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Antagonism between abscisic acid and ethylene in *Arabidopsis* acts in parallel with the reciprocal regulation of their metabolism and signaling pathways

Wan-Hsing Cheng · Ming-Hau Chiang · San-Gwang Hwang · Pei-Chi Lin

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Abstract Although abscisic acid (ABA) and ethylene have antagonistic functions in the control of plant growth and development, including seed germination and early seedling development, it remains unknown whether a convergent point exists between these two signaling pathways or whether they operate in parallel in *Arabidopsis thaliana*. To elucidate this issue, four ethylene mutants, *ctr1*, *ein2*, *ein3*, and *ein6*, were crossed with *aba2* (also known as *gin1-3*) to generate double mutants. Genetic epistasis analysis revealed that all of the resulting double mutants displayed *aba2* mutant phenotypes with a small plant size and wiltiness when grown in soil or on agar plates. Further ethylene sensitivity or triple response analyses demonstrated that these double mutants also retained the *ctr1* or *ein* mutant phenotypes, showing ethylene constitutive triple and insensitive responses, respectively. Our current data therefore demonstrate that ABA and ethylene act in parallel, at least in primary signal transduction pathways. Moreover, by microarray analysis we found that an *ACC oxidase* (*ACO*) was significantly upregulated in the *aba2* mutant, whereas the *9-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*) gene in *ein2* was upregulated, and both the *ABSCISIC ACID INSENSITIVE1* (*ABI1*) and *cytochrome P450*, *family 707*, *subfamily A*, *polypeptide 2* (*CYP707A2*) genes in *etr1-1* were downregulated. These data further suggest that ABA and ethylene may control the hormonal biosynthesis, catabolism, or signaling of each other to enhance their antagonistic effects upon seed germination and early seedling growth.

Keywords Abscisic acid · Ethylene · Antagonism · ABA2 · EIN2

Abbreviations

ABA	Abscisic acid
ABI	Abscisic acid insensitive
ACO	ACC oxidase
CPK	Calcium-dependent protein kinase
CYP707A2	Cytochrome P450, family 707, subfamily A, polypeptide 2
ERA1	Enhanced response to ABA1
ERF	Ethylene response factor
gin	Glucose insensitive
glo	Glucose oversensitive
Lox2	Lipoxygenase
NCED	9-cis-epoxycarotenoid dioxygenase
SnRK	SNF1-related protein kinase
VSP1	Vegetative storage protein 1
THI2.1	Thionin

Introduction

Abscisic acid (ABA) is a classic phytohormone that plays an important role in various aspects of plant growth and development, including seed maturation and dormancy (Karssen et al. 1983; Koornneef et al. 1989), stomatal closure (Leung and Giraudat 1998), and adaptation to environmental stress (Qin and Zeevaart 2002; Xiong et al. 2002). In the past decade, significant advances have been made to better understand the expression and regulation of

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W.-H. Cheng (✉) · M.-H. Chiang · S.-G. Hwang · P.-C. Lin
Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan, Republic of China
e-mail: whcheng@gate.sinica.edu.tw

