Maintenance of shoot growth by endogenous ABA: genetic assessment of the involvement of ethylene suppression

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Abstract

Previous work demonstrated that normal levels of endogenous abscisic acid (ABA) are required to maintain shoot growth in well-watered tomato plants independently of effects of hormone status on plant water balance. The results suggested that the impairment of shoot growth in ABA-deficient mutants is at least partly attributable to increased ethylene production. To assess the extent to which ABA maintains shoot growth by ethylene suppression, the growth of ABA-deficient (aba2-1) and ethylene-insensitive (etr1-1) single- and double-mutants of Arabidopsis was examined. To ensure that the results were independent of effects of hormone status on plant water balance, differential relative humidity regimes were used to achieve similar leaf water potentials in all genotypes and treatments. In aba2-1, shoot growth was substantially inhibited and ethylene evolution was doubled compared with the wild type, consistent with the results for tomato. In the aba2-1 etr1-1 double mutant, in which ABA was equally as deficient as in aba2-1 and shoot growth was shown to be insensitive to ethylene, shoot growth was substantially, although incompletely, restored relative to etr1-1. Treatment with ABA resulted in the complete recovery of shoot growth in aba2-1 relative to the wild type, and also significantly increased the growth of aba2-1 etr1-1 such that total leaf area and shoot fresh weight were not significantly lower than in etr1-1. In addition, ABA treatment of aba2-1 etr1-1 restored the wider leaf morphology phenotype exhibited by etr1-1. The results demonstrate that normal levels of endogenous ABA maintain shoot development, particularly leaf expansion, in well-watered Arabidopsis plants, partly by suppressing ethylene synthesis and partly by another mechanism that is independent of ethylene.

Key words: ABA, aba2-1, abscisic acid, Arabidopsis thaliana, ethylene, etr1-1, shoot growth.

Introduction

Hormonal regulation of plant growth is complex. Interactions among hormones are widespread and counteracting effects of different hormones on a given developmental process are common. Early reports that applied abscisic acid (ABA) can inhibit ethylene production led to speculation that this interaction is involved in the effects of ABA status on plant growth (Wright, 1980; Bradford and Hsiao, 1982), although this topic was not pursued. The findings of several recent studies have renewed interest in the importance of the interaction between ABA and ethylene in plant development. Interactions between these hormones appear to occur at many levels, including both positive and negative reciprocal effects on synthesis (Riov et al., 1990; Ghassemian et al., 2000; Hansen and Grossman, 2000; Hussain et al., 2000; Sharp et al., 2000; Spollen et al., 2000), and both positive and negative interactions between signalling pathways (Beaudoin et al., 2000; Ghassemian et al., 2000; Gazzarrini and McCourt, 2001; Fedoroff, 2002; León and Sheen, 2003). In particular, studies of ethylene suppression by endogenous ABA have led to a reassessment of the role of ABA in shoot growth (Sharp, 2002; Sharp and LeNoble, 2002).
ABA has generally been regarded as an inhibitor of shoot growth (Trewavas and Jones, 1991; Davies, 1995; Munns and Cramer, 1996). This view was based on observations that (a) ABA accumulates to high concentrations in plants experiencing water deficits or other adverse conditions, often correlating with growth inhibition, and (b) applications of ABA usually result in growth inhibition. However, the interpretation of these results is complicated by uncertainty that the effects of applied ABA are predictive of the role of endogenous ABA (Trewavas and Jones, 1991; Sharp et al., 1994). Paradoxically, ABA-deficient mutants are often shorter and have smaller leaves than their corresponding wild types, and their growth can be substantially restored by applying ABA (Imber and Tal, 1970; Bradford, 1983; Quarrie, 1987). Because ABA-deficient mutants are typically wilty (due to high stomatal conductance and reduced root hydraulic conductance) even under well-watered conditions, their reduced shoot growth was attributed to impaired plant water balance (Bradford, 1983; Neill et al., 1986; Nagel et al., 1994; Léon-Kloosterziel et al., 1996a). However, it was also reported that ethylene production was greater in ABA-deficient mutants of tomato (Tal et al., 1979) and Arabidopsis (Rakitina et al., 1994), and that the mutants of tomato exhibited morphological symptoms characteristic of excess ethylene, such as leaf epinasty and adventitious rooting (Tal, 1966; Nagel et al., 1994). Despite these observations, the possibility that ethylene is a cause of shoot growth inhibition in ABA-deficient mutants was not considered until recently.

