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A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid

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Summary. We have screened a large population of M_2 seeds of *Arabidopsis thaliana* for plants which are resistant to exogenously applied indole-acetic acid (IAA). One of the resistant lines identified in this screen carries a dominant mutation which we have named *axr2*. Linkage analysis indicates that the *axr2* gene lies on chromosome 3. Plants carrying the *axr2* mutation are severe dwarfs and display defects in growth orientation of both the shoot and root suggesting that the mutation affects some aspect of gravitropic growth. In addition, the roots of *axr2* plants lack root hairs. Growth inhibition experiments indicate that the roots of *axr2* plants are resistant to ethylene and abscisic acid as well as auxin.

Key words: Plant hormone – Mutant – *Arabidopsis* – Auxin – Plant development

Introduction

During the last 50 years, numerous investigators have described the effects of exogenously applied plant hormones on a variety of plants and plant tissues. These studies have established a role for hormones in many aspects of plant growth and development, including germination, stem elongation, xylem differentiation, gravitropism, abscission and senescence (see Davies 1987 for reviews). Although the molecular details of hormone action remain largely unknown, it is believed that each hormone acts by first binding to a specific protein receptor. The strongest support for this model comes from the study of the plant hormone indole-3-acetic acid (IAA) or auxin. A putative auxin receptor from maize has been purified to homogeneity and a gene encoding this protein has been isolated (Hesse et al. 1989; Inohara et al. 1989). In addition, experiments with tobacco protoplasts suggest that at least one species of receptor is located on the plasma membrane and that an early biochemical response to auxin treatment is stimulation of a plasma membrane ATPase (Barbier-Brygoo et al. 1989). Receptors for the other growth hormones have not been unequivocally identified and nothing is known about the proteins involved in their respective signal transduction pathways.

In animal and fungal systems, the isolation of hormone-insensitive mutants has been an effective way of studying hormone action. For example, yeast mutants insensitive to mating pheromones have been used to identify the pheromone receptors (Hartwell 1980) as well as components of a G-protein involved in transduction of the pheromone signal (Nakafuku et al. 1987; Dietzel and Kurjan 1987; Whiteway et al. 1989). Several groups have now adopted a similar strategy for the study of plant hormone action, and hormone-resistant mutants have been identified in a number of species (King 1988). In *Arabidopsis thaliana*, mutants have been isolated which are resistant to ethylene (Bleecker et al. 1988), abscisic acid (ABA) (Koornneef et al. 1984), gibberellic acid (GA) (Koornneef et al. 1985) and auxin (Maher and Martindale 1980; Estelle and Somerville 1987). The functions of the genes identified in this way have not been determined for any of these mutants. However, the generation of restriction fragment length polymorphism (RFLP) maps in *Arabidopsis* (Chang et al. 1988; Nam et al. 1989) as well as the development of techniques for cloning large segments of *Arabidopsis* DNA into yeast artificial chromosome (YAC) vectors (Guzman and Ecker 1988) should permit the isolation and molecular characterization of the locitor hormone resistance (Meyerowitz 1987).

We are interested in using auxin-resistant mutants of *Arabidopsis* to study the mechanism of auxin action. A number of resistant mutants (*aux1*, *dwf* and *axr1*) have already been isolated by screening for resistance to the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) (Maher and Martindale 1980; Estelle and Somerville 1987). In this report we present the results of a screen for IAA-resistant mutants. One of the resistant

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lines recovered carries a new dominant mutation that we have called *axr2*.

Materials and methods

Plant material. Arabidopsis plants were grown at 23° C on a commercially available peat-lite mixture such as Metro-Mix™, with continuous illumination supplied at an intensity of 85–105 $\mu E/m^2$ per second. Every 2 weeks plants were given 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 $CoCl_2$. For certain experiments, plants were grown under sterile conditions on petri plates. Seeds were surface sterilized for 20 min in 30% (v/v) bleach and 0.01% Triton-X-100 and then placed on petri plates containing the nutrient solution mentioned above, supplemented with 8 g/l agar and 10 g/l sucrose (minimal medium). Hormones were added to the media after autoclaving. Sterile plants were grown at 22° C to 24° C with a 16 h light cycle at a light intensity of 20 to 60 $\mu E/m^2$ per second. All plants were Columbia ecotype unless stated otherwise. The lines used for mapping studies were obtained from Dr. Chris Somerville (Michigan State University).

