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Interactions between Abscisic Acid and Ethylene Signaling Cascades

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We screened for mutations that either enhanced or suppressed the abscisic acid (ABA)–resistant seed germination phenotype of the Arabidopsis *abi1-1* mutant. Alleles of the constitutive ethylene response mutant *ctr1* and ethylene-insensitive mutant *ein2* were recovered as enhancer and suppressor mutations, respectively. Using these and other ethylene response mutants, we showed that the ethylene signaling cascade defined by the *ETR1*, *CTR1*, and *EIN2* genes inhibits ABA signaling in seeds. Furthermore, epistasis analysis between ethylene- and ABA-insensitive mutations indicated that endogenous ethylene promotes seed germination by decreasing sensitivity to endogenous ABA. In marked contrast to the situation in seeds, *ein2* and *etr1-1* roots were resistant to both ABA and ethylene. Our data indicate that ABA inhibition of root growth requires a functional ethylene signaling cascade, although this inhibition is apparently not mediated by an increase in ethylene biosynthesis. These results are discussed in the context of the other hormonal regulations controlling seed germination and root growth.

INTRODUCTION

Abscisic acid (ABA) regulates various aspects of plant growth and development, including seed maturation and dormancy, as well as adaptation to abiotic environmental stresses (Zeevaart and Creelman, 1988; Davies and Jones, 1991). Substantial progress has been made in the characterization of ABA signaling pathways (Busk and Pagès, 1997; Bonetta and McCourt, 1998; Leung and Giraudat, 1998; MacRobbie, 1998). In particular, mutational analyses in Arabidopsis have led to the identification of several genes that control ABA responsiveness. These genetic screens were based primarily on the inhibition of seed germination by applied ABA. The ABA-insensitive (*abi*) mutants *abi1* to *abi5* are able to germinate in the presence of ABA concentrations that are inhibitory to the wild type (Koornneef et al., 1984; Ooms et al., 1993; Finkelstein, 1994; Nambara et al., 1995). In contrast, germination of the *era1* (enhanced response to ABA) to *era3* mutant seed is prevented by low concentrations of ABA that ordinarily permit germination of wild-type seed (Cutler et al., 1996). As judged from their impact on seed dormancy, these two sets of mutations also alter the regulation of seed germination by endogenous ABA. Like ABA-deficient mutants (Karssen et al., 1983; Léon-Kloosterziel et al., 1996a), the ABA-insensitive mutants *abi1* to *abi3* display marked reductions in seed dormancy (Koornneef et al., 1984; Ooms et al., 1993; Nambara et al., 1995). Conversely, the ABA-supersensitive *era1* mutation confers enhanced seed dormancy (Cutler et al., 1996).

The *abi3*, *abi4,* and *abi5* mutants exhibit additional defects in various aspects of seed maturation (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Ooms et al., 1993; Finkelstein, 1994; Parcy et al., 1994; Nambara et al., 1995). The *ABI3* and *ABI4* genes have been cloned and encode putative transcriptional regulators. ABI3 is orthologous to the maize Viviparous 1 protein (McCarty et al., 1991; Giraudat et al., 1992). ABI4 contains an APETALA2-like DNA binding domain (Finkelstein et al., 1998).

The *abi1*, *abi2*, and *era1* mutations clearly affect ABA responses in vegetative tissues as well. The *era1* mutation enhances sensitivity to ABA in stomatal guard cells and hence reduces transpirational water loss during drought (Pei et al., 1998). The *ERA1* gene encodes the β subunit of the protein farnesyl transferase, a negative regulator of ABA action (Cutler et al., 1996). The *ABI1* and *ABI2* genes encode homologous protein serine/threonine phosphatases 2C (Leung et al., 1994, 1997; Meyer et al., 1994; Rodriguez et al., 1998). The *abi1-1* and *abi2-1* mutants carry the same amino acid substitution at equivalent positions in the ABI1 and

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ABI2 proteins, respectively (Leung et al., 1997; Rodriguez et al., 1998). These two mutations are dominant and lead to largely overlapping sets of ABA-insensitive phenotypes, including reduced seed dormancy, defective stomatal regulation, altered ABA regulation of gene expression, and ABA-resistant seed germination and seedling growth (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Leung et al., 1997; Allen et al., 1999). In contrast to the ABA-resistant *abi1-1* mutant, loss-of-function alleles of *ABI1* are supersensitive to ABA, indicating that the ABI1 phosphatase (and, by homology, possibly ABI2 as well) is a negative regulator of ABA responses (Gosti et al., 1999).

More recently, genetic screens based on ABA-regulated reporter genes in vegetative tissues have been developed. The *ade1* (ABA-deregulated gene expression) mutation enhances gene expression in response to ABA but not to cold (Foster and Chua, 1999). The *hos5* (high expression of osmotically responsive genes) mutant displays an increased sensitivity of gene expression to ABA and osmotic stress but not to cold (Xiong et al., 1999). The *ade1* and *hos5* mutations have little effect on seed germination.

To identify additional loci controlling sensitivity to ABA, we screened for mutations that either enhance or suppress the ABA-resistant seed germination phenotype of *abi1-1*. Remarkably, in each of these two screens we recovered alleles of mutants that were previously known to affect responsiveness to another plant hormone, ethylene. Using these and other ethylene response mutants, we demonstrate the existence of extensive interactions between the ABA and ethylene signaling cascades. These interactions, however, differ markedly in seed and in root. The ethylene pathway regulates seed dormancy negatively by inhibiting ABA signaling. In contrast, these two hormonal cascades act synergistically in inhibiting root growth.

RESULTS

Isolation of *ctr1* Mutants as Phenotypic Enhancers of *abi1-1*

Seeds homozygous for the *abi1-1* mutation were treated with ethyl methanesulfonate (Gosti et al., 1999), and the M_2 population was screened for mutants that showed enhancement of the ABA-resistant seed germination phenotype M_2 seeds were plated on agar medium containing 100 μM ABA, a concentration that inhibits germination of *abi1-1*. After 5 days at 21°C, germinated seedlings with green cotyledons or an elongated root with hairs were selected as candidate enhancers. These individuals were propagated in soil, and their phenotype was retested in the next (M_3) generation. From an estimated total of 4000 M_1 plants and 185,000 M_2 seeds screened, we finally retained 34 independent lines (originating from distinct pools of M_1 plants) that were markedly more resistant than *abi1-1* to ABA inhibition of seed germination.

Among these enhancer lines, 16 displayed a characteristic morphological phenotype. Light-grown seedlings had small cotyledons and a short hairy root (Figure 1A), and the adult plants were severely stunted with dark green leaves (not shown). Six of these dwarf enhancer lines were randomly

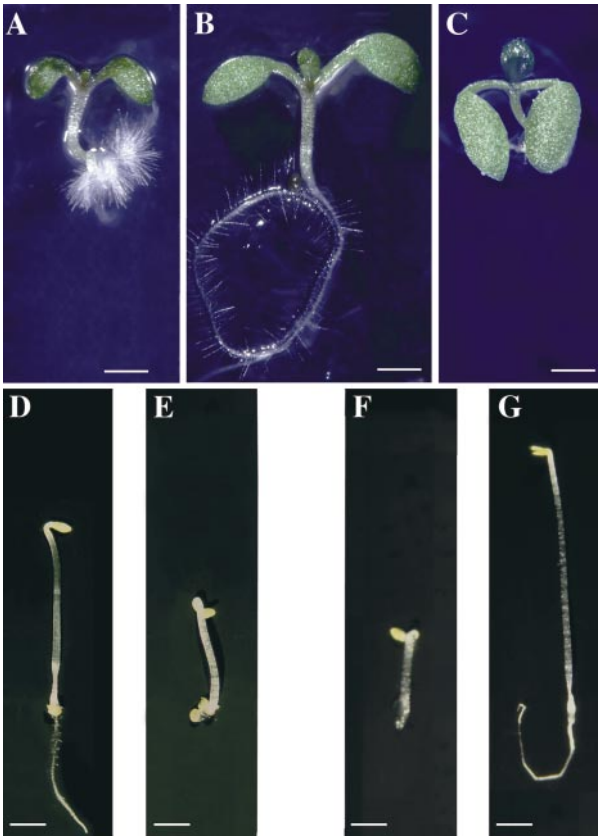

Figure 1. Morphological Phenotypes of Mutants.

