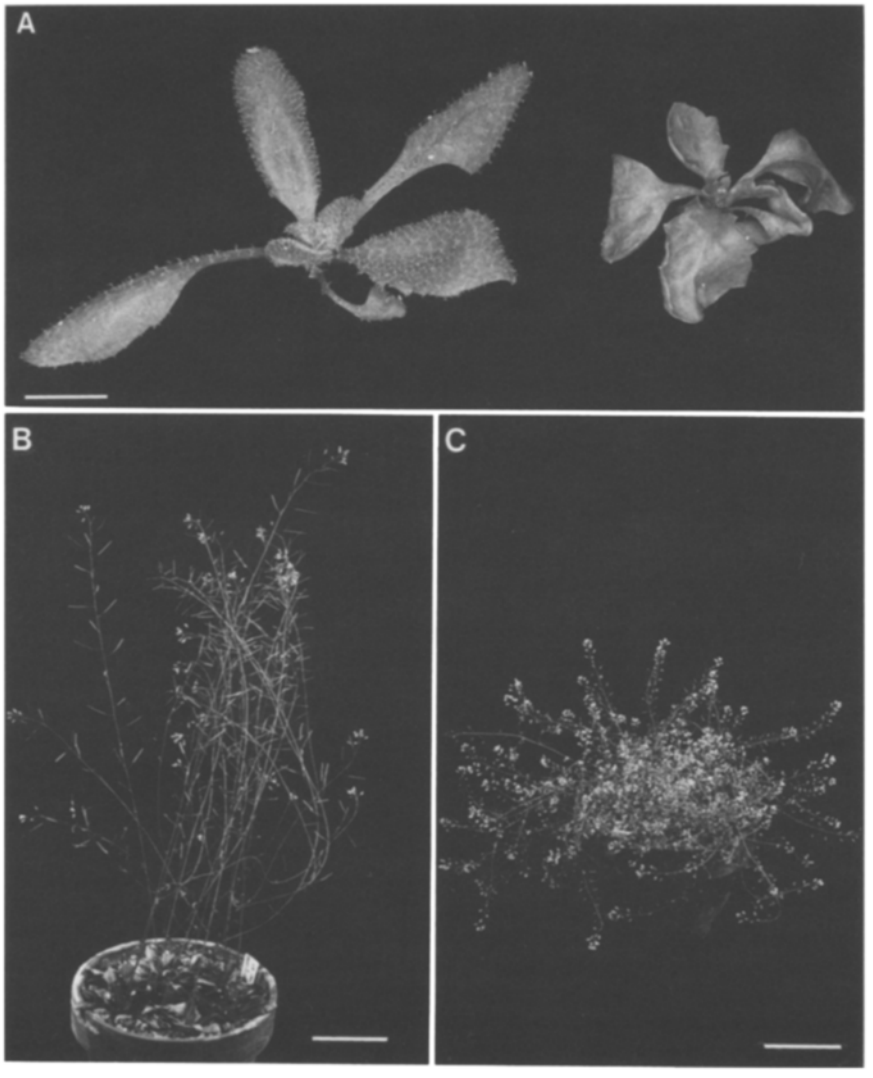

Fig. 1A–C. Morphology of wild-type and mutant plants. **A** Wild-type (*a*) and ME-3 (*b*) plants 3 weeks after sowing. The ME-3 plant is also homozygous for the glabrous marker *gl-1*. The *bar* indicates 3 cm. **B** Wild-type plants 8 weeks after sowing. **C** ME-3 plants 8 weeks after sowing. The pots in frames **B** and **C** both contain 8 plants. The bars in frames **B** and **C** indicate 5 cm

screened and yielded an additional seven mutants (ME-7 to ME-13). In all 13 mutants recovered, resistance was stably inherited. One mutant, ME-10, was only weakly resistant and has not been further characterized.

Developmental phenotype

The physical appearance of the 12 2,4-D resistant mutants is dramatically different from wild-type at every stage of development. Figure 1 illustrates wild-type plants and mutant line ME-3 at the rosette stage and at maturity. All 12 mutants have a very similar appearance. The mutant rosettes are small compared to wild type and have small crinkled leaves with shortened petioles. The roots are thinner than wild type and not as highly branched (data not shown). When the plants mature and begin to flower, wild-type plants typically produce between 1 and 5 primary inflorescences, whereas ME-3 plants produce up to 25 or 30 thin stemmed primary inflorescences. This growth behavior results in the formation of a short bush instead of the vertically oriented wild-type plant and suggests that the mutation causes a reduction in apical dominance.

