The aux1 Mutation of Arabidopsis Confers Both Auxin and Ethylene Resistance1,2

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ABSTRACT

Mutagenized populations of Arabidopsis thaliana seedlings were screened for plants capable of root growth on inhibitory concentrations of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid. Four of the mutant lines recovered from this screen display a defect in root gravitropism as well as hormone resistance. The aerial portions of these plants are similar to wild-type in appearance. Genetic analysis of these four mutants demonstrated that hormone resistance segregated as a recessive trait and that all four mutations were alleles of the auxin-resistant mutation aux1 [Maher HP, Martindale SJU (1980) Biochem Genet 18: 1041–1053]. These new mutants have been designated aux1–7, 1–12, 1–15, and 1–19. The sensitivity of wild-type and aux1–7 roots to indole-3-acetic acid, 2,4-dichlorophenoxyacetic acid, and ethylene was determined. The results of these assays show that aux1–7 plants require a 12-fold (indole-3-acetic acid) or 18-fold (2,4-dichlorophenoxyacetic acid) higher concentration of auxin than wild-type for a 50% inhibition of root growth. In addition, ethylene inhibition of root growth in aux1–7 plants is approximately 30% that of wild-type at saturating ethylene concentrations. These results indicate that aux1 plants are resistant to both auxin and ethylene. We have also determined the effect of ethylene treatment on chlorophyll loss and peroxidase activity in the leaves of aux1 and wild-type plants. No difference between mutant and wild-type plants was observed in these experiments, indicating that hormone resistance in aux1 plants may be limited to root growth. Our studies suggest that the AUX1 gene may have a specific function in the hormonal regulation of gravitropism.

The results of many physiological studies have shown that most plant developmental processes are regulated by several hormones acting in concert (14). Many specific examples of interactions between hormones have now been documented. Among the best-characterized hormone interactions are those involving auxin and ethylene. Early studies demonstrated that auxin treatment induced ethylene biosynthesis (4, 20). Later it was shown that this stimulation was due to an increase in the level of ACC,1 the immediate biosynthetic precursor of ethylene (9). This discovery led to the realization that some auxin responses may be due to the action of auxin-induced ethylene. Other types of interactions have also been described. In the case of leaf abscission, ethylene appears to promote abscission by inhibiting transport of auxin from the leaf through the abscission zone (1). Inhibition of auxin transport by ethylene has also been demonstrated in stem segments, suggesting that this phenomenon may have more general significance (23). The mechanism of hormone interaction has not been determined for either of these examples.

To identify the genes and proteins involved in hormone action, we and others have screened for mutants that are resistant to exogenously applied hormone (11). In Arabidopsis thaliana, mutants have been isolated that are resistant to auxin, ethylene, abscisic acid, and gibberellic acid (3, 7, 8, 12, 13, 16). It is believed that the molecular characterization of these mutants will be facilitated by the map-based cloning strategies that are feasible in Arabidopsis (17). In this report we describe a screen for new ethylene-resistant mutants of Arabidopsis. One class of mutants recovered in this screen carries a mutation in the previously identified auxin-resistant locus, aux1 (16). Our analysis shows that a single lesion in the AUX1 gene confers both auxin and ethylene resistance to the roots of seedlings.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All plant lines used were derived from the wild-type Columbia ecotype of Arabidopsis thaliana. Plants were grown in a support medium consisting of Metromix medium saturated with a mineral salts solution containing 5 mM KNO3, 2.5 mM KH2PO4 (pH 5.6), 2 mM MgSO4, 2 mM Ca(NO3)2, 50 μM CuSO4, 1 μM ZnSO4, 0.2 μM NaMoO4, 10 mM NaCl, and 0.01 μM CoCl2. Growing conditions were 21° to 24°C with continuous illumination at an intensity of 75 to 110 μE/m2/s. Plants grown under sterile conditions were surface-sterilized for 15 min in a 30% v/v bleach and 0.02% Triton X-100 (Sigma) solution and placed in Petri dishes containing the nutrient solution described above supplemented with 7 g/L agar and 10 g/L sucrose. Hormones were added after the medium was autoclaved. Plants grown under sterile conditions were incubated at 21° to 24°C with 16 h/d of illumination at an intensity of 55 to 60 μE/m2/s. All mutant lines used for physiological experiments were backcrossed twice to wild-type Columbia strains. The aux1 mutant was obtained from P. Maher (Edinburgh). We have renamed this mutant aux1-1 to conform to

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2 Genetic nomenclature: According to guidelines established at the Third International Arabidopsis Meeting, Michigan State University, April 1987, wild-type gene symbols are capitalized (example, AUX1) and mutants are represented with lower case letters (example, aux1).