(A) Eight-day-old seedling of mutant line *H3* (subsequently renamed *abi1-1 ctr1-10*) grown with a 16-hr-light photoperiod.
(B) Eight-day-old *abi1-1* seedling grown with a 16-hr-light photoperiod.
(C) Eight-day-old seedling of mutant line *84B* (subsequently renamed *abi1-1 ein2-45*) grown with a 16-hr-light photoperiod. The root is out of focus but was similar to that of *abi1-1.*
(D) Three-day-old *abi1-1* seedling grown in the dark.
(E) Three-day-old seedling of mutant line *H3* (subsequently renamed *abi1-1 ctr1-10*) grown in the dark.
(F) Three-day-old Landsberg *erecta* (L*er*) seedling grown in the dark on medium supplemented with 10 μM 1-aminocylcopropane-1-carboxylic acid (ACC).
(G) Three-day-old seedling of mutant line *84B/1* (subsequently renamed *ein2-45*) grown in the dark on medium supplemented with 10 μM ACC.
Bars = 500 μm.

selected for genetic analysis. In germination assays, these enhancer lines could be reliably distinguished from *abi1-1* by their resistance to 30 μM ABA (Figure 2A). Backcrosses to *abi1-1* showed that in all six lines analyzed, the enhancer phenotype (germination on 30 μM ABA) and the dwarf phenotype were recessive. In complementation tests, the F_1 progeny displayed both phenotypes, indicating that the six lines are allelic to each other and that these enhancer mutations are also responsible for the dwarf phenotype. We analyzed one of these enhancer lines (isolation number *H3*) in more detail.

The morphological characteristics described above for light-grown *H3* seedlings and adult plants were very similar to those reported for the constitutive ethylene response mutant *ctr1* (Kieber et al., 1993). The F_1 progeny of a complementation cross between *H3* and the recessive *ctr1-1* mutant displayed the same characteristic dwarf phenotype as the parents, indicating that the enhancer mutation contained in line *H3* is indeed a *ctr1* mutant allele. This allele was named *ctr1-10*, and line *H3* thus corresponds to the *abi1-1 ctr1-10* double mutant. When exposed to ethylene, dark-grown Arabidopsis seedlings develop a characteristic triple response: inhibition of hypocotyl and root elongation, radial swelling of the hypocotyl, and exaggeration of the apical hook (Guzman and Ecker, 1990). Dark-grown *H3* seedlings, like *ctr1-1* (Kieber et al., 1993), displayed a constitutive triple response in the absence of exogenous ethylene (Figure 1E), whereas *abi1-1* seedlings had a wild-type etiolated morphology (Figure 1D). The *CTR1* gene encodes a Raf-like protein kinase that is a negative regulator of ethylene signaling (Kieber et al., 1993). DNA sequence analysis showed that the *ctr1-10* mutation is a G-to-A transition at nucleotide 4511 in the *CTR1* gene (GenBank accession number L08790), which converts amino acid Asp-676 to Asn in the CTR1 kinase. Because Asp-676 corresponds to an invariant residue of kinase domains, the *ctr1-10* mutation probably disrupts the catalytic activity of CTR1, as was predicted for all other *ctr1* mutant alleles (Kieber et al., 1993). For subsequent studies, line *H3* was outcrossed to the Landsberg *erecta* (L*er*) wild type, and the *ctr1-10* mutation was isolated in a wild-type *ABI1* background.

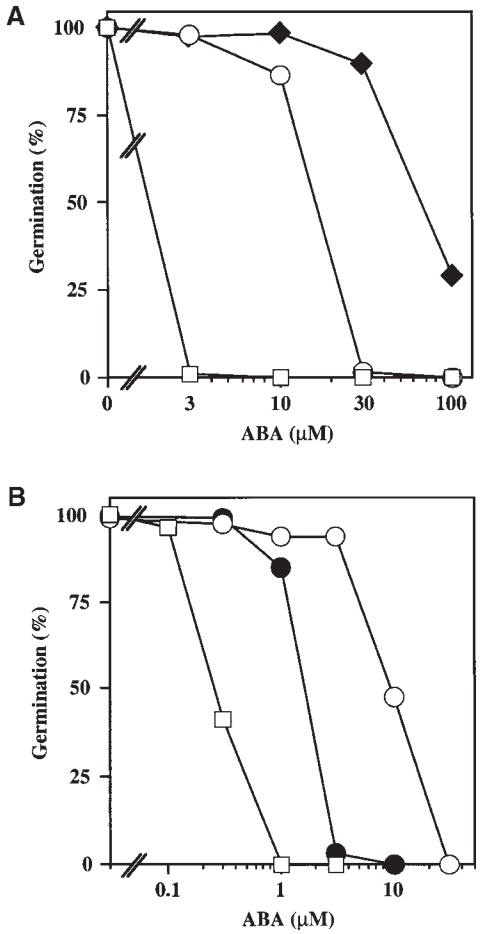

Figure 2. ABA Dose Response for Germination Inhibition.

(A) Seeds of L*er* wild type (open squares), *abi1-1* (open circles), and *H3* (subsequently renamed *abi1-1 ctr1-10*; filled diamonds) were plated on medium supplemented with the indicated concentrations of ABA, chilled for 4 days at 4°C in darkness, and incubated for 5 days at 21°C with a 16-hr-light photoperiod. The number of germinated seeds (with green cotyledons, elongated root with hairs, or both) was expressed as the percentage of the total number of seeds plated (100 to 200).

(B) Seeds of L*er* wild type (open squares), *abi1-1* (open circles), and *84B* (subsequently renamed *abi1-1 ein2-45*; filled circles) were plated on medium supplemented with the indicated concentrations of ABA, chilled for 4 days at 4°C in darkness, and incubated for 3 days at 21°C with a 16-hr-light photoperiod. The number of germinated seeds (with green cotyledons) was expressed as the percentage of the total number of seeds plated (100 to 200).