The flowers on the mutant are smaller than wild-type and 8 of the 12 mutants are self-sterile. In reciprocal crosses with wild-type the mutants are normally fertile as the female parent but are very poor male parents. One reason for this effect is suggested by experiments in which the pollen yield was measured in three samples of five flowers each, from both wild-type and the self-sterile mutant line ME-5, just prior to dehiscence. Mutant flowers produced 389 ± 82 pollen grains/flower whereas wild-type flowers had $3{,}950 \pm 280$ grains/flower. Although pollen yield is dramatically reduced in the mutant the amount of pollen produced should be sufficient to allow some self-fertilization. Indeed, hand pollination of mutants with their own pollen produced normal seed set.

Measurement of pistil and stamen length at various stages during flower development reveals why selfing does not occur. As the wild-type flower matures the filaments elongate so that the anthers are in direct contact with the stigma during dehiscence. In Fig. 2 this elongation is illustrated by an increase in the stamen/pistil ratio between stage 1 and stage 2 wild-type flowers. In mutant line ME-5 the stamens are always substantially shorter than the pistil (Fig. 2) so that the pollen is released some distance below

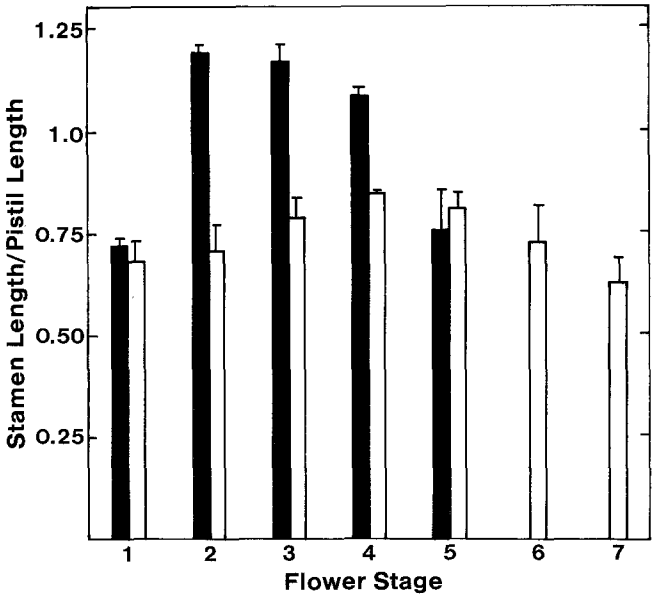

Fig. 2. Relative length of stamens and pistil in developing wild-type and ME-5 flowers. The length of the tallest stamen was measured and expressed relative to the length of the pistil in staged flowers as described in Materials and methods. Anther dehiscence begins in stage 2 flowers. The *solid bars* represent the values for wild-type flowers and the *open bars* the values for ME-5 flowers. Each value is the mean of at least 4 measurements ± standard error

Table 1. Genetic segregation of 2,4-D resistance in mutant lines ME-3 and ME-6

Cross	Number of plants		
	Resistant	Sensitive	x^{2a}
ME-6 × ME-6	153	0	
ME-6 × wild-type F_1	0	52	
ME-6 × wild-type F_2	37	83	2.15[b]
ME-3 × ME-3	122	0	
ME-3 × wild-type F_1	0	41	
ME-3 × wild-type F_2	43	131	0.008[b]

[a] x^2 calculated based on an expected ratio of 3 sensitive to 1 resistant
[b] $P > 0.5$

the stigma. This defect in combination with reduced pollen production adequately explains the observed self-sterility.

Genetic analysis

Because of the self-sterility of eight of the mutant lines it was convenient to maintain these lines as heterozygotes. F1 plants were generated by crossing the original resistant M2 plant to wild-type using the mutant as the female parent. The seed was collected from each F1 plant and used as a source of segregating homozygous mutant plants. These plants were identified by germinating on medium containing 0.5 μM 2,4-D, as described below.