3 Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; IAA, indole-3-acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.
AUXIN AND ETHYLENE RESISTANCE IN ARABIDOPSIS

Mutagenesis

Approximately 50,000 seeds were mutagenized with ethyl methyl sulfonate (Sigma) as described previously (7). This M₁ (first mutagenized generation) seed was sown at a density of approximately one plant per cm². At maturity, the progeny of these plants were harvested to generate an M₂ population.

Determination of Auxin and Ethylene Sensitivity in the Root and Hypocotyl of aux1 and Wild-Type Plants

Sterile wild-type and aux1 seedlings were grown as described above. To measure the sensitivity of roots to the auxins IAA and 2,4-D, 5-d-old seedlings were transferred to medium supplemented with various concentrations of either IAA or 2,4-D. The position of the root tip of each seedling was marked on the plate. The amount of new root growth was measured after 3 d. Percent inhibition of root growth was determined relative to growth of roots on medium not supplemented with auxin.

To measure ethylene sensitivity in roots, 5-d-old seedlings were placed in sealed jars containing various levels of ethylene. The ethylene concentration in each jar was measured using a Varian 3300 gas chromatograph equipped with a flame ionization detector (10). The jars were opened daily and after resealing were recharged with ethylene. After 3 d, root growth of treated seedlings was measured and percent inhibition of root growth determined relative to the root elongation of plants not exposed to ethylene.

The effect of ethylene on hypocotyl growth was determined in etiolated plants. Surface sterilized wild-type and aux1 seedlings were incubated on agar at 4°C for 7 d, exposed to white light for 40 min to promote uniform germination, and then placed into sealed jars charged with various levels of ethylene. All jars were placed in a dark chamber and seedlings were allowed to grow for 3 d after germination. Hypocotyls were then measured and hypocotyl elongation was expressed as the average percent inhibition of hypocotyl growth of ethylene-treated seedlings compared with hypocotyl growth in untreated seedlings.

Measurement of Ethylene-Induced Peroxidase Activity

To assay ethylene induction of peroxidase activity, wild-type and aux1 Arabidopsis plants were grown in pots for 18 d and then split into two groups. One group was placed into a flow-through chamber maintained at 20.0 μL/L ethylene. The second group was placed into a chamber containing air. After 48 h, peroxidase activity was measured in rosettes of both treated and untreated plants. Plants were homogenized in 0.05 M sodium acetate (pH 6.0) at a concentration of 1 g fresh weight to 3 mL of buffer. The crude homogenate was centrifuged for 15 min and the supernatant assayed for peroxidase activity as described previously (24).

Ethylene-Induced Chl Loss From Excised Leaves

Ethylene-induced Chl loss was measured in leaves from 18-d-old plants. The three largest rosette leaves from seven wild-type and seven mutant plants were removed. Three leaves were immediately assayed for Chl content as described previously (15). The remaining leaves were floated on Milipore filtered water in Petri dishes and then divided into two groups, one of which was placed into a chamber maintained at 5.5 μL/L ethylene while the other was placed into a chamber containing only air. Chl content was determined at 1, 2, and 3 d after excision in groups of three leaves chosen at random from the treated and untreated groups. Data are expressed as percent Chl retained relative to Chl content in leaves on the day of excision.

RESULTS

Isolation of ACC Resistant Mutants

A 1 × 10⁻⁴ M concentration of the ethylene biosynthetic precursor, ACC, inhibits the growth of the roots of wild-type seedlings. This inhibition is thought to be due to an endogenous enzyme that converts ACC into ethylene (26). By screening for mutants that are resistant to the inhibition of root growth by ACC, we hoped to recover mutations that either prevented the normal conversion of ACC to ethylene or affected the response of the plant to ethylene. A total of 10,000 M₂ seedlings from a single M₁ population were screened on medium supplemented with 1 × 10⁻⁴ M ACC. Nine seedlings were recovered, which elongated roots on this medium. These seedlings were transplanted to soil and allowed to self, and M₃ lines were established. All of the M₃ lines were resistant to both ACC and ethylene.