Isolation of an *ein2* Mutant as Phenotypic Suppressor of *abi1-1*

We described previously a screen for phenotypic suppressors of *abi1-1* (Gosti et al., 1999). In brief, seeds homozygous for the *abi1-1* mutation were treated with ethyl methanesulfonate, and the M_2 population was screened for mutants that showed a reversion of the ABA-resistant seed germination phenotype of *abi1-1.* Mutant line *84B* is one of the extragenic suppressors isolated in that screen. Line *84B*, which showed a partial reversion of the resistance of *abi1-1* to the ABA inhibition of seed germination, could be conveniently distinguished from *abi1-1* by its sensitivity to 3 μM ABA (Figure 2B). In addition, the cotyledons of 1-week-old *84B* seedlings grown on ABA-free medium curled downward (Figure 1C) and were markedly bigger than both *abi1-1* (Figure 1B) and the L*er* wild type (not shown). *84B* plants also had rosette leaves that were larger than those of *abi1-1*

and the L*er* wild type (not shown). Backcrossing *84B* to *abi1-1* showed that the suppressor phenotype (sensitivity to 3 μM ABA) and the cotyledon phenotype were both monogenic and recessive. Furthermore, these phenotypes cosegregated with each other in the 79 F_2 individuals analyzed, indicating that both phenotypes most likely result from the same mutation.

To separate this suppressor mutation from the *abi1-1* mutation, *84B* was outcrossed to the L*er* wild type. We selected an F_2 line (*84B/1*) that was homozygous for the wild-type *ABI1* allele (as determined by polymerase chain reaction analysis; Leung et al., 1997) and had larger cotyledons and rosette leaves than did L*er*. Ethylene-insensitive mutants of Arabidopsis grown in air have bigger rosette leaves than does the wild type (Bleecker et al., 1988; Guzman and Ecker, 1990). Furthermore, we mapped the *84B/1* mutation to the top of chromosome 5 in a region that contains the ethylene-insensitive *EIN2* locus (data not shown). Hence, we investigated whether *84B/1* was allelic to the *ein2-1* mutant (Guzman and Ecker, 1990). Like the *84B/1* plants, 1-week-old *ein2-1* seedlings grown in the light had enlarged and downward-curling cotyledons (data not shown). Dark-grown *ein2-1* seedlings fail to develop the ethylene-evoked triple response (Guzman and Ecker, 1990). Similarly, *84B/1* seedlings, when germinated in the dark on medium supplemented with the immediate ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), did not display the triple response (Figure 1G), whereas the wild-type control did (Figure 1F). As in *ein2-1*, this ACC-insensitive phenotype was recessive in *84B/1*, and the F_1 progeny of a complementation cross between *84B/1* and *ein2-1* was unresponsive to ACC. These data demonstrate that *84B/1* is a novel *ein2* mutant allele, which we call *ein2-45* (hence, the initial suppressor line *84B* corresponds to the *abi1-1 ein2-45* double mutant). The *EIN2* gene encodes a novel integral membrane protein of unknown molecular function (Alonso et al., 1999). DNA sequencing revealed that the *ein2-45* mutation is a G-to-A transition at nucleotide 5195 of the *EIN2* gene (GenBank accession number AF141202), which converts amino acid Gly-1138 to Glu in the hydrophilic C-terminal domain of the EIN2 protein.

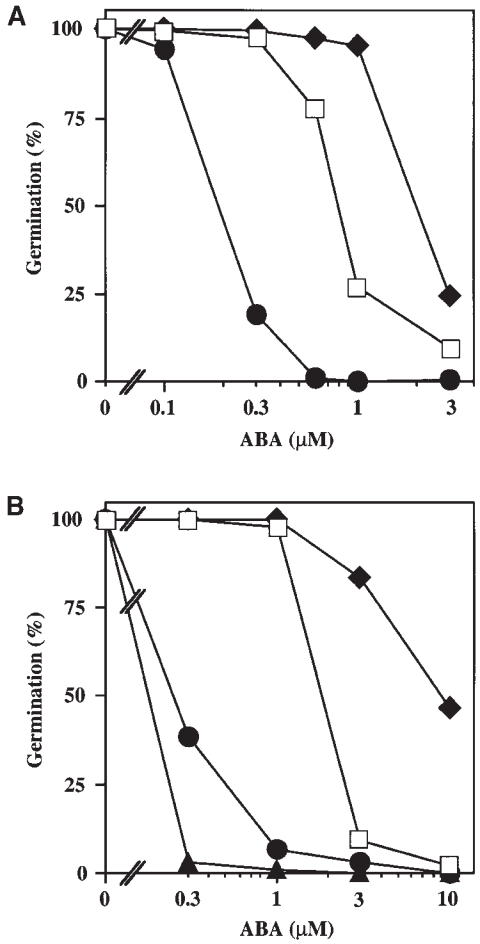

Figure 3. ABA Dose Response for Germination Inhibition.

Seeds were plated on medium supplemented with the indicated concentrations of ABA, chilled for 4 days at 4°C in darkness, and incubated at 21°C with a 16-hr-light photoperiod. The number of germinated seeds (with fully emerged radicle tip) was expressed as the percentage of the total number of seeds plated (100 to 200).
(A) Seeds of L*er* wild type (open squares), *ctr1-10* (filled diamonds), and *ein2-45* (filled circles) were incubated for 2 days at 21°C.
(B) Seeds of the Col wild type (open squares), *ctr1-1* (filled diamonds), *ein2-1* (filled circles), and *etr1-1* (filled triangles) were incubated for 3 days at 21°C.

Seeds of Ethylene-Insensitive Mutants Are Supersensitive to ABA

As described above, the ABA-resistant phenotype of *abi1-1* seeds is enhanced and suppressed by the *ctr1-10* and *ein2-45* mutations, respectively. These mutations also modify the ABA sensitivity of seed germination in the absence of the *abi1-1* mutation. Figure 3A shows that *ctr1-10* seeds were less sensitive to ABA than were L*er* wild-type seeds, and, conversely, that *ein2-45* seeds were supersensitive to ABA. These results do not seem to be biased by the fact that *ctr1-10* and *ein2-45* were initially selected in screens based on ABA sensitivity. Indeed, similar results were obtained when we tested the distinct *ctr1-1* and *ein2-1* alleles, which have been isolated solely on the basis of their ethylene-related phenotypes (Guzman and Ecker, 1990; Kieber et al., 1993). Compared with the wild-type Columbia (Col) strain from which these mutants are derived, ABA sensitivity of seed germination was decreased in *ctr1-1* but enhanced in *ein2-1* (Figure 3B). Moreover, a complementation test between *ein2-45* and *era3-1* showed that the latter mutant, which

was selected by Cutler et al. (1996) because of its enhanced sensitivity to ABA during seed germination, is also allelic to *ein2* (data not shown). The dominant ethylene-insensitive *etr1-1* mutation inhibits ethylene binding to the ETR1 receptor (Bleecker et al., 1988; Schaller and Bleecker, 1995), and hence the step of the ethylene signaling cascade it affects differs from that affected by *ein2*. Nevertheless, like *ein2*, *etr1-1* seeds were markedly more sensitive to ABA than were wild-type Col seeds (Figure 3B).

Endogenous ABA plays a major role in promoting Arabidopsis seed dormancy. Seed dormancy is diminished in various ABA-deficient or ABA-insensitive mutants (Koornneef et al., 1984; Ooms et al., 1993; Léon-Kloosterziel et al., 1996a) but is enhanced in the ABA-supersensitive *era1* mutant (Cutler et al., 1996). Because ethylene response mutations altered sensitivity to ABA in the germination assay described above, we analyzed the effect of these mutations on seed dormancy. Freshly harvested seeds of the *ctr1-1* mutant germinated only slightly faster than did wild-type Col seeds (Figure 4B), and the *ctr1-10* response did not differ substantially from that of the L*er* wild type (Figure 4A). Hence, under our experimental conditions, *ctr1* mutations had at most a limited effect on seed dormancy. In contrast, the ethylene-insensitive *ein2* and *etr1-1* mutants displayed markedly enhanced seed dormancy. When assayed without any pretreatment, freshly harvested seeds of *ein2-45* (Figure 4A), *ein2-1*, and *etr1-1* (Figure 4B) had a much lower germination rate than did seeds of the corresponding wild type. For *etr1-1*, this result is consistent with the previous observation by Bleecker et al. (1988). However, after a cold treatment (stratification) that breaks dormancy, *ein2* and *etr1-1* mutant seeds displayed full germination.