The genetic basis for 2,4-D resistance and the associated morphological phenotype was determined by crossing each mutant line to wild-type and scoring both phenotypes in the F1 and F2 generations. The 2,4-D resistant phenotype was scored by placing seeds on agar solidified minimal media containing 0.5 μM 2,4-D. Root growth of the wild-type is completely inhibited under these conditions while the mutants all show significant root growth. Segregation data for the mutant lines ME-3 and ME-6 is presented in Table 1. The heterozygous F1 plants were not resistant to 2,4-D and segregation of the resistant phenotype in the F2 fits the 3 sensitive to 1 resistant ratio expected for a single recessive mutation. All 12 of the mutants analysed behaved in this manner. In addition, in a total of 1,170 F2 plants scored, the altered growth habit co-segregated with resistance to 2,4-D. This result plus the isolation of two independent sets of mutants with the same phenotype indicates that the developmental aberrations are caused by the same mutation that causes 2,4-D resistance.

Table 2. Complementation analysis of 2,4-D resistant mutants

Cross	Number of plants		
	Resistant	Sensitive	X^{2a}
ME-1/+ × ME-5/+	7	37	1.93[b]
ME-2/+ × ME-5/+	3	20	1.47[b]
ME-3/+ × ME-5/+	19	85	2.45[b]
ME-4/+ × ME-5/+	5	19	0.25[b]
ME-6/+ × ME-5/+	8	47	3.20[b]
ME-13/+ × ME-5/+	11	35	0.20[b]

[a] x^2 Calculation for an expected segregation of 1 resistant to 3 sensitive
[b] $P > 0.05$

Complementation analysis was performed with the lines recovered in screen A (ME-1 to ME-6) as well as ME-13, isolated in screen B. Because most of the mutants have reduced male fertility this analysis was performed by crossing F1 heterozygotes and scoring the progeny for resistance. The recovery of resistant plants among the progeny at a ratio of 1 resistant to 3 sensitive indicates non-complementation. The results are presented in Table 2. By this criterion the seven mutations lie in the same complementation group. We have designated the gene defined by this complementation group the *Axr-1* gene for auxin resistance.

Sensitivity to 2,4-D and IAA

When wild-type *Arabidopsis* plants are grown on minimal medium supplemented with sufficiently high concentrations of either 2,4-D or IAA, root growth is inhibited. This inhibition was used as the basis of an assay for sensitivity to both these auxins. Mutant lines ME-3 and ME-6 are both less sensitive as measured by this assay (Fig. 3a, b). The concentration of 2,4-D required for 50% inhibition of root growth is 0.037 μM in the wild-type and between 1 and 2 μM for ME-3 and ME-6. The sensitivity to IAA is also altered in ME-3 and ME-6 relative to wild-type although the difference is not as great as for 2,4-D. A 50% inhibition of root growth by IAA is achieved with 0.11 μM in the wild-type, 0.68 μM for ME-3 and 0.84 μM for ME-6.

Metabolism of radiolabelled 2,4-D

In order to determine whether or not the resistance phenotype is due to a change in uptake or metabolism of 2,4-D