Mutagenesis. Approximately 25000 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 M_1 seed was sown at a density of approximately $1/cm^2$. The resulting M_2 seed was bulk harvested in a single lot. Three independent M_2 populations were prepared in this way.

Determination of auxin, ethylene and ABA sensitivity. To assay for auxin, ethylene and ABA sensitivity, seedlings were germinated and grown on petri plates containing minimal medium. Plates were oriented vertically so that the roots would grow along the agar surface. After 5 days, seedlings were transferred to minimal medium supplemented with various concentrations of either IAA, 2,4-D or ABA. Ethylene sensitivity was measured by transferring seedlings to minimal medium and then placing the plates in air-tight jars containing various levels of ethylene. For all experiments, the root tips of the transferred seedlings were placed on a line marked on the plate. Three days later, the amount of root growth after transfer was measured and root growth inhibition at each hormone concentration was determined by comparing with growth on minimal medium. Temperature and light regimes were those described for sterile plants. During the ethylene assay, ethylene levels were measured daily by gas chromatography (Kende and Hanson 1976).

Linkage studies. The *axr2* mutation was mapped by crossing homozygous *axr2* plants to a mapping line carrying a visible marker on each chromosome. F_1 plants were allowed to self and the F_2 progeny were scored for segregation of *axr2* and the visible markers. The results were analyzed using the Linkage-1 computer program (Suiter et al. 1983). The degree of linkage between *axr2* and RFLPs on chromosome 3 (Chang et al. 1988) was determined by crossing *axr2* plants (ecotype Columbia) to wild-type plants (ecotype Niederzenz). Seeds from individual F_2 plants from this cross were collected in order to establish F_3 families. The genotype of each F_2 plant was determined by examining F_3 families for segregation of the *axr2* phenotype. In order to score the RFLP genotype, DNA was isolated from each F_3 family using the procedure of Dellaporta et al. (1983) and digested with the appropriate restriction enzyme. The digested DNA was run on an agarose gel and blotted onto Hybond-N™ (Amersham) membranes (Maniatis et al. 1982). Hybridization probes were prepared by labeling DNA with ^{32}P using the random priming method (Feinberg and Vogelstein 1983). The segregation of RFLPs was scored in the F_3 families and linkage determined using the Linkage-1 program.

Results

Isolation of IAA-resistant mutants

When wild-type *Arabidopsis* seeds are placed on minimal medium containing 5×10^{-5} M IAA, the seeds germinate and the cotyledons expand, but the roots fail to elongate. In order to isolate IAA-resistant mutants, M_2 *Arabidopsis* seeds were spread onto minimal medium supplemented with 5×10^{-5} M IAA at a density of 5000 seeds per plate. After germination, the plates were examined daily under a dissecting microscope to identify M_2 seedlings with elongating roots.

A total of 250000 M_2 seeds, from three distinct M_2 populations (designated M_2-1 through 3), was screened for IAA-resistant mutants. Twenty-seven IAA-resistant lines were isolated in these screens. Table 1 shows a summary of genetic studies performed on these mutants. Twenty resistant lines carry recessive mutations at the *aux1* locus, previously defined by Maher and Martindale (1980), and one line carries a recessive allele of the *axr1* locus, originally identified by Estelle and Somerville (1987). Five of the mutants have not been assigned to a locus (uncharacterized mutants). The last mutant has a unique phenotype (see below) suggesting that it may represent a new class of auxin-resistant mutant. This mutant has been named *auxin-resistant-2* (*axr2*) and is the subject of the remainder of this report.