These results suggested that the *ein2* and *etr1-1* mutations may increase seed dormancy by enhancing the sensitivity to endogenous ABA. This model predicts that the effect of *ein2* and *etr1-1* on seed dormancy should be masked by mutations that inactivate the ABA signaling cascade. Such was not the case when the *abi1-1* and *ein2-45* mutations were combined because *abi1-1 ein2-45* double mutant seeds were more dormant than wild-type seeds (Figure 5). However, as judged from the ABA dose required to achieve 50% inhibition of seed germination, the ABA sensitivity in *abi1-1* seeds is reduced from wild-type sensitivity by only ~10-fold (Figure 2; Koornneef et al., 1984; Finkelstein and Somerville, 1990). We thus tested mutants with seeds even more extremely insensitive to ABA. Both the *abi3-4* mutant and the *abi1-1 abi3-1* double mutant display >1000-fold reductions in ABA sensitivity at germination (Figures 6A and 6C; Finkelstein and Somerville, 1990; Ooms et al., 1993). Remarkably, the *abi3-4 ein2-45* double mutant and the *abi1-1 abi3-1 ein2-45* triple mutant were as nondormant as the *abi3-4* and *abi1-1 abi3-1* parents (Figures 6B and 6D). As discussed above, these results are consistent with the hypothesis that the EIN2 cascade regulates negatively the degree of seed dormancy by inhibiting ABA action.

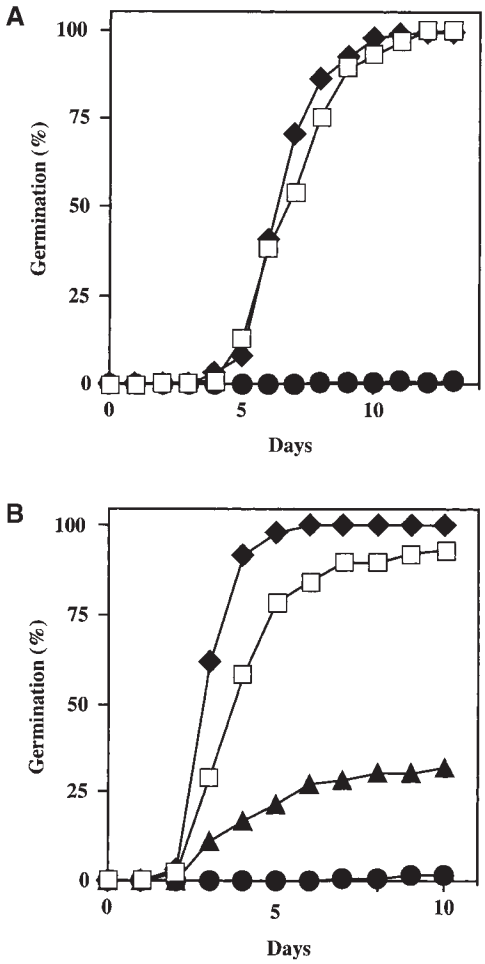

Figure 4. Seed Dormancy.

Freshly harvested seeds were plated on ABA-free medium and incubated directly (without cold pretreatment) at 21°C with a 16-hr-light photoperiod. The number of germinated seeds (with fully emerged radicle tip) at a given time was expressed as the percentage of the total number of seeds plated (100 to 200). In simultaneous experiments in which seeds from the same batches were first chilled for 4 days at 4°C in darkness to break dormancy, all genotypes displayed 100% germination after 5 days at 21°C (data not shown).

(A) Seeds of the L*er* wild type (open squares), *ctr1-10* (filled diamonds), and *ein2-45* (filled circles).

(B) Seeds of the Col wild type (open squares), *ctr1-1* (filled diamonds), *ein2-1* (filled circles), and *etr1-1* (filled triangles).

Root Growth of Ethylene-Insensitive Mutants Is Resistant to ABA Inhibition

We investigated whether, besides increasing ABA sensitivity in seeds, *ein2* mutations also affect ABA responses in vegetative tissues. Detached leaves of *ein2-45* and wild-type L*er* displayed similar kinetics of water loss, suggesting that

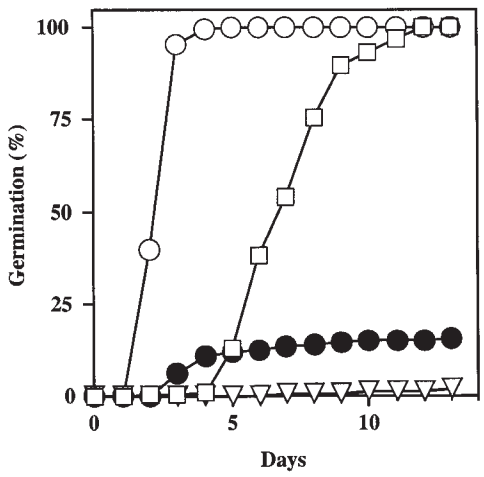

Figure 5. Seed Dormancy.

Freshly harvested seeds of the L*er* wild type (open squares), *abi1-1* (open circles), *ein2-45* (open triangles), and *abi1-1 ein2-45* (filled circles) were plated on ABA-free medium and incubated directly (without cold pretreatment) at 21°C with a 16-hr-light photoperiod. The number of germinated seeds (with fully emerged radicle tip) at a given time was expressed as the percentage of the total number of seeds plated (100 to 200). In simultaneous experiments in which seeds from the same batches were first chilled for 4 days at 4°C in darkness to break dormancy, all genotypes displayed 100% germination after 5 days at 21°C (data not shown).

ein2-45 does not alter ABA regulation of stomatal aperture (data not shown). Likewise, induction of the *RAB18* (Lang and Palva, 1992) and *RD29A* (Yamaguchi-Shinozaki and Shinozaki, 1993) mRNAs by exogenous ABA was not substantially modified in *ein2-45* (data not shown). In contrast, the *ein2-45* mutation markedly decreased the sensitivity of root growth to inhibition by ABA. Root growth in *ein2-45* was essentially as ABA resistant as in *abi1-1* (Figure 7). This effect was not specific to the *ein2-45* allele, however, because *ein2-1* as well as *etr1-1* roots displayed less sensitivity to ABA than did wild-type Col (Figure 8A). As expected, the ethylene-insensitive *ein2* and *etr1-1* mutants were also resistant to inhibition of root growth by applied ACC (Figure 8B). In contrast, the ABA-insensitive *abi1-1* mutant had a wild-type sensitivity to ACC (Figure 8B).