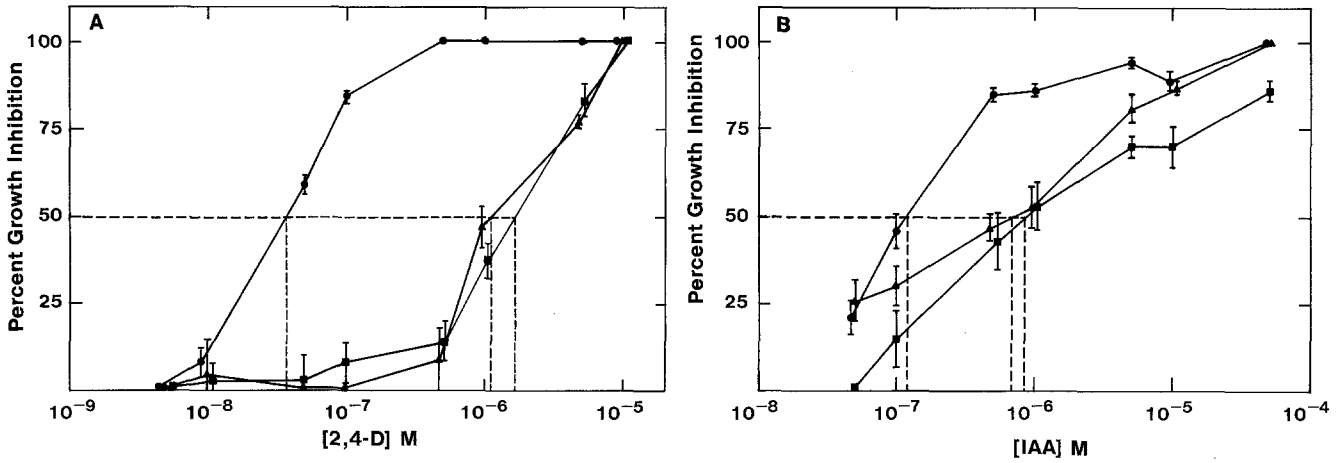

Fig. 3A, B. The effect of exogenous auxin on root growth of wild-type and mutant seedlings. Inhibition of root growth in wild-type (●), ME-3 (▲) and ME-6 (■) plants is expressed relative to root growth on non-supplemented medium. Each value represents the mean of measurements from at least six plants. *Error bars* describe the standard error. *Dashed lines* indicate the level of compound producing a 50% inhibition of root growth. **A** The levels of 2,4-D producing 50% inhibition were: wild-type, 0.037 μM; ME-3, 1.1 μM; ME-6, 1.6 μM. **B** The levels of IAA producing 50% inhibition were: wild-type, 0.12 μM; ME-3, 0.68 μM; ME-6, 0.84 μM

Table 3. Metabolism of 2,4-D

	Wildtype		ME-6	
	pmol/gfwt[a]	% total	pmol/gfwt[a]	% total
Ether soluble metabolites				
Et-1	348.8± 25.6	8.7±1.1	739.1±138.0	13.8±3.1
Et-2	124.1± 8.1	3.3±0.8	230.8± 17.0	4.3±0.3
Et-3	152.7± 52.9	4.4±2.6	78.1± 8.5	1.5±0.2
Et-4	1,752.0±127.3	43.6±4.2	2,378.5±152.7	44.4±2.3
Aqueous soluble metabolites				
Aq-1	207.7± 36.2	4.9±0.3	301.1± 54.3	5.7 ±1.3
Aq-2	32.5± 5.4	0.8±0.1	44.7± 4.2	0.83±0.1
Aq-3	619.1±151.7	14.2±1.9	957.5±109.9	18.0 ±2.7
Aq-4	91.2± 21.7	2.1±0.2	131.1± 15.4	2.5 ±0.3
Un-digested	832.0±304.5	17.9±6.1	487.3± 41.8	9.1 ±1.0
Total	4,159.9±754.5		5,348.2±163.5	

[a] Inferred by assuming no change in specific activity of each metabolite relative to applied 2,4-D

Metabolites were extracted and analyzed by thin layer chromatography as described in Materials and methods. Et-4 is 2,4-D and Aq-3 is 2,4-D released during β-glucosidase digestion. The other metabolites were not identified

both wild-type and ME-6 plants were germinated and grown on medium containing (^{14}C)2,4-D for 3 days. The labelled metabolites were extracted, fractionated into ether soluble and aqueous soluble metabolites and further separated by thin layer chromatography. The levels of each metabolite detected is shown in Table 3. Only the 2,4-D present in the ether and water soluble fractions was identified. The other metabolites were given an arbitrary designation with a higher number indicating a higher mobility during chromatography. Significantly higher levels of radioactive metabolites were found in the mutant seedlings after the incubation period. This is thought to be due to the fact that at the concentration of 2,4-D used in this experiment (1 μM), growth of the wild-type seedlings is severely inhibited while the resistant plants are only moderately affected. The percentage of the total label represented in each fraction is similar in wild-type and ME-6 tissue. In particular, there is little difference between the two lines in the relative amounts of unmetabolized 2,4-D (fraction Et-4). As in other dicotyledonous species (Feung et al. 1975) a major route of detoxification of 2,4-D in *Arabidopsis* may be by conjugation with sugar residues. Glycoconjugates are represented in the aqueous fraction (Aq) and comprise a significant proportion of the total label extracted in both wild-type and ME-6 plants. We have not eliminated the possibility that there is an alteration in compartmentalization within the cell that is not evident when looking at total cellular pools. However this possibility aside, the labelling studies suggest that mutations in the *Axr-1* gene do not result in changes in the metabolism of 2,4-D.