Table 1. Mutants isolated on 5×10^{-5} M indole-3-acetic acid (IAA)[a]

Locus	M_2-1	M_2-2	M_2-3
aux1	11	8	1
axr1	0	1	0
axr2	1	0	0
Uncharacterized mutants	2	1	2

[a] 100000 seeds were screened from M_2-1; 100000 seeds were screened from M_2-2 and 50000 seeds were screened from M_2-3

Table 2. Genetic segregation of 2,4-dichlorophenoxyacetic acid (2,4-D) resistance in *axr2* plants

Cross	Number of plants		
	Resistant	Sensitive	χ^{2a}
axr2 × wild type F_1	140	0	–
axr2 × wild type F_2	130	42	0.03*

* $P > 0.5$

[a] χ^2 was calculated based on an expected ratio of 3 resistant to 1 sensitive

Genetic analysis of axr2

To determine the genetic basis for auxin resistance in the *axr2* mutant, homozygous *axr2* plants were crossed to wild-type plants and the resulting F_1 seeds were scored for segregation of the auxin-resistant phenotype. Resistance to auxin was determined by plating the seeds onto minimal medium supplemented with 10^{-7} M 2,4-D. The synthetic auxin 2,4-D was used instead of the more labile IAA since *axr2* plants also display resistance to 2,4-D. The results of the F_1 and F_2 analysis are shown in Table 2. All of the 140 F_1 plants scored were resistant to 2,4-D and also had the *axr2* morphological phenotype. Several F_1 plants were transplanted to pots and allowed to self to produce an F_2 population. The segregation ratio of auxin resistance to auxin sensitivity in the F_2 was 3:1. In all cases, auxin resistance co-segregated with the *axr2* morphological phenotype. The *axr2* mutant has now been backcrossed to wild-type seven times and the morphological phenotype has never been observed to segregate away from hormone resistance. The results of the F_1 and F_2 analysis indicate that auxin resistance and the co-segregating morphological phenotype of the *axr2* mutant are caused by a single dominant mutation.

Map position of axr2

In order to determine the chromosomal location of *axr2*, homozygous mutant plants were crossed to several multiply marked lines. Examination of 774 F_2 plants indicated that the *axr2* mutation lies 12.5 ± 2.6 centimorgans from *glabrous-1* (*gl-1*) on chromosome 3. To position the *axr2* gene more accurately on chromosome 3, the degree of linkage between *axr2* and the RFLP markers 249 and 105 (Chang et al. 1988) was determined. Segregation of RFLP 105 was scored in 142 F_3 families and RFLP 249 was scored in 88 F_3 families. The results of visible marker and RFLP mapping are shown in Fig. 1. The combined mapping data indicate that *axr2* lies on chromosome 3, at a site near RFLP 255 (Chang et al. 1988).

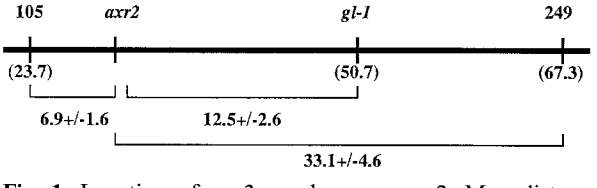

Fig. 1. Location of *axr2* on chromosome 2. Map distances are shown in centimorgans. *Numbers* in *parentheses* are from Chang et al. (1988)

Morphological phenotype of axr2 *mutants*

Mutant plants are dark green, vigorous, and have normal fertility. However, striking differences can be observed between wild-type and *axr2* plants in the roots, rosettes and inflorescences. The morphology of hetero-

Fig. 2A and B. Morphology of wild type and mutant plants. **A** Wild type (left) and *axr2*/*axr2* (right) rosettes 24 days after sowing. **B** Wild type (left) and *axr2*/*axr2* plants 6 weeks after sowing