ABA biosynthesis and its signaling genes at the molecular level. Quite recently, most of the key components involved in ABA biosynthesis have been isolated and characterized through genetic and biochemical screens for ABA-deficient mutants in *Arabidopsis* that show an early germination phenotype (North et al. 2007; Dall'Osto et al. 2007; for reviews, see Finkelstein et al. 2002; Seo and Koshiba 2002; Schwartz et al. 2003; Xiong and Zhu 2003). For instance, ABA1, a zeaxanthin epoxidase (ZEP), catalyzes the epoxidation of zeaxanthin and antheraxanthin to violaxanthin in plastids (Marin et al. 1996; Xiong et al. 2002). After structural modification, violaxanthin is converted to 9-cis-epoxycarotenoid through ABA4 activity and/or other isomerase(s) (North et al. 2007). The epoxycarotenoids 9′-cis-neoxanthin and/or 9-cis-violaxanthin are then oxidized by 9-cis-epoxycarotenoid dioxygenase (NCED) to generate a C15 intermediate, xanthoxin (Schwartz et al. 1997). The product xanthoxin is then transported to the cytosol and further converted to abscisic aldehyde by a short-chain dehydrogenase/reductase 1, encoded by *ABA2* in *Arabidopsis* (Rook et al. 2001; Cheng et al. 2002; González-Guzmán et al. 2002). In the last step of ABA biosynthesis, abscisic aldehyde is oxidized to form abscisic acid by *Arabidopsis* aldehyde oxidase 3 (AAO3) (Seo et al. 2000), which needs a molybdenum cofactor sulfurase encoded by ABA3 (Bittner et al. 2001; Xiong et al. 2001) for its activity. Of these genes, ABA2 acts as a link between sugar and ABA signaling (Cheng et al. 2002) and its expression is upregulated by prolonged stress. Thus, it is proposed that ABA2 has a fine-tuning function in mediating ABA biosynthesis through primary metabolic changes in response to stress (Lin et al. 2007).

Likewise, genetic screens for reduced ABA inhibition of seed germination have identified several components that participate in ABA signaling including ABI1 to ABI5 and ABI8. *ABI1* and *ABI2* encode homologous serine/threonine phosphatase 2C proteins (Leung et al. 1997) that play a negative role in ABA signal transduction (Sheen 1998; Gosti et al. 1999). *ABI3* is an ortholog of maize *VP1*, a B3-domain containing transcription factor (Brady et al. 2003). The ABI4 and ABI5 proteins belong to an APETALA2-domain and a β-basic leucine zipper transcription factors, respectively (Finkelstein et al. 1998; Finkelstein and Lynch 2000). ABI8 is a novel protein of unknown function (Brocard-Gifford et al. 2004). In addition to genetic screens for mutants with reduced ABA sensitivity, ABA hypersensitive mutants have also been isolated and characterized. Most of the corresponding genes in these cases are involved in RNA processing and protein metabolism (for review, see Finkelstein et al. 2008). For instance, *Enhanced Response to ABA1* (*ERA1*), encoding a farnesyl transferase, is involved in the farnesylation of signaling proteins (Cutler et al. 1996). Mutation of RPN10, a subunit of the 26S proteosome, also causes ABA hypersensitivity (Smalle et al. 2003). However, the mechanisms by which RNA processing and protein metabolism alter ABA sensitivity in response to stress remain largely unknown. Recently, two SNF1-related protein kinases (SnRK2.2 and SnRK2.3) (Fujii et al. 2007) and two calcium-dependent protein kinases (CPK4 and CPK11) (Zhu et al. 2007) in *Arabidopsis* have been shown to regulate ABA signal transduction and to affect seed germination, root or seedling growth, and other phenotypes. These data further support the involvement of protein kinases in the ABA signaling effects that subsequently regulate plant growth and development.