In a study of the ABA-deficient flacca and notabilis mutants of tomato, it was demonstrated that normal levels of ABA are required to maintain shoot development, particularly leaf expansion, in well-watered plants, independently of effects on water balance (Sharp et al., 2000). This result was achieved by using differential relative humidity (RH) regimes so that the mutants had the same leaf water potentials as the wild type throughout development. Under these conditions the mutants continued to exhibit severely impaired shoot growth together with increased rates of leaf ethylene evolution, leaf epinasty and adventitious rooting. Treatment of flacca with silver thiosulphate to inhibit ethylene action partially restored leaf and stem growth, indicating that the impairment of shoot growth caused by ABA deficiency was at least partly attributable to ethylene. However, toxicity problems associated with the long-term use of chemical inhibitors prevented assessment of the full extent to which ethylene accounted for the inhibition of growth in the mutants.

In this study, the degree to which maintenance of shoot growth by endogenous ABA in well-watered plants involves the suppression of ethylene synthesis and/or signalling has been assessed by examining the growth of ABA-deficient (aba2-1; Léon-Kloosterziel et al., 1996a) and ethylene-insensitive (etr1-1; Bleecker et al., 1988) single- and double-mutants of Arabidopsis. The experimental strategy of equalizing leaf water potentials of all genotypes and treatments was used so that the results were independent of effects of hormone status on plant water balance. The results demonstrate that the maintenance of shoot growth by ABA involves both ethylene suppression and an ethylene-independent function.

Materials and methods

Plant material

The Arabidopsis thaliana L. (Heynh) aba2-1 and etr1-1 mutants are in the Columbia ecotype. Seeds of the wild type and of etr1-1 were obtained from the Arabidopsis Biological Resource Center, The Ohio State University, Columbus, USA. Seeds of aba2-1 were kindly provided by Jan AD Zeevaart (Michigan State University, East Lansing, USA). The aba2-1 mutant is impaired in ABA biosynthesis, being blocked in the conversion of xanthoxin to ABA-aldehyde (Schwartz et al., 1997), and exhibits substantial ABA deficiency and impairment of shoot growth in well-watered plants (Léon-Kloosterziel et al., 1996a). The mutant appears to be specific for ABA biosynthesis; accordingly, as demonstrated in this study, it does not exhibit pleiotropic effects on shoot growth. The dominant etr1-1 mutation inhibits ethylene binding to the ETR1 receptor, conferring ethylene insensitivity (Schaller and Bleecker, 1995). The aba2-1 etr1-1 double mutant was selected in the F2 progeny of crosses between the homozygous parents. Selection of double mutant plants took advantage of two features of the ethylene-insensitive phenotype. First, ethylene-insensitive seedlings do not usually germinate without a cold treatment of several days (Bleecker et al., 1988). It was observed that ABA-deficiency could substitute for these treatments, consistent with the findings of Beaudoin et al. (2000) for ABA- and ethylene-insensitive double mutants of Arabidopsis. Second, ethylene-insensitive seedlings do not show the typical triple response to applied ethylene: shortening and radial swelling of the hypocotyl, inhibition of root elongation, and exaggerated curvature of the apical hook in dark-grown seedlings (Guzman and Ecker, 1990). ABA-deficiency was also tracked by wiltiness of the inflorescence under wild-type growth conditions. Homozygosity was established by testing for lack of segregation for these characteristics in subsequent generations, and further checked by several independent test-crosses. In the selected double mutant line, ABA-deficiency and ethylene-insensitivity of shoot development were confirmed by direct assessment, as described below.