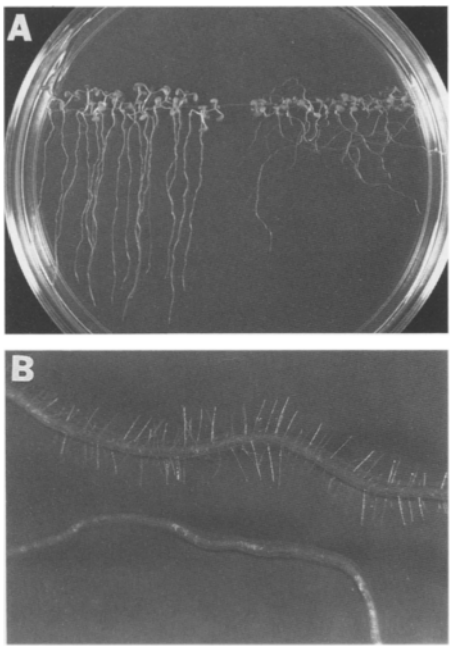

Fig. 3A and B. Wild type and mutant seedling roots. **A** Root gravitropism in wild type (left) and *axr2/axr2* (right) seedling roots. Seedlings were grown on vertically oriented agar plates and photographed after 6 days. **B** Morphology of wild type (upper) and *axr2/axr2* (lower) seedling roots

zygous *axr2* plants is indistinguishable from that of homozygous *axr2* plants, with the exception of the rate of root growth (see below). Figure 2A illustrates wild-type and *axr2/axr2* plants at the rosette stage. Mutant leaves are smaller, rounder and have shorter petioles than wild-type leaves. Inflorescences of *axr2* plants have a morphology very different from that of wild-type plants (Fig. 2B). While both wild-type and mutant plants have approximately the same number of nodes, the internode length is considerably shorter in the mutant plants. As a result, *axr2* plants have much shorter inflorescences. The height of the main inflorescence of 7 week old wild-type plants ranges from 40–60 cm while the main inflorescence of *axr2/axr2* and *axr2/+* plants is only 8–10 cm. In addition, the stems of mutant plants are not oriented properly. Mutant inflorescences are curved and twisted and frequently grow back toward the soil suggesting a defect in gravitropism.

There are several differences between the roots of wild-type and *axr2* plants. The roots of homozygous mutant plants have a significantly slower growth rate than either heterozygous or wild-type plants. In addition, wild-type roots grown vertically on minimal medium have numerous root hairs while *axr2/axr2* plants almost completely lack root hairs (Fig. 3B). Heterozygous mutant plants also lack root hairs (data not shown). It is interesting to note that both *axr2/axr2* and *axr2/+* roots have root hairs when grown on high concentrations of auxin (data not shown) suggesting that absence of root hairs in the mutant is due to a reduction in auxin sensitivity.

Finally, the roots of *axr2* plants do not respond normally to gravity. Figure 3A shows the behavior of wild-type, *axr2/axr2* and *axr2/+* roots when they are grown on vertically oriented agar plates. Wild-type roots grow straight down, while *axr2/axr2* and *axr2/+* roots show an altered gravitropic response. Initially the direction of root growth in the mutant depends on the orientation of the seed on the agar. Several days after germination the mutant roots begin to grow downward but in a very erratic fashion.

Analysis of hormone resistance

To determine the level of auxin resistance in wild-type and mutant plants, inhibition of root growth was measured at various concentrations of auxin. Figure 4 shows the dose response curve for wild-type and *axr2/axr2* plants grown on 2,4-D and IAA. The results indicate that *axr2* plants are resistant to both IAA and 2,4-D. The concentration of IAA which causes 50% inhibition of root growth is 3×10^{-8} M for wild type and 2×10^{-6} M for *axr2/axr2*. On 2,4-D, 50% inhibition of root growth occurs at a concentration of 1.9×10^{-8} M for wild type and 2.1×10^{-7} M for *axr2/axr2*. Thus, *axr2* mutants are 10-fold more resistant to 2,4-D than wild-type plants and 65-fold more resistant to IAA than wild-type. The dose response curve of *axr2/+* plants is almost identical to that of *axr2/axr2* plants on IAA and 2,4-D (data not shown). Homozygous *axr2* mutants were also tested for resistance to naphthalene acetic acid (NAA) and the tryptophan analog α-methyl tryptophan. The *axr2/axr2* mutants are 10-fold more resistant to NAA than wild type plants but show no resistance to α-methyl tryptophan (data not shown).