Genetic Analysis

Four of the mutants recovered in our screen for ACC resistance have similar phenotypes. We initially designated these lines accr (for ACC-resistant) 7, 12, 15, and 19. The rosettes and inflorescences of plants from these lines are similar to wild type in morphology, but their roots display a defect in gravitropism when grown on agar plates (data not shown). To determine the genetic basis for ACC-resistance in these four lines, plants of each mutant line were crossed to wild-type, and ACC-resistance was scored in the F₁ and F₂ generations by germinating seed on agar medium containing 1 × 10⁻⁴ M ACC. Segregation data for two of the mutant lines are displayed in Table I. These data show that ACC resistance segregates as a recessive trait in these lines. Similar re-

Table I. Genetic Segregation of ACC Resistance in Mutant Lines accr7 and accr15

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<tr>
<th>Cross</th>
<th>No. of Plants</th>
<th>χ²</th>
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<tr>
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<tr>
<td>accr7 × wild-type F₁</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>accr7 × wild-type F₂</td>
<td>392</td>
<td>1168</td>
</tr>
<tr>
<td>accr15 × wild-type F₁</td>
<td>61</td>
<td>0</td>
</tr>
<tr>
<td>accr15 × wild-type F₂</td>
<td>231</td>
<td>74</td>
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⁹⁺ χ² calculated based on an expected ratio of three sensitive to one resistant.

⁸⁻ P > 0.8 in both instances.
results were obtained with the other lines. Analysis of the other five phenotypically distinct ACC-resistant mutations is in progress.

Complementation analysis was performed between the four phenotypically similar ACC-resistant lines. The F1 progeny of crosses between each of the four lines were all resistant to 1 × 10^-4 M ACC. Greater than 200 F2 progeny from each cross were tested on ACC to determine if second site noncomplementation was responsible for the observed ACC resistance in the F1. All F2 plants were ACC-resistant, indicating that the four mutant lines carry mutations in the same locus.

The aux1 Mutation Confers Resistance to Ethylene and Auxin

When accr seedlings are grown on vertically oriented agar plates, the root growth occurs in a random direction. Because this root phenotype is similar to that reported for the recessive auxin-resistant mutation aux1 (16), we tested for complementation between the ACC-resistant lines and the original aux1 allele (renamed aux1-1). The F1 progeny resulting from these crosses were all resistant to ACC, indicating that the ACC-resistant mutations are alleles of the AUX1 gene. The original isolates acc7, 12, 15, and 19 have been redesignated aux1-7, aux1-12, aux1-15, and aux1-19, respectively. We had previously isolated 12 auxin-resistant lines that displayed the same phenotype as the aux1-1 mutation (25). Complementation analysis showed that these mutants also were alleles of the AUX1 gene.

The results of the complementation analyses show that recessive mutations at the AUX1 locus confer both auxin and ethylene resistance to the roots of seedlings. Dose-response assays were performed with increasing concentrations of IAA, 2,4-D, or ethylene to compare the hormone sensitivities of wild-type and aux1-7 roots. The roots of the aux1 mutants are highly resistant to ethylene and the two auxins by this assay. Representative data for the mutant aux1-7 is shown in Figures 1 and 2. In the wild-type strain, ethylene levels greater than 10 μL/L inhibit root growth by 85%, while the response of aux1 plants does not exceed 30% inhibition of root growth, even at an ethylene concentration of 1000 μL/L (Fig. 1).

We found that aux1-7 roots were also resistant to both auxins (Fig. 2). The concentration of IAA required to produce a 50% inhibition of root growth was 12-fold higher for the mutant than for wild-type plants. Similarly, an 18-fold higher concentration of 2,4-D was required to achieve 50% root growth inhibition in aux1-7 plants.

To determine if all of our aux1 alleles and the original aux1-1 allele were resistant to the inhibition of root growth by auxin and ethylene, we treated aux1 seedlings with 5 μL/L ethylene or 1.0 μM 2,4-D. All of the aux1 isolates we have tested were able to grow roots when treated with either ethylene or 2,4-D at these concentrations.

Ethylene Resistance in aux1 Seedlings Is Specific to the Roots

To determine if hormone resistance was expressed in all tissues of aux1 plants, we measured ethylene sensitivity in the hypocotyl and the leaves of wild-type and mutant plants. Ethylene inhibited growth of etiolated hypocotyls to the same extent in both genotypes (Fig. 3).

Chl levels in ethylene-treated and untreated leaves were measured over a 3-d period. The time course of Chl loss was similar in wild-type and mutant leaves (Fig. 4). Peroxidase activity was measured in crude extracts from two groups of
aux1 and wild-type plants. One group of each line was treated with 20.0 μL/L ethylene while the other group was left untreated. The ethylene-induced increase in peroxidase activity was the same in aux1 and wild-type plants (Table II).