Germination and seedling triple response assays

Non-cold-treated seeds were treated with 1.6% sodium hypochlorite solution for 20 min, rinsed six times with equal volumes of double deionized water, and plated onto Petri plates containing agar plus nutrients. The medium comprised 1.2% agar, 0.5% sucrose, 4 mM KNO₃, 1 mM Ca(NO₃)₂, 0.3 mM MgSO₄, 2 mM KH₂PO₄, 89 mM iron citrate, 10 μM H₃BO₃, 2 μM MnCl₂, 0.77 μM ZnSO₄, 0.31 μM CuSO₄, 0.13 μM MoO₃, and 0.1 μM NiCl₂. To allow gas exchange, the plates were wrapped with one layer of surgical tape (Microtome 3M Company, St Paul, Minnesota, USA). The plates were placed in an air-tight glass container in the dark. The ethylene concentration in the container was maintained at 100 ppm by daily injection of pure ethylene (after briefly opening and closing the container to allow air exchange). Seedling morphology was examined 3–4 d after plating. The germination and triple response characteristics of the wild type and mutant lines used for this study are summarized in Table 1.
Table 1. Germination and triple response characteristics of non-cold treated seeds of wild type, aba2-1, etr1-1 and aba2-1 etr1-1

<table>
<thead>
<tr>
<th>Genotype (number of seed tested)</th>
<th>Germination (%)</th>
<th>Lacking triple response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (109)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>aba2-1 (63)</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>etr1-1 (55)</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>aba2-1 etr1-1 (104)</td>
<td>70</td>
<td>100</td>
</tr>
</tbody>
</table>

Ethylene treatment throughout shoot development

Experiments were conducted in which all genotypes were treated with 1 ppm ethylene for 5 d per week from days 2–23. Seeds were germinated as described below. Pots (in groups of 12) were then placed in 36 l plastic containers with clear Plexiglas lids that were sealed to the containers using petrolatum. Pure ethylene was injected through a septum at the beginning and mid-point of each light period to achieve an ethylene concentration in the container of 1 ppm. Prior to each ethylene injection the lid was removed to allow air exchange (in a fume hood) and then resealed. The lids were left off for the remaining 2 d per week. Control plants were treated similarly except that air was injected instead of ethylene. For this experiment only, the day/night RH regime in the growth chamber was 94/98% for all genotypes; all other conditions were as described below. Plants were harvested on day 23 for measurements of total leaf area and total shoot fresh and dry weights.

Shoot growth under controlled relative humidity regimes

Seeds were treated with 1.6% sodium hypochlorite solution for 20 min, rinsed six times with equal volumes of double deionized water, and sown in 10 cm diameter, 360 cm³ plastic pots (six seeds per pot) filled with a 2:1 (v:v) mixture of Green Formula Growing Mix (Lambert Peat Moss, Inc., Quebec, Canada) and sand which was wetted thoroughly with deionized water. The pots were covered with clear plastic wrap (with slits for aeration) and subjected to a cold treatment of 2 °C for 5 d to break seed dormancy in all genotypes. The pots were then transferred to growth chambers with a day/night temperature of 24/18 °C and a 14 h light photoperiod. The photon flux density at the pot surface was 150 μmol photons m⁻² s⁻¹ photosynthetically active radiation supplied by cool-white fluorescent and incandescent lamps. The plastic wrap was removed on day 1 (emergence was defined as day 0), and seedlings were thinned to one per pot. The day/night RH was 94/98% until day 2 and then varied according to the genotype and treatment as detailed below. Pots were kept well watered by sub-irrigation with deionized water (as needed, depending on the humidity conditions within the chamber). Nutrient solution (Peters Professional 20-10-20 with micronutrients, 0.5 g l⁻¹, Grace-Sierra Horticultural Products Co., Milpitas, California, USA) was supplied by sub-irrigation once during the first week, and then every 3 d (50 ml per pot) throughout the remainder of the 23 d experiments.

Application of ABA

In some experiments, plants of aba2-1 and aba2-1 etr1-1 were sprayed with 1 μM (±)-ABA (Sigma) daily, from emergence. In preliminary experiments, this treatment was determined to result in complete recovery of shoot growth of aba2-1. The solution contained ethanol and Tween 20 at final concentrations of 0.1% and 0.01% (v/v), respectively. Shoots were sprayed to the drip point 30 min before the end of the photoperiod. Spray control (sc) plants were sprayed with deionized water containing the same concentrations of ethanol and Tween.

Relative humidity regimes

A series of preliminary experiments determined the RH regimes that were necessary to achieve equivalent leaf water potentials among the genotypes and treatments. Accordingly, plants of wild type, etr1-1, aba2-1, aba2-1 + ABA, and aba2-1 etr1-1 + ABA were maintained at a day/night RH of 70/75% throughout the experiments. The other genotypes and treatments were grown under the following regimes.

aba2-1 and aba2-1 (sc): day 2–23, 94/98%.
aba2-1 etr1-1 and aba2-1 etr1-1 (sc): day 2–17, 94/90%; day 18–23, 94/98%.