Resistance of *axr2* mutants to the plant hormones ethylene and ABA was also analyzed. The effect of ethyl-

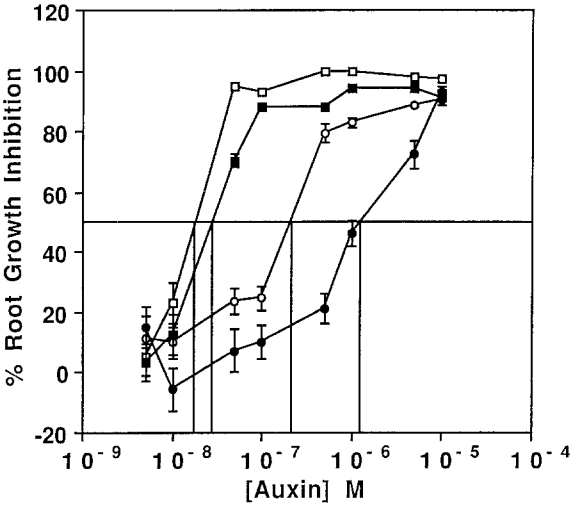

Fig. 4. Dose-response curve for wild type and *axr2/axr2* seedling roots on IAA and 2,4-D. Inhibition of root growth by auxin is expressed relative to growth on non-supplemented medium. *Filled symbols* represent plants grown on IAA, (■) wild type and (●) *axr2/axr2*, and *open symbols* represent plants grown on 2,4-D, (□) wild type and (○) *axr2/axr2*. Each value represents the mean of measurement for at least ten plants. Error bars indicate the standard error. *Lines* are drawn to indicate the hormone concentrations which result in 50% inhibition of root growth

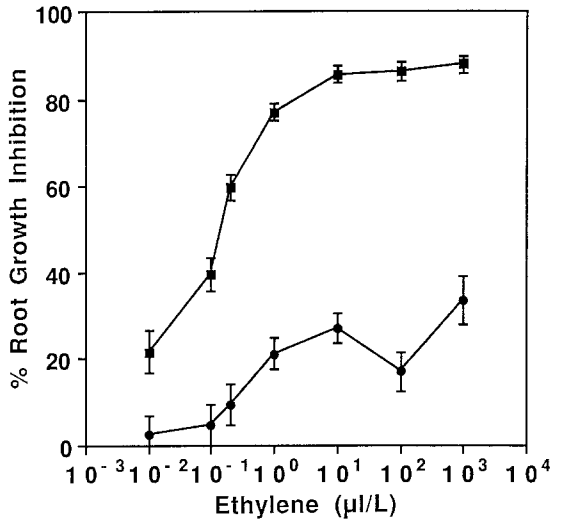

Fig. 5. Dose-response curve for wild-type and *axr2/axr2* seedling roots treated with ethylene. Inhibition of root growth by ethylene is expressed relative to growth of untreated seedlings. (■), wild type plants; (●), *axr2/axr2* plants. Each value represents the mean of ten plants. Error bars indicate the standard error