Discussion

We have isolated 13 lines of *Arabidopsis* that are resistant to the artificial auxin 2,4-D. Two of these lines are approximately 50-fold more resistant to 2,4-D than the wild-type and about 8-fold more resistant to the natural auxin IAA. In the 12 lines we have studied, 2,4-D resistance is due to a single recessive mutation. Complementation analysis among seven of these lines indicates that the mutation conferring resistance lies in the same gene in all seven lines. Although complementation tests were not performed for the remaining five lines, the similarity of phenotype suggests that these mutants also carry mutations in the same gene. We have named the gene defined by this complementation group the *Axr-1* gene for altered auxin response.

Each of the resistant lines displays a characteristic syndrome of growth abnormalities. Several of the affected growth processes, such as lateral root growth, apical dominance, and elongation of stamens are thought to be mediated, at least in part, by auxin (Blakely et al. 1972; Phillips 1975; Koning 1983). That these morphological changes are

manifestations of the same mutation that confers resistance to auxin is indicated by the isolation of plants with the same phenotype from two independently mutagenized populations of seed and by the co-segregation of the phenotype in 1,170 F2 plants. The association of 2,4-D resistance with these developmental abnormalities suggests that the herbicidal action of 2,4-D is related to its auxin activity and that the developmental processes affected require auxin to occur normally.

Auxin resistance could, in principle, be due to a mutation causing a gain of function such as an increase in the activity of a detoxifying enzyme or a loss of function such as a reduction in the affinity of an auxin receptor for auxin. Because the mutations described here are recessive they probably result in a loss of function. This view is also supported by the fact that we did not observe any alteration in the metabolism of applied 2,4-D.

Although the resistance mutations in the *Axr-1* gene appear to result in the loss of some function, the frequency at which they were isolated indicates that they are not nulls. In other studies in our laboratory null mutations at several other loci were recovered at a frequency of approximately 1 in 2,000 M2 plants (Browse et al. 1985; Caspar et al. 1985). In contrast we have recovered only 1 mutation in the *Axr-1* gene in every 25,000 M2 plants. The relatively low rate of recovery of these auxin resistant mutations and the fact that they probably result in a loss of function suggests that null mutations in the *Axr-1* gene are lethal.

Clearly, the normal function of the *Axr-1* gene cannot be ascertained at this preliminary stage. However the evidence described above suggests to us that this gene encodes an essential function associated with auxin action, and that mutations in *Axr-1* that produce resistance are accompanied by a reduction in function of the gene product and a disruption of normal development. The relatively low level of resistance to IAA is consistent with maintenance of normal IAA function and suggests that the mutation causes a change that specifically discriminates against 2,4-D. An attractive possibility is that the *Axr-1* gene codes for an auxin receptor and that resistance is due to an alteration that has a greater effect on the affinity of this receptor for 2,4-D than for IAA.

Auxin is thought to be involved in virtually every aspect of plant development. In attempting to determine the mechanism of auxin action it will become important to know which components are shared between tissues and which are specific for a particular tissue. Auxin-resistant mutants of tobacco (Muller et al. 1985) and *Arabidopsis* (Mirza et al. 1984) have been isolated which have defects in root development but are otherwise morphologically normal, suggesting that some components of the cellular machinery involved in auxin response are tissue specific. In contrast, the auxin-resistant lines described here are altered in every tissue, including roots, rosette, inflorescences and flowers. In addition a preliminary description of another and possibly related *Arabidopsis* mutant with a dwarf phenotype has been described (Mirza and Maher 1980). It is therefore possible that the various auxin responses are all related by a common mechanism and that the *Axr-1* gene encodes a function involved in this common mechanism.

The identification of proteins that are specifically involved in auxin biosynthesis and response has not been accomplished. Because the concentration of IAA in plant tissues is very low and the abundance of biosynthetic enzymes is correspondingly low it has not been possible to identify or isolate any enzymes involved in auxin biosynthesis. Attempts to identify an auxin receptor by isolating molecules that bind radiolabelled auxin have also been equivocal, partly because a suitable control tissue, one lacking a detectable auxin response, has not been available. The 2,4-D resistant mutants described here, as well as other mutants with defects related to auxin levels or auxin response should help to identify these proteins. For example in vitro binding of 2,4-D in extracts prepared from wildtype and mutant tissue may help to identify an auxin receptor. In addition, techniques currently being developed in several laboratories (Meyerowitz and Pruitt 1985) should make it possible to clone any gene in *Arabidopsis* that can be identified by mutation (Estelle and Somerville 1986). Once cloned, the *Axr-1* gene can be used to identify the protein product and this in turn should facilitate elucidation of the cellular function.

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