In addition to ABA, ethylene is another stress-induced hormone with fundamental roles in germination, sex determination, leaf abscission, flower senescence, fruit ripening, and responses to biotic and abiotic stress (for review, see Johnson and Ecker 1998). It has been shown that a subset of the functions of ethylene overlaps with those of ABA. Ethylene, for instance, also participates in seed germination and in early seedling establishment, albeit with opposite effects to ABA (Zhou et al. 1998). The *enhanced response to ABA3* (*era3*) mutant was shown to be a new allele of *ein2* that shows hypersensitivity to ABA in seed germination, but an insensitivity to ABA in root growth (Ghassemian et al. 2000). Similarly, *ctr1* and *ein2* were recovered as an enhancer and a suppressor, respectively, of the ABA-resistant seed germination of *abi1-1* (Beaudoin et al. 2000). CTR1 belongs to the Raf family of Ser/Thr protein kinases and negatively regulates ethylene signaling (Kieber et al. 1993). The mutation of CTR1 in the *ctr1-1* mutant causes an ethylene constitutive triple response and insensitivity to sugar (Zhou et al. 1998). EIN2 is a central component of ethylene signaling and plays important roles in crosslinking multiple hormones and stress (Alonso et al. 1999; Wang et al. 2007). It was also reported that ABA-deficient mutants of *Arabidopsis aba2* and tomato *flacca* and *notabilis* reveal inhibition of shoot growth, largely because of high ethylene production in these mutants (Sharp et al. 2000; LeNoble et al. 2004). Hence, the ABA and ethylene signaling pathways have a close interplay in plant growth, development, and stress response. However, it remains unknown whether their respective signal transduction pathways have any convergent points or function only in parallel. To elucidate this issue, four double mutants were generated by crossing the ethylene mutants, *ctr1*, *ein2*, *ein3*, and *ein6*, with the *aba2* (or *gin1-3*) mutant. The reason that we used *aba2* in this study rather than an ABA signal mutant is that the ABA has multiple sites of perception and signaling pathways. All *ABI* genes identified to date only respond to parts, but not all, of ABA or stress signal transduction pathways. However, the use of *aba2* lacking ABA biosynthesis will thus block all of ABA signal transduction pathways. Genetic

epistasis analysis revealed that these double mutants displayed both an *aba2* and a *ctr1*/or *ein* mutant phenotypes, i.e., a small plant size and either a constitutive triple response or insensitivity to ethylene. This indicates that ABA and ethylene act in parallel, at least during primary (or early) signal transduction. Further microarray analysis of the *aba2*, *ein2*, and *etr1* mutants suggested that ABA and ethylene may control the hormonal biosynthesis, catabolism, or signaling of each other to enhance their mutually antagonistic effects upon seed germination and early seedling growth.

Materials and methods

Plant materials and growth conditions

Plant materials used in this study were the *Arabidopsis* (*Arabidopsis thaliana*) ecotypes Columbia (Col) and Landberg *errata* (L*er*). The Col ecotype was used in most experiments. The *aba2* mutant is the *glucose insensitive1-3* (*gin1-3*) allele with a 53-bp deletion at the start of exon 2 (Cheng et al. 2002). The ethylene mutants included in this study were *etr1-1* (Col), *ctr1-1* (Col), *ein2-1* (Col), *ein3-1* (Col), and *ein6-1* (L*er*), all of which harbor mutations in genes involved in the ethylene signal transduction pathway; the corresponding mutant seeds were requested from the Arabidopsis Biological Resource Center (ABRC). The *eto1-4* (Col) mutant, which causes ethylene overproduction, was also used in this study. All seeds were sterilized and subjected to cold pretreatment at 4°C for 3 days in the dark, and then grown on agar plates or in soil at the first day of germination or planting. Seed germination and seedling growth were performed at 24°C under a long day (16-h light/8-h dark cycle) with a light intensity of $\sim$80 $\mu Es^{-1}m^{-2}$. Details of the seed sterility and medium preparation methods have been described previously (Lin et al. 2007).

Isolation of the *ctr1aba2* or *einaba2* double mutants

For the double mutant isolation, the *ctr1* and *ein* mutants (*ein2*, *ein3*, and *ein6*) were crossed with the *aba2* mutant to generate F1 seeds. These F1 seeds were then germinated in soil, and mature plants were self pollinated to produce F2 seeds. Subsequently, the F2 seeds were grown on 1% sucrose medium supplemented with or without 10 μM ACC for 5 days in the dark. For the *ctr1aba2* double mutant, the F2 etiolated seedlings grown on 1% sucrose medium without ACC and showing an ethylene triple response were transferred to the same fresh medium under light conditions for 14 days prior to transplantation to soil. For the *einaba2* double mutants, etiolated seedlings grown on medium with 10 μM ACC and showing an ethylene-insensitive phenotype were transferred to the same fresh medium without ACC under light conditions for 14 days prior to transplanting to soil. The genotypes of the double mutants were further confirmed by genomic PCR using primers that amplified the 53-bp deletion region in exon 2 of the *aba2* allele as described previously (Lin et al. 2007). This testing was performed for at least one further generation to validate the double mutants with an ethylene-responsive phenotype and an *aba2* background.