Leaf water potentials

Leaf water potentials were measured during the light and dark periods on days 12 and 20. Water potentials on day 12 were measured by the Shardakov dye method (Slavik, 1974) because at this stage of development there were no fully expanded leaves. (Preliminary measurements indicated that leaf water potentials measured at this developmental stage by isopisiatic thermocouple psychrometry were erroneously low due to cell wall relaxation after excision.) One leaf from each of four plants was placed into 40 μl of dyed (methyl blue, Sigma) sucrose solution of known water potential. A second leaf from the same four plants was placed into a sucrose solution of different water potential. Routinely, the water potentials of the two solutions were within 0.05 MPa of each other. The solutions were precooled and maintained at 4 °C to prevent tissue growth. After 30 min to allow water exchange accompanying water potential equilibration between the leaves and solutions (the duration required for equilibration was determined in preliminary measurements), a 1 μl aliquot was removed from each solution and deposited in the centre of 1 ml of non-dyed solution of the same concentration as the original dyed solution. The upward or downward movement of the droplet indicated whether it had decreased or increased in density during the incubation, and therefore whether the mean leaf water potential of the four plants was higher or lower than that of the original solution. As necessary, measurements were repeated using new sets of plants and dyed solutions of different water potentials (as dictated by the results from previous sets) to determine the solution water potential that resulted in very little or no movement of the droplet.

Water potentials on day 20 were measured on expanded leaves by isopisiatic thermocouple psychrometry (Boyer and Knapling, 1965). Two leaves per plant were used for each measurement.

Table 2. Shoot growth response of wild type, etr1-1, aba2-1 and aba2-1 etr1-1 plants to ethylene treatment throughout development

<table>
<thead>
<tr>
<th>Genotype and days of treatment</th>
<th>Total leaf area (% control)</th>
<th>Shoot fresh wt. (% control)</th>
<th>Shoot dry wt. (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type: 2–23 d</td>
<td>64.8</td>
<td>70.2</td>
<td>67.1</td>
</tr>
<tr>
<td>etr1-1: 2–23 d</td>
<td>104.1</td>
<td>102.1</td>
<td>114.0</td>
</tr>
<tr>
<td>aba2-1: 2–23 d</td>
<td>35.3</td>
<td>33.6</td>
<td>35.5</td>
</tr>
<tr>
<td>aba2-1 + ABA: 14–23 d</td>
<td>57.9</td>
<td>55.9</td>
<td>49.2</td>
</tr>
<tr>
<td>aba2-1 etr1-1: 2–23 d</td>
<td>104.3</td>
<td>114.9</td>
<td>115.0</td>
</tr>
</tbody>
</table>
Growth analysis, ethylene evolution and ABA content

Plants were harvested on day 23 for measurements of whole shoot ethylene evolution rate, total leaf area (using a Li-Cor leaf-area meter, Lincoln, Nebraska, USA), blade length and width of the largest rosette leaf, leaf number, and total shoot fresh and dry weights. For ethylene evolution measurements, individual whole shoots were harvested 5–8 h into the light period and placed in a 60 ml syringe, which was then sealed and maintained in the growth chamber for 5–7 min. Evolved ethylene was measured by GC as described previously (Sharp et al., 2000). The measurements were made before the induction of wound-induced ethylene, and are considered to be good estimates of the rate of ethylene evolution by the shoots of intact plants.

In separate experiments, plants were grown under the same conditions as for the growth experiments and used for measurements of whole shoot ABA content. Between 70–105 plants per genotype were harvested 5–8 h into the light period on day 23, immediately frozen in liquid nitrogen, freeze-dried and finely ground. Duplicate measurements of ABA content were made by GC, as described previously (León-Kloosterziel et al., 1996).

Statistical analysis

Analyses of variance were performed with means compared using Fischer’s least significant difference test at the $P=0.05$ level (except where noted).