We investigated whether ABA inhibition of root growth might be mediated by an ABA-induced increase in ethylene biosynthesis. Under the experimental conditions of our root growth bioassay, treating wild-type plantlets with as much as 100 μM ABA failed to induce the ethylene-responsive *GST2* mRNA (Zhou and Goldsbrough, 1993), whereas this transcript did accumulate in response to as little as 0.2 μM ACC (data not shown). Moreover, ethylene production was similar in plantlets treated with 100 μM ABA (7.9 ± 4.2 pL hr^{-1} $seedling^{-1}$) and in untreated controls (7.7 ± 3.8 pL hr^{-1} $seedling^{-1}$), whereas, as expected, ethylene production was enhanced in plantlets exposed to 10 μM ACC (100.2 ± 8.3 pL hr^{-1} $seedling^{-1}$). Hence, the present data indicate that ABA inhibition of root growth requires a functional ethylene signaling cascade; however, this inhibition is apparently not mediated by an increase in ethylene biosynthesis.

The only mutant previously described as being cross-resistant to ethylene and ABA is the *axr2-1* mutant. This dominant mutation was originally recovered in a screen for auxin-resistant seedlings and was subsequently shown to decrease the sensitivity of root growth to inhibition by auxin, ethylene, and ABA (Wilson et al., 1990). Like *ein2* and *etr1-1*, *axr2-1* roots were indeed resistant to both ABA and ACC (Figure 8). However, in marked contrast to *ein2* and *etr1-1*, germination of *axr2-1* seeds was not supersensitive to ABA (Figure 9). This observation is consistent with the view that the *axr2-1* mutant has a primary defect in the action of auxin rather than that of ethylene (Wilson et al., 1990; Timpte et al., 1994).

DISCUSSION

Mutational and molecular analyses in Arabidopsis have identified several of the elements in the ethylene signal transduction pathway (Johnson and Ecker, 1998). Ethylene is perceived by a family of receptors related to ETR1, and ethylene binding inhibits the signaling activities of these receptors (Hua and Meyerowitz, 1998). In the absence of ethylene, the receptors activate CTR1, which regulates negatively the downstream signaling components, including EIN2 (Kieber et al., 1993; Clark et al., 1998; Alonso et al., 1999). We isolated the *ein2-45* mutation as a suppressor of the ABA-resistant seed germination phenotype of *abi1-1*. Furthermore, in the wild-type *ABI1* background, *ein2-45* mutant seeds were also supersensitive to ABA. This phenotype is not specific to the *ein2-45* allele because we found that the *ein2-1* mutation similarly enhances ABA sensitivity of seed germination and that the ABA-supersensitive *era3-1* mutant described by Cutler et al. (1996) is also an *ein2* mutant allele. Conversely, like *ein2-1* (Guzman and Ecker, 1990), *ein2-45* and *era3-1* mutants were insensitive to ethylene, as shown by their failure to develop the characteristic triple response evoked by ethylene (or ACC) in dark-grown seedlings. These data show that, in addition to its role as a positive regulator of ethylene signaling, *EIN2* is a negative regulator of ABA sensitivity in seeds.

ein2 mutants have also been recovered in screens for mutants resistant to cytokinins (Cary et al., 1995) or inhibitors of auxin transport (Fujita and Syono, 1996) and in screens for delayed leaf senescence (Oh et al., 1997). None of the other ethylene-insensitive loci was recovered in these screens. Furthermore, expression of the EIN2 C-terminal domain in *ein2* mutant plants restored responsiveness to jasmonic acid and paraquat-induced oxygen radicals but was not suf-

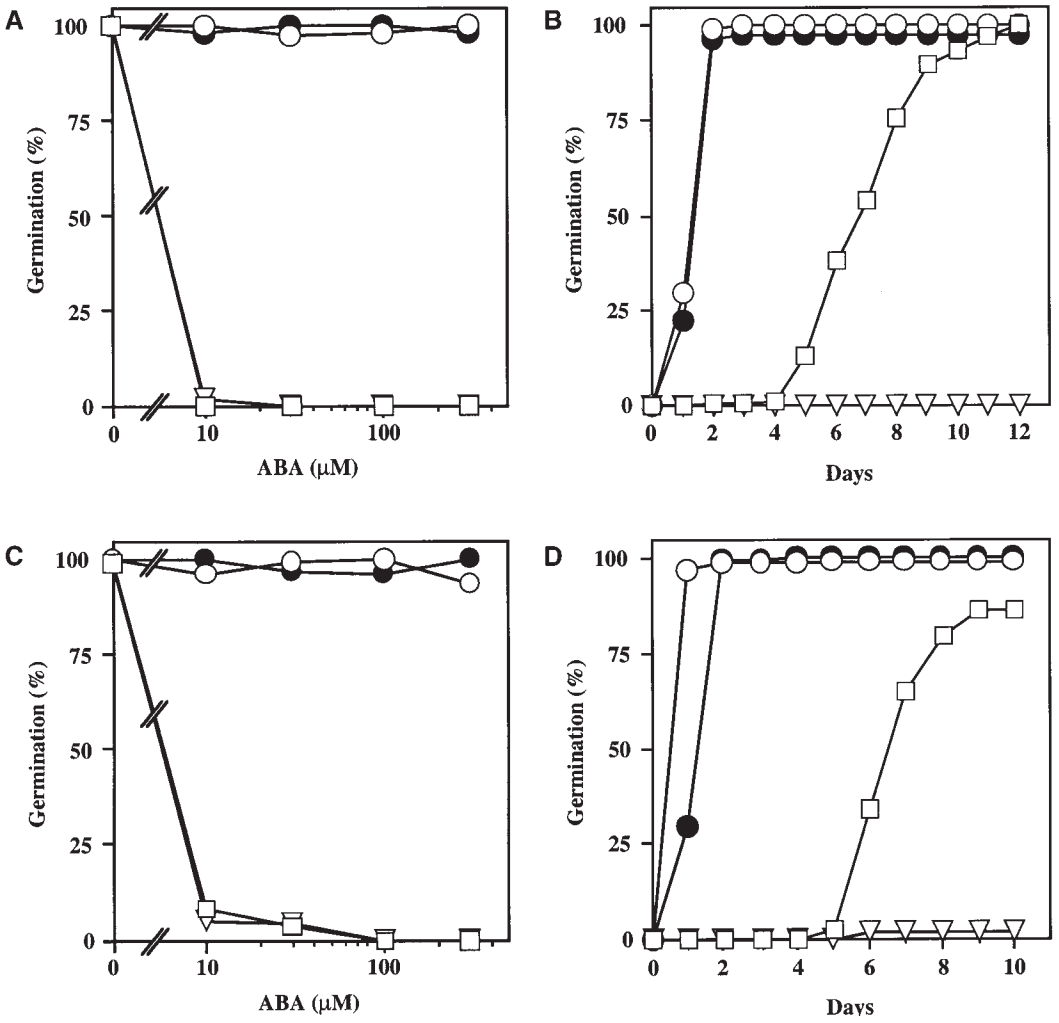

Figure 6. Effect of the *ein2-45* Mutation on the Seed Phenotypes of Severely ABA-Insensitive Mutants.