Isolation of the *aco* and *acoaba2* double mutants

The *aco* mutant seeds for a SALK T-DNA line (Alonso et al. 2003) were requested from the ABRC stock center (Columbus, OH) with the accession no. SALK_082132. The T-DNA insertion at the *ACO* (*At1g12010*) locus was confirmed by genotyping. RT-PCR analysis confirmed that the *aco* mutant is a null mutant with an undetectable transcript. For the *acoaba2* double mutant isolation, the *aco* plant was crossed with *aba2*. In the resulting F2 segregating population, the *acoaba2* double mutant was identified by genotyping for the respective mutant alleles. The putative double mutant was thus confirmed and showed no segregation in the F3 generation.

Germination and root elongation tests

For germination testing, seeds were sterilized, cold pretreated, and then grown on medium supplemented with sucrose or glucose. The medium was autoclaved and cooled to 50–60°C prior to the addition of filter-sterilized ABA [(±)-abscisic acid; Sigma; catalog no. A-1049], ACC (1-aminocyclopropane-carboxylic acid; Sigma; catalog no. A3903), or AVG [L-α-(2-aminoethxyvinyl)glycine hydrochloride; Sigma; catalog no. A6685] at the various concentrations indicated in the text. The procedure for the root elongation analysis has been described previously (Lin et al. 2007).

Dehydration and water loss experiments

For dehydration, seeds subjected to cold pretreatment were grown in soil for 21 days, and subsequently pots were soaked in water for 5 min. The excess water was removed by transferring pots to a clean tray for another 5 min, and the plants were transferred to a new clean tray where watering was withheld for the periods indicated in the text. For water loss tests, the soil-grown plants at 21 days of age were excised and the aerial parts of tissues were placed on plastic weigh boats and kept in an electronic dry box (model-DX-76, Taiwan-DX-76, Taiwan Dry Tech Corp.) with a relative humidity of approximately 40%. The fresh

weights of the tissues were measured at 30-min intervals for 3 h.

Ethylene and ABA measurements

To assay for ethylene, the sterilized seeds having undergone a cold pretreatment were sown in 20-ml flat-bottom Headspace vials (catalog no. 5182-0837, Agilent Technologies, Santa Clara, CA), each with 33 seeds. Each vial containing 10 ml of modified MS medium supplemented with 1% sucrose was sealed using a crimp cap (catalog no. 5183-4477, Agilent Technologies) and placed in a culture room at 24°C in the dark for 3 or 4 days. Subsequently, the vials containing etiolated seedlings were arranged in a Headspace Sampler (Aglient G1888 Network Headspace Sampler) with a connection to gas chromatography (Hewlett Packard HP6890 GC System) to measure the ethylene contents. The measurement protocols essentially followed the manufacturer's instructions.

For the ABA assay, seedlings grown on 1% sucrose agar plates for 12–14 days were harvested, followed by ABA extraction, purification, and measurement of ABA as described previously (Lin et al. 2007).

Microarray assay

Cold pretreated seeds were grown on 1% sucrose agar plates for 12–14 days. Total RNAs were extracted as described (Lin et al. 2007), and 10 μg aliquots were then used for cDNA synthesis and labeling by in vitro transcription followed by fragmentation according to the GeneChip Expression Analysis Technical Manual rev5, Affymetrix. Each labeled sample (11 μg) was hybridized to an ATH1 GeneChip at 45°C for 16.5 h. The washing and staining steps were performed using a Fluidic Station-450, and the ATH1 slides were scanned using the Affymetrix GeneChip Scanner 7G. Subsequent data processing and analysis were performed using the Affymetrix Microarray Suit, version 5.0 software. Two independent sets of microarray analyses were performed in this study.