Results and discussion

Ethylene insensitivity of $etr1-1$ and $aba2-1$ $etr1-1$ throughout shoot development

Based on the triple response assay, both $etr1-1$ and the $aba2-1$ $etr1-1$ double mutant exhibited lack of growth responses to ethylene at the seedling stage (Table 1). However, to enable a definitive assessment of the extent to which maintenance of shoot growth by ABA involves the suppression of ethylene synthesis and/or signalling, it was essential to verify that shoot growth in both mutants remained insensitive to ethylene throughout the developmental stage studied. In particular, it was conceivable that combining with $aba2-1$ might have altered the effectiveness of the $etr1-1$ mutation in conferring ethylene insensitivity.

Treatment with 1 ppm ethylene from days 2–23 resulted in 30–35% inhibition of shoot development in the wild type, and 65% inhibition in $aba2-1$ (Table 2). In addition, beginning the treatment of $aba2-1$ at day 14 resulted in 40–50% inhibition, confirming that 1 ppm ethylene was an effective growth-inhibitory concentration throughout.
development (i.e. the inhibition observed in plants treated from day 2 was not caused only at the seedling stage). By contrast, both etr1-1 and aba2-1 etr1-1 showed complete insensitivity to ethylene for all of the parameters examined.

The greater inhibition of shoot growth in response to the ethylene treatment in aba2-1 than in the wild type suggests that, in addition to increasing ethylene production (see below), ABA deficiency may also have increased the sensitivity of shoot growth to ethylene. Such an effect would not be unexpected given recent evidence for interactions between ABA- and ethylene-signalling pathways (Beaudoin et al., 2000; Ghassemian et al., 2000). However, it should be noted that these results do not allow an accurate assessment of this possibility. An alternative explanation is that the greater inhibition of growth in aba2-1 by applied ethylene was because of an additive effect with the increase in endogenous ethylene production. Definitive assessment of whether ABA deficiency alters the sensitivity of shoot growth to applied ethylene requires a comparison of ABA-deficient and wild-type plants at the same rate of endogenous ethylene production (by chemically or genetically decreasing ethylene synthesis in the ABA-deficient plants).

**Leaf water potentials under differential RH regimes**

By using different RH regimes to compensate for differences in transpiration rate and hydraulic conductance between genotypes, similar leaf water potentials were achieved in all genotypes and treatments (Table 3). Importantly, the remaining small differences in water potential between treatments were of a conservative nature with regard to the interpretation of the growth results (shoot growth characteristics are reported below). For example, in the second half of the experiment the daytime water potential of the double mutant was 0.13 MPa lower than that of aba2-1 despite both genotypes being exposed
Table 4. Leaf blade width and length of wild type, etr1-1, aba2-1 and aba2-1 etr1-1 plants

Measurements were made of the largest rosette leaf 23 d after emergence in the experiments presented in Figs 1–5 and Table 3. ABA-treated plants were sprayed with 1 μM ABA daily from emergence. Data are means ± standard error (n=7–16). Independent statistical comparisons were made between sub-groups of genotypes and treatments as indicated by font style (roman, bold, italic). Within each column, different letters indicate significant differences at the 0.01 level; sc, spray control.

<table>
<thead>
<tr>
<th>Genotype and treatment</th>
<th>Blade width (mm)</th>
<th>Blade length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>14.9±0.3 b</td>
<td>31.1±0.8 a</td>
</tr>
<tr>
<td>etr1-1</td>
<td>17.6±0.5 a</td>
<td>25.5±0.5 b</td>
</tr>
<tr>
<td>aba2-1</td>
<td>8.9±0.3 c</td>
<td>21.3±0.6 c</td>
</tr>
<tr>
<td>aba2-1 etr1-1</td>
<td>13.6±0.4 b</td>
<td>24.1±0.7 b</td>
</tr>
<tr>
<td>aba2-1 (sc)</td>
<td>7.9±0.2 b</td>
<td>20.1±0.6 b</td>
</tr>
<tr>
<td>aba2-1 + ABA</td>
<td>14.4±0.2 a</td>
<td>28.9±0.5 a</td>
</tr>
<tr>
<td>aba2-1 etr1-1 (sc)</td>
<td>12.3±0.3 b</td>
<td>22.0±0.8 b</td>
</tr>
<tr>
<td>aba2-1 etr1-1 + ABA</td>
<td>16.3±0.5 a</td>
<td>26.6±0.8 a</td>
</tr>
</tbody>
</table>

to the same RH of 94% (probably because of the greater transpiring surface area of the double mutant), and thus improved water balance was not a contributing factor in the greater growth of the double mutant. Similarly, the water potential of the aba2-1 + ABA treatment was 0.11 MPa lower than that of the aba2-1 (sc) plants, and thus improved water balance did not contribute to ABA-induced growth recovery. It should be noted that the RH regime required for ABA-treated plants of both aba2-1 and aba2-1 etr1-1 (70/75%) was the same as that for wild type and etr1-1 plants, indicating that normal stomatal conductance and root hydraulic properties were restored by the ABA treatment.