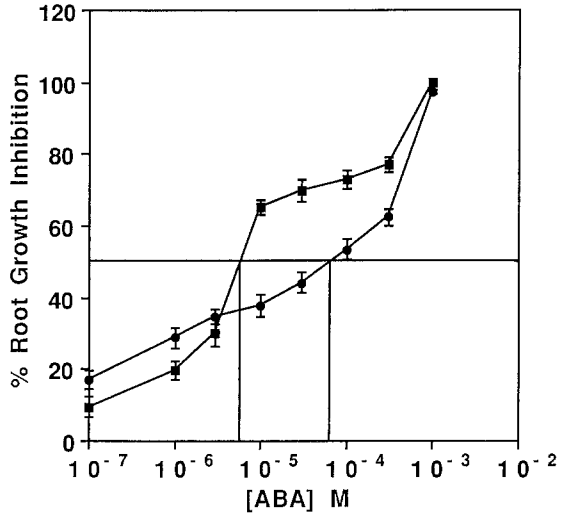

Fig. 6. Dose-response curve for wild type and *axr2/axr2* seedling roots on abscisic acid (ABA). Inhibition of root growth by ABA is expressed relative to growth of untreated seedlings. (■), wild type plants; (●), *axr2/axr2* plants. Each value represents the mean of ten plants. Error bars indicate the standard error. *Lines* are drawn to indicate the hormone concentrations which result in 50% inhibition of root growth

ene on root growth of wild type and *axr2* plants is shown in Fig. 5. The shapes of the two dose response curves are very similar. However, inhibition of root growth in the mutant does not exceed 30%, while the maximum level of inhibition in the wild type is approximately 85%. Figure 6 shows the effect of ABA on root growth of wild type and mutant seeds. In this experiment 50% inhibition of root growth occurred at an ABA concentration of 6.0×10^{-6} M for wild type seeds and 6.0×10^{-7} M for *axr2* seeds. Thus, in addition to conferring resistance to the auxins, IAA, NAA and 2,4-D, the *axr2* mutation also produces significant resistance to ethylene and ABA.

Discussion

In previous studies, auxin-resistant mutants of *Arabidopsis* have been isolated by screening for plants that are resistant to the synthetic auxin 2,4-D (Maher and Martindale 1980; Estelle and Somerville 1987). We reasoned that by screening for resistance to the natural auxin IAA, we might recover a different spectrum of mutations. After screening a total of 250000 M_2 seedlings we have isolated 27 lines which are resistant to IAA. Complementation analysis indicates that in 20 of these lines, the mutation conferring resistance is an allele of the *aux1* mutation on chromosome 2 (Maher and Martindale 1980). In another line, resistance is due to a mutation at the *axr1* locus on chromosome 1 (Estelle and Somerville 1987). Of the remaining resistant lines, 5 are uncharacterized and 1 carries a dominant mutation which we have called *axr2*. The roots of *axr2* seedlings are resistant to auxin, ethylene and ABA. Mapping studies place the *axr2* mutation on chromosome 3, indicating that it is not allelic to the previously identified auxin-resistant mutations, *axr1* or *aux1*. An additional dominant auxin-resistant mutant, designated *dwf*, has been isolated and partially characterized (Mirza and Maher 1985). This mutant lies approximately 36 map units from *glabrous* on chromosome 3 (J. Mirza and E. Maher, personal communication) indicating that *dwf* and *axr2* are distinct genes.

The *axr2* mutation has dramatic affects on the development of roots, leaves and stems. It is not known if the developmental phenotype is a consequence of hormone resistance. However, many of the affected growth processes, such as root gravitropism and stem elongation, are known to be hormonally regulated (Jacobs and Ray 1976; Shen-Miller 1973) suggesting that a defect in hormone action is responsible for some aspects of the mutant phenotype. Alternatively, the mutant phenotype could be a consequence of decreased solute uptake due to the absence of root hairs. This hypothesis does not seem likely because *axr2* plants are healthy and show no signs of nutrient stress. In addition, mutants of *Arabidopsis* with severely reduced root hairs do not display any significant growth abnormalities (J. Schiefelbein and C. Somerville, personal communication).