(A) and **(C)** ABA dose response for germination inhibition. Seeds were plated on medium supplemented with the indicated concentrations of ABA, chilled for 3 days at 4°C in darkness, and incubated for 3 days at 21°C with a 16-hr-light photoperiod. The number of germinated seeds (with fully emerged radicle tip) was expressed as the percentage of the total number of seeds plated (70 to 100). Data from seeds of the L*er* wild type (open squares), *abi3-4* (open circles), *ein2-45* (open triangles), and *abi3-4 ein2-45* (filled circles) are shown in **(A)**. Data from seeds of the L*er* wild type (open squares), *abi1-1 abi3-1* (open circles), *ein2-45* (open triangles), and *abi1-1 abi3-1 ein2-45* (filled circles) are shown in **(C)**.
(B) and **(D)** Seed dormancy. Freshly harvested seeds were plated on ABA-free medium and incubated directly (without cold pretreatment) at 21°C with a 16-hr-light photoperiod. The number of germinated seeds (with fully emerged radicle tip) at a given time was expressed as the percentage of the total number of seeds plated (100 to 200). In simultaneous experiments in which seeds from the same batches were first chilled for 4 days at 4°C in darkness to break dormancy, all genotypes displayed 100% germination after 5 days at 21°C (data not shown). Data from seeds of the L*er* wild type (open squares), *abi3-4* (open circles), *ein2-45* (open triangles), and *abi3-4 ein2-45* (filled circles) are shown in **(B)**. Data from seeds of the L*er* wild type (open squares), *abi1-1 abi3-1* (open circles), *ein2-45* (open triangles), and *abi1-1 abi3-1 ein2-45* (filled circles) are shown in **(D)**.

ficient to induce the triple response (Alonso et al., 1999). Hence, EIN2 has been proposed to lie at the crossroads of multiple hormone response pathways (Alonso et al., 1999). However, EIN2 is not the sole ethylene signaling element that controls ABA responsiveness in seeds. As did the *ein2* mutations, the ethylene-insensitive *etr1-1* mutation in the ETR1 receptor enhanced ABA sensitivity in seeds. Conversely, seeds of the constitutive ethylene response mutants *ctr1* were less sensitive than the wild type to ABA. Hence, our results indicate that the entire pathway defined

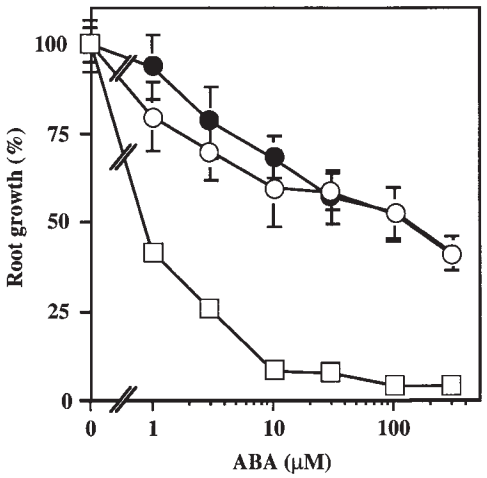

Figure 7. ABA Dose Response for Root Growth Inhibition.

Seeds of the L*er* wild type (open squares), *abi1-1* (open circles), and *ein2-45* (filled circles) were germinated and grown for 8 days on ABA-free medium. These seedlings were then incubated vertically on medium supplemented with the indicated concentrations of ABA, and their root length was scored after 5 days. Root growth of ABA-treated seedlings was expressed as a percentage relative to controls incubated on ABA-free medium. Values shown are mean ±SD from samples consisting of 15 to 20 seedlings each.

by ETR1, CTR1, and EIN2 impinges on ABA transduction in seeds.

It is currently unclear whether the ETR1–EIN2 cascade mediates ethylene signaling exclusively. For instance, various observations suggest that ETR1 (Hua and Meyerowitz, 1998), and possibly a whole subset of the ethylene receptors (Gamble et al., 1998), may have additional, ethylene-independent functions. Nevertheless, the simplest interpretation of the present data is that the enhanced dormancy of *etr1-1* and *ein2* seeds actually results from impaired ethylene signaling, and hence that endogenous ethylene regulates negatively the dormancy of Arabidopsis seeds. Ethylene has long been suspected to play a role in seed dormancy, but direct evidence has been scarce (Abeles et al., 1992; Kepczynski and Kepczynska, 1997). Results of studies with biosynthesis inhibitors were ambiguous because in many cases these inhibitors markedly lowered ethylene production without affecting seed germination (Kepczynski and Kepczynska, 1997). However, studies with 2,5-norbornadiene, a competitive antagonist of ethylene, indicated that endogenous ethylene is necessary for the germination of dormant seeds from *Chenopodium album* (lamb's-quarters) (Machabée and Saini, 1991) and *Amaranthus retroflexus* (Kepczynski et al., 1997). Similarly, our genetic data indicate that endogenous ethylene regulates negatively the degree of seed dormancy in Arabidopsis. Under our experimental conditions, endogenous ethylene content in the wild type was at or close to saturating values for its action on seed dormancy because constitutive activation of the ethylene pathway in the *ctr1* mutants or treating wild-type seeds with 10 μM ACC (data not shown) barely decreased dormancy.

Seed dormancy mutants of Arabidopsis can be classified into two groups depending on whether or not they are affected in ABA action (biosynthesis or sensitivity) (Léon-Kloosterziel et al., 1996b). As discussed above, the *etr1-1* and *ein2* mutations markedly enhanced the sensitivity of seed germination to exogenous ABA. Furthermore, epistasis analyses between *ein2-45* and severely ABA-insensitive mutants indicated that ethylene regulates negatively the degree of seed dormancy by decreasing the sensitivity to endogenous ABA. The correlation between dormancy and ABA sen-

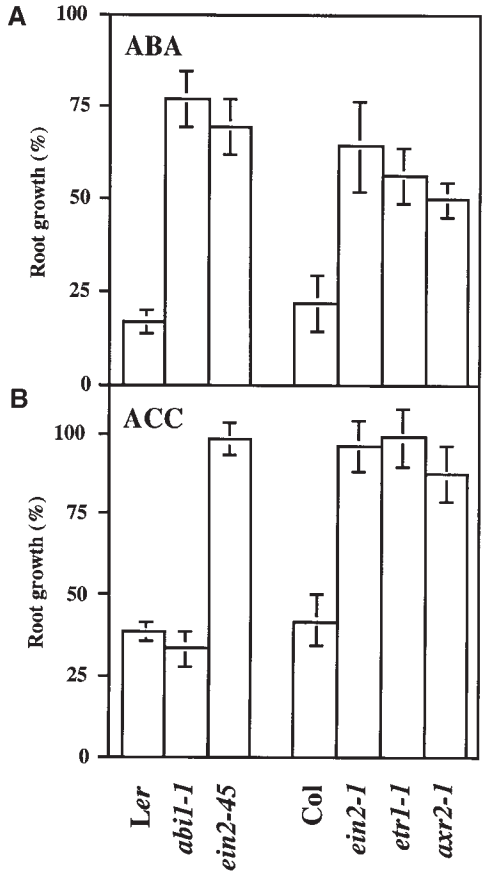

Figure 8. Sensitivity of Root Growth to Applied ABA and ACC.

(A) and **(B)** Seeds of the indicated genotypes were germinated and grown for 6 days on medium containing no additional supplements. These seedlings were then incubated vertically on medium supplemented with 100 μM ABA **(A)** or 0.2 μM ACC **(B)**, and their root length was scored after 4 days. Root growth of ABA- or ACC-treated seedlings was expressed as a percentage relative to controls incubated on normal medium. Values shown are mean ±SD from samples consisting of 15 to 20 seedlings each.