**ABA content and ethylene evolution under differential RH regimes**

When grown at equivalent leaf water potentials, the total shoot ABA content of aba2-1 was 8% of the wild type value at the day 23 harvest (Fig. 1, inset). For both genotypes, the values are of the same order of magnitude as those reported previously for well-watered plants (Léon-Kloosterziel et al., 1996a). The ABA content of etr1-1 was 47% higher than in the wild type, consistent with a report that the shoot ABA content of the ein2 (ethylene-insensitive) mutant of Arabidopsis is approximately double that of the wild type (Ghassemian et al., 2000). Importantly, however, the aba2-1 etr1-1 double mutant was almost as deficient in ABA as aba2-1; the ABA content of the double mutant was 11% of the value in etr1-1.

The rate of leaf ethylene evolution of aba2-1 was doubled compared with the wild type (Fig. 1), consistent with results for the flacca mutant of tomato when grown with the same leaf water potentials as the wild type (Sharp et al., 2000). Enhanced ethylene production in ABA-deficient mutants has also been reported in earlier studies of Arabidopsis (aba1; Rakitina et al., 1994) and tomato (flacca; Tal et al., 1979), although the extent to which the increase in those studies was a direct result of ABA deficiency or an indirect result of decreased plant water status was not known (there are many reports that ethylene production can be increased by plant water deficits). The ethylene evolution rate of etr1-1 was similar to that of the wild type, consistent with previous findings (Bleecker et al., 1988). (Rates of evolution in that report were an order of magnitude greater than in this study. However, ethylene evolution was measured on leaves that had been excised for 12 h and, therefore, probably included wound-induced ethylene production.) In the aba2-1 etr1-1 double mutant, ethylene evolution was increased to the same level as in aba2-1, consistent with the equivalent ABA deficiency in the two mutants.

**Shoot growth maintenance by ABA: involvement of ethylene suppression**

When grown with equivalent leaf water potentials, aba2-1 exhibited substantial inhibition of leaf area development and shoot fresh and dry weights compared to the wild type (Fig. 2; representative plants are shown in Fig. 3). Leaf blades were significantly narrower and shorter in aba2-1 (Fig. 3; Table 4), and the total leaf area of aba2-1 was only 45% of the wild type at the end of the 23 d experiments. These results demonstrate that non-hydraulic effects of ABA-deficiency are a major cause of shoot growth inhibition in aba2-1, in agreement with findings for the flacca and notabilis mutants of tomato (Sharp et al., 2000).

By contrast with aba2-1, leaf blades of etr1-1 were significantly wider as well as shorter than in the wild type (Fig. 3; Table 4), although total leaf area and shoot fresh weight were not significantly different in the two genotypes (shoot dry weight was slightly smaller in etr1-1) (Fig. 2). A similar leaf morphology phenotype has been observed in the ein2 mutant of Arabidopsis (Alonso et al., 1999).

The aba2-1 etr1-1 double mutant exhibited significantly greater total leaf area and shoot fresh and dry weights compared to aba2-1 (Fig. 2). However, growth restoration was incomplete relative to either the wild type or etr1-1, with all parameters remaining significantly smaller in the double mutant. Restoration of total leaf area (55% relative to etr1-1) was greater than for shoot fresh or dry weights. Notably, the leaf morphology of aba2-1 etr1-1 resembled that of the wild type rather than etr1-1 (Fig. 3; Table 4). Blade width of the double mutant was not significantly different from the wild type, but significantly smaller than in etr1-1. By contrast, leaf length was significantly shorter than in the wild type, but not significantly different from etr1-1. The number of leaves was not significantly different among the four genotypes (data not shown).
The partial growth restoration of aba2-1 etr1-1, taken together with the demonstrated ABA-deficiency and ethylene insensitivity of this double mutant, shows that the impairment of shoot growth in aba2-1 is substantially attributable to the increase in ethylene production (together with a possible increase in ethylene sensitivity). Accordingly, it is concluded that normal levels of ABA maintain shoot development, particularly leaf expansion, partly by suppression of ethylene synthesis.