**DISCUSSION**

The recessive aux1 mutation of Arabidopsis was originally identified by Maher and Martindale (16) in a screen for mutants that could develop roots on medium that contained a high concentration of 2,4-D. In addition to auxin resistance, the aux1 mutation affects the gravitropic response of seedling roots. When aux1 seeds are germinated on a vertically oriented agar plate, the direction of root growth is random. The aerial portions of mutant plants are normal in appearance and growth behavior. We have isolated four mutant lines of Arabidopsis with a gravitropic defect similar to that of the aux1 mutant by screening for resistance to the ethylene biosynthetic precursor ACC. Each of these four lines is homozygous for a recessive aux1 mutation. Our studies show that the aux1 mutation confers resistance to auxin, ACC, and ethylene. Since ACC is rapidly converted to ethylene by plant tissues (26), we believe that ACC resistance in these mutants is a consequence of ethylene resistance.

The only visible effects of the aux1 mutation are a slight increase in root elongation and a disruption of root gravitropism (16, 19). Our experiments show that ethylene resistance is restricted to the root. Together these results suggest that the AUX1 gene functions primarily in the root. The anatomy and ultrastructure of mutant roots is indistinguishable from wild-type (22), indicating that the AUX1 gene is not required for root differentiation and development. Instead, we believe that the AUX1 gene may function specifically in the hormonal regulation of gravitropism.

There are several possible models that might explain how the aux1 mutation causes both auxin and ethylene resistance. For example, the mutant may have a membrane defect, which decreases permeability to a number of molecules, including auxin and ethylene. We believe that this explanation is unlikely because mutant plants display a normal sensitivity to other growth-inhibiting molecules such as ABA and the tryptophan analog α-methyl tryptophan (data not shown). In addition, a defect that alters membrane permeability would be expected to have some effect on the health of the plant. The aux1 plants are completely normal in appearance.

Because auxin treatment stimulates ethylene production, auxin inhibition of root growth may be due in part to auxin-induced ethylene. Thus, an ethylene-resistant mutation may also confer auxin resistance. We have done experiments with the ethylene-insensitive mutant of Arabidopsis, etr (3), which suggest that this is not the case in Arabidopsis. The roots of etr seedlings are completely insensitive to ethylene but display

<table>
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<th>Plant Line</th>
<th>Peroxidase Activity</th>
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<td></td>
<td>Treated</td>
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<tr>
<td>Wild-type</td>
<td>0.057 ± 0.013</td>
</tr>
<tr>
<td>aux1-7</td>
<td>0.067 ± 0.006</td>
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only slight resistance to auxin (data not shown). Recent studies analyzing the effects of inhibitors of ethylene biosynthesis on auxin inhibition of root growth also suggest that auxin-induced ethylene plays at most a small role in the auxin response (6).

It is possible that the mutant is defective in a function required for response to both hormones. This function may be required for signal transduction of the auxin and ethylene signals or it may be directly involved in cell growth. However, the dose-response curves of wild-type and aux1 roots treated with auxin and ethylene suggest that the mutation confers resistance to the two hormones by different mechanisms. The auxin response of aux1 roots requires higher concentrations of auxin compared with wild-type roots but is otherwise normal. In contrast, ethylene sensitivity in the mutant is approximately 30% that of wild-type at saturating ethylene concentrations, indicating that a function required for ethylene response is limiting in aux1 roots. This result could be explained if ethylene sensitivity in roots is regulated by auxin. According to this model, ethylene resistance in aux1 plants is a consequence of reduced auxin sensitivity. We intend to test this hypothesis by determining if low levels of auxin will enhance the sensitivity of aux1 roots to saturating concentrations of ethylene.

Multiple hormone resistance is not unique to the aux1 mutant. The aux2 mutation in Arabidopsis confers resistance to auxin, ethylene, and ABA (25). In tobacco, the ibal mutant is resistant to both auxin and abscisic acid (2). These mutants provide genetic evidence for the integration of hormone signals during plant growth and development, and may be useful in the study of hormone interactions. The aux1 locus has already been mapped to the second chromosome (16). We intend to identify a closely linked restriction fragment length polymorphism marker (5, 21) on chromosome two, and use this marker to initiate a chromosome walk to the aux1 locus. The cloning and molecular characterization of this gene may lead to new insights into the nature of hormone interactions and the role of hormones during gravitropism.

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LITERATURE CITED