There are a number of ways of explaining the multiple hormone resistance exhibited by the *axr2* mutant. Because auxin is known to induce ethylene biosynthesis (Jones and Kende 1979), and ethylene will inhibit root growth, it is possible that auxin resistance is a consequence of ethylene resistance. However, experiments with the ethylene insensitive mutant *etr* (Bleecker et al. 1988), suggest that this hypothesis is not correct. Ethylene inhibition of root growth does not occur in the *etr* mutant, even at very high ethylene concentrations (F. Pickett and M. Estelle, unpublished). Hence if auxin inhibition of root growth is partly due to auxin-induced ethylene, the *etr* mutant should be significantly resistant

to auxin. Our studies show that the *etr* mutant is only slightly resistant to auxin indicating that auxin resistance in the *axr2* mutant is not solely a consequence of ethylene resistance (F. Pickett and M. Estelle, unpublished).

Another explanation for multiple hormone resistance is a general decrease in uptake of small molecules. We feel that this explanation is unlikely because ethylene is known to diffuse freely through the plasma membrane. In addition, mutant plants are not resistant to the tryptophan analog α-methyl tryptophan (data not shown).

It is also possible that the *axr2* mutation disrupts a function which is required for normal response to all three hormones. For example, the mutant may be defective in some aspect of signal transduction that is common to the different hormones. In animal systems, intracellular messengers such as inositol triphosphate (IP_3) and Ca^{++} participate in transduction of many different extracellular signals (Sekar and Hokin 1986). In plants, similar roles for these two second messengers have been proposed (Guern 1987; Poovaiah et al. 1987). Several studies suggest that auxin, and perhaps other hormones, act by stimulating release of IP_3 from phosphatidyl inositol (Poovaiah et al. 1987). By analogy to animal systems, IP_3 may then cause an increase in intracellular Ca^{++} concentration and the consequent activation of a variety of Ca^{++} dependent enzymes. The validity of this particular model for plant hormone action remains to be determined. However if the *axr2* mutation does affect the action of a second messenger such as calcium, the result might be resistance to several hormones.

It is interesting to note that the *axr2* mutant displays a significantly higher level of resistance to the natural auxin IAA, than it does to either 2,4-D or 1-NAA. One explanation for this difference is that the *axr2* gene product binds directly to auxin. The gene may code for a hormone receptor or a protein involved in transport or metabolism of IAA. The roots of mutant plants could be more resistant to IAA because this auxin does not bind to the *axr2* gene product as efficiently as do 2,4-D or 1-NAA. If this is the case, resistance to ethylene and ABA could be due to cellular changes which occur as a result of the auxin defect.

Multiple hormone resistance is not unique to *axr2* plants. We have recently found that the recessive *aux1* mutant of *Arabidopsis*, originally identified in a screen for auxin resistance (Maher and Martindale 1980) is also resistant to ethylene (F. Pickett and M. Estelle, unpublished). In tobacco the *iba1* mutant appears to be resistant to auxin, ABA, and the GA biosynthetic inhibitor, paclobutrazol (Bitoun et al. 1990). We expect that the study of this class of mutants will provide insight into how plant hormones act together to regulate plant development.

In this report we describe the characterization of a new hormone-resistant mutant of *Arabidopsis*. This mutant is novel because it is resistant to several plant hormones and has a dramatic developmental phenotype. Linkage analysis places the gene in close proximity to a number of RFLPs (Chang et al. 1988; Nam et al. 1989). In order to isolate the *axr2* gene we intend to use these molecular markers as starting points for a chromosome walk with either YAC or cosmid clones. To identify cosmids carrying the *axr2* gene we plan to use the root phenotype of *axr2* plants. In experiments with *Agrobacterium rhizogenes* we have found that hairy root tumors generated on *axr2* plants lack root hairs. By co-transformation of wild type plants with cosmid clones carrying fragments of *axr2* DNA and an *A. rhizogenes* Ri T-DNA (Van Sluys et al. 1987) we hope to confer the dominant root hairless phenotype on a tumor growing on a wild-type plant. The advantage of this approach compared with other procedures involving whole plant transformation is that it does not require time-consuming regeneration of transgenic plants and should permit a more facile isolation of the mutant gene.

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