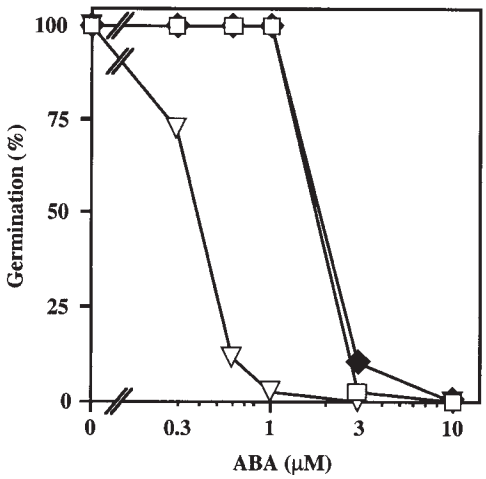

Figure 9. ABA Dose Response for Germination Inhibition.

Seeds of the Col wild type (open squares), *ein2-1* (open triangles), and *axr2-1* (filled diamonds) were plated on medium supplemented with the indicated concentrations of ABA, chilled for 4 days at 4°C in darkness, and incubated for 5 days at 21°C with a 16-hr-light photoperiod. The number of germinated seeds was expressed as the percentage of the total number of seeds plated (70 to 100).

sitivity, however, was not perfect in all the genotypes analyzed here. *etr1-1* seeds were somewhat less dormant but were more sensitive to exogenous ABA than *ein2-1* seeds. Conversely, *abi1-1 ein2-45* double-mutant seeds were more dormant but less sensitive to exogenous ABA than were wild-type L*er* seeds. It is unclear whether these few discrepancies suggest that ethylene has in addition some ABA-independent effect on seed dormancy or whether they simply result from the fact that inhibition of seed germination by exogenous ABA mimics only imperfectly the action of endogenous ABA on seed dormancy (Steber et al., 1998).

Our results support that ethylene regulates seed dormancy largely by counteracting the effect of ABA (Figure 10A). In this respect, there are many similarities between the roles of ethylene and gibberellin (GA). GA is absolutely required for the germination of wild-type Arabidopsis seed because GA-deficient mutants such as *ga1* fail to germinate (unless supplied with exogenous GA) (Koornneef and van der Veen, 1980). In contrast to the *ga1* single mutant, however, double mutants that combine *ga1* with ABA-deficient or ABA-insensitive mutations do not require exogenous GA to germinate (Koornneef et al., 1982; Nambara et al., 1992; Steber et al., 1998). Finally, the GA-deficient *ga1* and GA-insensitive *sly1* mutations increase the sensitivity of seed germination to ABA inhibition. *ga1* and *sly1* mutants were isolated by Steber et al. (1998) as suppressors of *abi1-1* in a screen very similar to the one in which we recovered *ein2-45*. Hence, both endogenous GA and ethylene appear to control seed germination by decreasing ABA responsiveness (Figure 10A). GA and ethylene can replace each other to a certain extent because germination of the *ga1* mutant could be induced by exogenous ethylene (Karssen et al., 1989), and conversely, a GA treatment stimulated germination of the *etr1-1* mutant (Bleecker et al., 1988). The roles of endogenous GA and ethylene are unlikely to be completely redundant, however, because mutations that impair the action of either of these two hormones lead to tangible alterations in seed germination.

Primary seed dormancy is established on the mother plant during the late stages of seed development (seed maturation). Studies on ABA-deficient mutants clearly demonstrated that embryonic ABA is required for the onset of dormancy during Arabidopsis seed maturation (Karssen et al., 1983; Léon-Kloosterziel et al., 1996a). Because ABA content markedly decreases at the end of seed maturation, Karssen et al. (1983, 1989) postulated that after the onset of dormancy, endogenous ABA may not be required for its maintenance. However, recent experiments in barley (Wang et al., 1995) and in *Nicotiana plumbaginifolia* (Grappin et al., 2000) indicate that the maintenance of dormancy in imbibed seeds is actually an active process involving de novo ABA

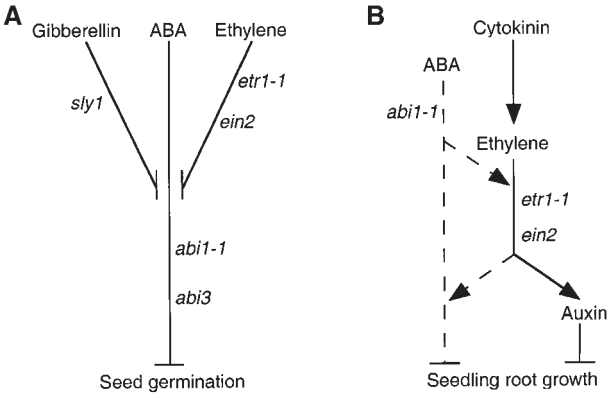

Figure 10. Schematic Representations of the Interactions between the ABA and Ethylene Signaling Cascades.

(A) Regulation of seed germination. Ethylene and GA signaling cascades promote seed germination by decreasing sensitivity to endogenous ABA in imbibed seeds. The *abi1-1* and *abi3* mutants are ABA insensitive, the *sly1* mutant is GA insensitive, and the *etr1-1* and *ein2* mutants are ethylene insensitive. The positions of the intersections between the ABA, ethylene, and GA pathways are speculative.

(B) Inhibition of seedling root growth. Inhibition of root growth in response to increased amounts of ethylene is largely mediated through internal accumulation of auxin. Cytokinin inhibits root growth by stimulating ethylene biosynthesis. ABA inhibition of root growth is apparently not mediated by an increase in ethylene biosynthesis but requires an active ethylene signaling cascade. Either the basal activity of the ethylene pathway is required to synergize ABA action or ABA stimulates the ethylene signaling cascade.

synthesis. Ethylene and GA do not seem to regulate the induction of dormancy during seed maturation and are thought to act mainly during imbibition to break dormancy and trigger germination (Karssen et al., 1989; Nambara et al., 1991; Abeles et al., 1992; Kepczynski and Kepczynska, 1997). The observation that seeds of ethylene-insensitive (this study) and GA-insensitive (Steber et al., 1998) mutants are supersensitive to exogenous ABA suggests that ethylene and GA may counteract directly the action of ABA in dormancy maintenance by inhibiting ABA signaling in imbibed seeds.

Whereas *etr1-1* and *ein2* seeds were supersensitive to ABA, however, the roots of these mutants were cross-resistant to ethylene and ABA. Treatment with exogenous ethylene (or ACC), or constitutive activation of the ethylene cascade by the *ctr1* mutation, inhibits the growth of seedling roots. Ethylene inhibits auxin transport in both shoots and roots, and evidence suggests that ethylene inhibition of root growth is largely mediated through internal auxin accumulation (Timpte et al., 1995; Fujita and Syono, 1996; Luschnig et al., 1998; Marchant et al., 1999). In particular, the ethylene resistance of *axr2* (Wilson et al., 1990), *aux1* (Pickett et al., 1990), *axr1* (Timpte et al., 1995), and *eir1* (Luschnig et al., 1998) mutant roots seems to be a secondary consequence of the auxin insensitivity of these mutants (Figure 10B). Roots of the ethylene-insensitive mutants *ein1* (allelic to *etr1*) and *ckr1* (allelic to *ein2*) are resistant to cytokinin (Su and Howell, 1992; Cary et al., 1995) because cytokinin inhibits root growth by stimulating ethylene biosynthesis (Cary et al., 1995; Vogel et al., 1998) (Figure 10B). Whereas *etr1-1* and *ein2* roots were resistant to both ABA and ethylene, *abi1-1* root was resistant to ABA but had normal sensitivity to ethylene. This suggested that ethylene acts "downstream" of ABA. However, ABA-treated seedlings did not display any detectable change in ethylene production or in abundance of the ethylene-responsive *GST2* mRNA. This is consistent with previous studies also indicating that ABA has at most only small effects on ethylene production (Abeles et al., 1992; Woeste et al., 1999). Hence, unlike cytokinin, ABA inhibition of root growth is apparently not mediated by an ABA-induced stimulation of ethylene production. Nevertheless, the decreased ABA sensitivity of *etr1-1* and *ein2* roots shows that ethylene signaling is required for efficient ABA inhibition of root growth. This implies either that ABA can stimulate the ethylene signaling pathway or that the seedling's content of endogenous ethylene at rest has a synergistic effect on root growth inhibition by ABA (Figure 10B). Whether ABA inhibition of root growth requires auxin action, however, is currently unclear. Roots of the *axr2-1* mutant are cross-resistant to auxin, ethylene, and ABA (Wilson et al., 1990). In contrast, *aux1* and *eir1* roots are resistant to auxin and ethylene but are as sensitive as the wild type to ABA (Pickett et al., 1990; Luschnig et al., 1998).