Semiquantitative RT-PCR

Seedlings grown on 1% sucrose agar plates for 12–14 days were harvested and used for total RNA extraction. The extraction procedure and reverse transcriptase (RT)-PCR were followed the protocol described previously (Lin et al. 2007). For semiquantitative RT-PCR, at least three different program cycles were used to obtain the optimal PCR conditions. The resulting PCR products were separated and visualized on 1% agarose gels stained with ethidium bromide. The band intensity of RT-PCR products was quantified with Quantity One 4.5 version software (Bio-Rad Laboratories).

Results

Phenotypic comparisons of single and double mutants for the ABA and ethylene signaling pathways

Although ABA and ethylene function antagonistically in several aspects of plant growth and development, including seed germination and postgermination seedling establishment, it remains unknown whether there is any point of convergence between these two signaling pathways, or if they act in parallel only. To address this, four ethylene mutants, *ctr1*, *ein2*, *ein3*, and *ein6*, were crossed with the *aba2* (or *gin1-3*) mutant to generate double mutants. As *ctr1* (*constitutive triple response1*) is a loss-of-function mutant that manifests as a negative regulator of CTR1 in response to ethylene, the double mutant *ctr1aba2* displayed a *ctr1* phenotype with small leaves and plant size (Kieber et al. 1993) when grown on 1% sucrose agar plates for 14 days (Fig. 1A) or in soil for 21 or 42 days (Fig. 1B, C). Our detailed analysis further showed that the *ctr1aba2* double mutant developed a wilty phenotype (data not shown) and a smaller plant size than that of the *ctr1* single mutant (Fig. 1). Significantly, these are phenotypes that are typical of *aba2*. Similarly, combining a loss-of-function of the *ein2*, *ein3*, and *ein6* alleles with the *aba2* locus still produced a typical *aba2* appearance in the aerial portions of the plant, i.e., a small plant size and dark-green leaves, when grown on 1% sucrose agar plates or in soil (Fig. 1). Our previous data showed that the *aba2* mutant has smaller siliques and fewer seeds than those of the wild type (Cheng et al. 2002). Similarly, in our current analysis, the double mutants also displayed smaller siliques and produced less seeds, compared with the corresponding *ctr1* or *ein* single mutants (data not shown). Taken together, these data suggest that the double mutants, *ein2aba2* and *ein3aba2*, have phenotypes that closely resemble the *aba2* mutant, except that the *ctr1aba2* displays both the *ctr1* and *aba2* phenotype, because *ctr1* is a loss-of-function mutant and confers a constitutive ethylene signal transduction.

Comparison of the effects of drought treatment and water loss upon the single and double ABA/ethylene mutants

In addition to a small plant size, the ABA-deficient mutant *aba2* is characterized by a wilty phenotype. To confirm whether or not the double mutants also harbored this phenotype, 28-day-old plants were subjected to drought conditions. The results demonstrated that the