It should be noted that the gin1 (glucose insensitive) mutant of Arabidopsis has recently been shown to be allelic to aba2 (reviewed in León and Sheen, 2003). The gin1-1 etr1-1 double mutant was previously generated, but shoot growth characteristics were not quantified (Zhou et al., 1998). By contrast with the present results, photos of 4-week-old plants indicate that leaf growth of the double mutant was only slightly (if at all) greater than in gin1-1. However, plant water balance was not controlled, which complicates the growth comparison. In particular, under the greenhouse conditions in which the plants were grown, growth of both mutants may have been limited by adverse water relations.

Shoot growth maintenance by ABA also involves an ethylene-independent function

The fact that shoot growth was not completely restored in aba2-1 etr1-1 indicates that ABA has another function in maintaining growth in the wild type and the etr1-1 mutant, in addition to ethylene suppression. This hypothesis was tested by examining the ability of applied ABA to restore shoot growth of the aba2-1 and aba2-1 etr1-1 mutants. In both cases, improvement in shoot water status was prevented by adjustment of the RH regime (Table 3). The ABA treatment resulted in complete recovery of shoot growth in aba2-1 relative to the wild type (Fig. 4A–C), demonstrating that the growth impairment of the mutant results entirely from ABA deficiency and does not involve pleiotropic effects. The same treatment significantly increased the growth of aba2-1 etr1-1 such that total leaf area and shoot fresh weight were not significantly lower than in etr1-1 at the end of the experiment (Fig. 4E–G). Representative ABA-treated and spray control plants are illustrated in Fig. 5. Interestingly, the addition of ABA to aba2-1 restored leaf morphology to that of the wild type, whereas addition of ABA to aba2-1 etr1-1 restored the wider leaf blade observed in etr1-1 (compare Figures 3 and 5). In the ABA-treated plants, leaf width and length of aba2-1 and aba2-1 etr1-1 were not significantly different from the wild type and etr1-1, respectively (Table 4). Thus, expression of the wider leaf morphology phenotype of etr1-1 requires ethylene insensitivity and also normal levels of ABA. These results demonstrate that ABA also has an ethylene-independent function in promoting shoot development and leaf expansion.

The ABA treatment fully prevented the increase in leaf ethylene evolution in aba2-1 (Fig. 4D), consistent with results for the flacca mutant of tomato (Sharp et al., 2000).
It is noteworthy that the increase in ethylene evolution inaba2-1 etr1-1 was also prevented by ABA treatment (Fig. 4H). Thus, the suppression of ethylene synthesis by ABA does not appear to involve feedback regulation via the ethylene response pathway.

**Conclusion**

The results provide compelling evidence that normal levels of endogenous ABA are required to maintain shoot development, particularly leaf expansion, in well-watered *Arabidopsis* plants, partly by suppressing ethylene synthesis (and perhaps also sensitivity) and partly by another mechanism that is independent of ethylene. The ethylene insensitivity of shoot growth demonstrated in the aba2-1 etr1-1 double mutant, combined with the experimental strategy of avoiding variation in water status between plants with different levels of ABA, allowed a definitive assessment of the extent to which the inhibition of shoot growth resulting from ABA deficiency is attributable to ethylene. The finding that ethylene suppression is an important factor in the maintenance of shoot growth by ABA in well-watered plants confirms previous results in tomato (Sharp *et al.*, 2000). Similarly, restriction of ethylene synthesis and/or sensitivity is an important function of the accumulation of ABA in water-stressed maize seedlings (Spollen *et al.*, 2000; Sharp, 2002). ABA accumulation thereby maintains primary root elongation (Saab *et al.*, 1990; Sharp *et al.*, 1994), and plays either a growth-inhibitory or growth-promoting role in the shoot (due to a change during development in the effect of ethylene from promotive to inhibitory) (Saab *et al.*, 1990, 1992; Sharp, 2002). The previous studies relied on the use of chemical inhibitors of ethylene synthesis or action to assess the involvement of ethylene suppression in growth responses to ABA. The present study provides genetic confirmation that ethylene suppression is an important, although not the only role of endogenous ABA in shoot growth maintenance of well-watered plants.

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