This study revealed the existence of important interactions between ABA and ethylene signaling cascades in seeds and in roots. These interactions are antagonistic in the control of seed dormancy but synergistic in inhibiting root growth. Furthermore, in each of these two processes, the interaction between ABA and ethylene pathways is embedded in a specific and more complex web of multihormonal regulations. These results illustrate that hormone signaling cascades interact in elaborate networks highly dependent on the organ and the developmental stage of the plant. The signaling elements at the points at which these various pathways intersect and the mechanisms underlying the integration of the corresponding signals remain to be identified.

METHODS

Plant Material and Growth Conditions

The *Arabidopsis thaliana* mutants *abi1-1* (Koornneef et al., 1984), *abi3-1* (Koornneef et al., 1984), and *abi3-4* (Ooms et al., 1993) are in the Landsberg *erecta* (L*er*) ecotype and were obtained from Maarten Koornneef (Agricultural University, Wageningen, The Netherlands). The mutants *ein2-1* (Guzman and Ecker, 1990), *etr1-1* (Bleecker et al., 1988), *ctr1-1* (Kieber et al., 1993), and *axr2-1* (Wilson et al., 1990) are in the ecotype Columbia (Col) and were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK).

Plants were grown in a greenhouse (22°C with a 16-hr-light photoperiod) on soil irrigated with mineral nutrients. Seeds used for comparative studies were derived from plants grown and harvested simultaneously. For in vitro experiments, seeds were surface-sterilized and plated on medium containing Murashige and Skoog (1962) inorganic salts at half concentration, 100 mg/L *myo*-inositol, 1 mg/L thiamine-HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 1% sucrose, 2.5 mM MES-KOH, and 0.8% agar, pH 5.7. Except in dormancy tests, after the seeds were plated on agar medium, they were incubated for 3 to 4 days at 4°C in darkness to break dormancy before being transferred to 21°C. Unless otherwise indicated, plates were incubated at 21°C with a 16-hr-light photoperiod. When indicated, abscisic acid (ABA) (mixed isomers; Sigma) or 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma) was added to the medium. ACC stock solutions were prepared in water. ABA stock solutions were prepared in methanol, and equivalent volumes of methanol were added to the ABA-free control plates.

Screen for Suppressors and Enhancers of *abi1-1*

The screen for suppressors of *abi1-1* has been described previously (Gosti et al., 1999). The suppressor line *84B* (*abi1-1 ein2-45*) was backcrossed twice to *abi1-1* before detailed analysis. The screen for phenotypic enhancers of *abi1-1* was performed with the same M_2 population as for the suppressor screen (Gosti et al., 1999). Approximately 1000 seeds from each of the 185 independent M_2 pools (each pool corresponding to ~15 viable M_1 plants) were surface-sterilized and plated on agar plates containing 100 μM ABA. Seeds were kept for 4 days at 4°C in darkness to break dormancy and then were transferred to 21°C with a 16-hr-light photoperiod for 5 days. Germinated seeds that had developed green cotyledons or an elongated root with hairs (or both) were selected. These candidates were transferred to ABA-free medium for 1 week before being propagated

in soil. M_3 seeds were retested for ABA resistance at germination. Enhancers originating from the same pool of M_2 seeds were assumed to be siblings, and only one representative was retained for further analysis. The enhancer line *H3* (*abi1-1 ctr1-10*) was backcrossed to *abi1-1* before detailed analysis.

DNA Sequencing

The original *CTR1* (Kieber et al., 1993) and *EIN2* (Alonso et al., 1999) sequences were from the Col ecotype, whereas the *ctr1-10* and *ein2-45* mutants are in the L*er* ecotype. To identify the *ctr1-10* mutation, we obtained overlapping genomic fragments encompassing the entire *CTR1* gene from both the L*er* wild type and *ctr1-10* DNA by polymerase chain reaction with specific primers, and the amplified products were sequenced directly by using appropriate primers. Comparing the sequences revealed that the *ctr1-10* mutation correlated with a single nucleotide change in the coding region. Moreover, the *CTR1* gene contains only silent nucleotide differences between the Col and L*er* wild-type ecotypes. A similar strategy was followed to identify the *ein2-45* mutation. In addition to various silent nucleotide differences, the *EIN2* gene contains two nonsilent differences between the Col and L*er* wild-type ecotypes: Tyr-160 in Col is replaced by Cys in L*er*, and Thr-1288 in Col is replaced by Pro in L*er*.

Genetic Mapping

The suppressor mutation in line *84B/1* was mapped by using simple sequence length polymorphisms (Bell and Ecker, 1994) and cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel, 1993), markers that detect polymorphism between the L*er* and Col Arabidopsis strains.

Construction of Double and Triple Mutants

The *abi1-1 abi3-1* and *abi3-4 ein2-45* double mutants were selected in the F_2 progeny of crosses between the two corresponding homozygous parents. The *abi1-1 abi3-1 ein2-45* triple mutant was selected in the F_2 progeny of a cross between *abi1-1 abi3-1* and *84B* (*abi1-1 ein2-45*). The *ein2-45* mutation was tracked through its inhibiting the formation of the triple response in dark-grown seedlings treated with 10 μM ACC. The *abi1-1* and *abi3-1* genotypes were verified by using polymerase chain reaction markers. A CAPS marker that distinguishes the wild-type *ABI1* allele from the *abi1-1* allele has been described previously (Leung et al., 1997). A dCAPs marker (Neff et al., 1998), which could distinguish *ABI3* from *abi3-1*, was generated. The oligonucleotide primers 5′-TCCTTCCGAGGTGACCCA-CGT-3′ and 5′-CGGAAGATTATACATTTGCAATGG-3′ amplify a DNA fragment that contains a PshA1 restriction site in *ABI3* but not in *abi3-1*. Finally, the *abi3-4* mutation was followed on the basis of its associated green seed phenotype (Ooms et al., 1993).

Measurement of Ethylene Production

Ethylene production was measured on seedlings grown at 21°C with a 16-hr-light photoperiod. After growing for 6 days on germination medium, wild-type L*er* seedlings were transferred to 12 × 12-cm Petri plates (~200 seedlings per plate) containing 45 mL of either normal medium or medium supplemented with either 100 μM ABA or 10 μM ACC. The lids of these Petri plates were equipped with a septum. Three days after transfer the plates were sealed, and 1-mL air samples were collected 1 hr later for analysis. Ethylene was measured with a gas chromatograph fitted with a flame ionization detector.

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