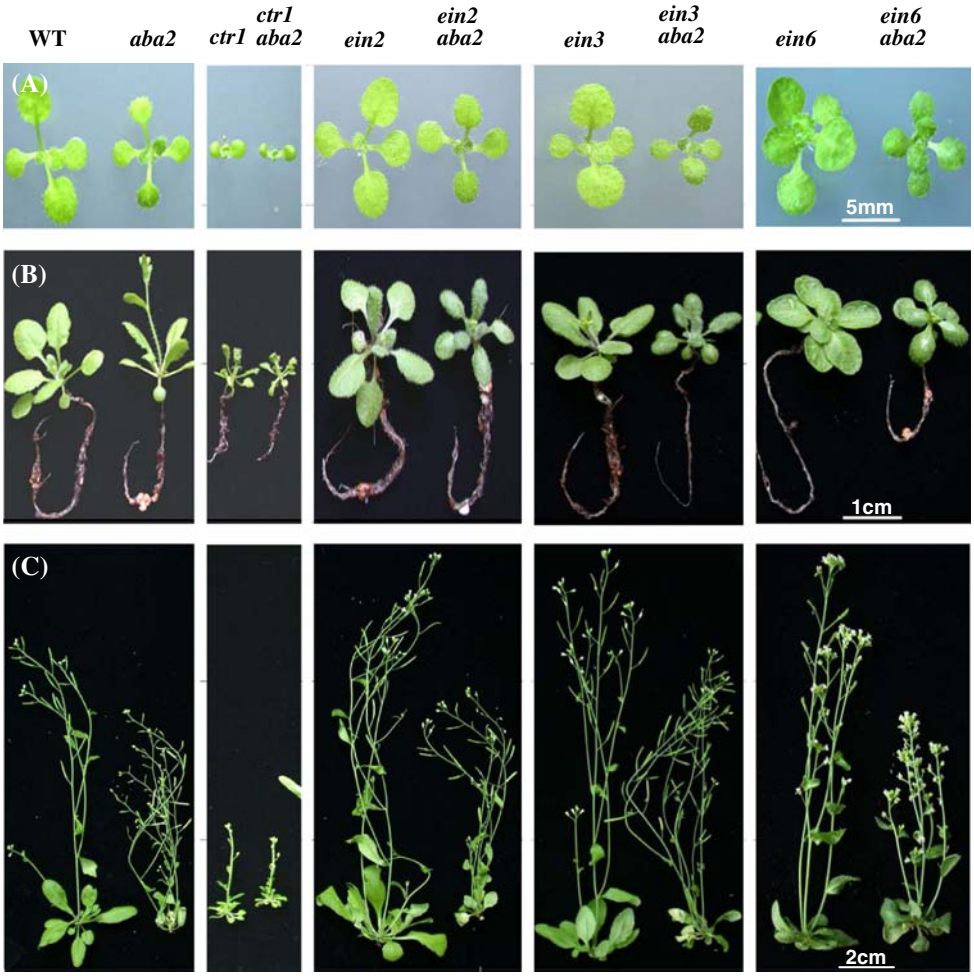

Fig. 1 Phenotypic comparison of single and double ABA/ethylene mutants. **A** Seeds were grown on 1% sucrose agar plates for 14 days. **B** Seeds were grown in soil for 21 days. **C** Seeds were grown in soil for 42 days. Three independent experiments were performed, each with at least 12 plants and giving consistent results. WT wild type

aba2, *ein2aba2*, and *ein3aba2* mutants developed a severe wilty phenotype, after the withholding of water for 4 days (Fig. 2A). In contrast, the wild type, *ein2*, and *ein3* plants retained a normal appearance even after 8 days of drought treatment. For relative water loss, the 28-day-old plants were removed, and the aerial regions were subjected to a water loss test. As shown in Fig. 2B and C, the *ein2* and *ein3* plants displayed a relative water loss pattern similar to the wild type, whereas the *ein2aba2* and *ein3aba2* double mutants showed a water loss pattern that resembled the *aba2* single mutant. Similar results were also observed for *ctr1* versus *ctr1aba2* and *ein6* versus *ein6aba2* comparisons (data not shown). Taken together, we conclude from these results that *aba2* has greater effects on aerial phenotypes (wiltiness and plant size) than *ein* mutants do.

Comparison of the ethylene responses of the ABA/ethylene mutants

Based on the aforementioned data, the ABA-signaling pathway has greater effects on aerial phenotypes than ethylene signaling does. As ethylene may induce root hair formation, we tested whether a constitutive triple ethylene response was gained in *ctr1aba2* and whether insensitivity to the ethylene phenotype arose in the *einaba2* mutants. All mutants were grown on 1% sucrose agar plates with or without ACC (10 μM). As shown in Fig. 3, in the absence of ACC, only the *ctr1* and *ctr1aba2* plants exhibited hairy roots, whereas the other genotypes showed no apparent induction of root hair formation (Fig. 3A). In the presence of ACC, in addition to *ctr1* and *ctr1aba2*, the wild-type and *aba2* plants also displayed hairy roots at the newly growing roots, whereas the *ein* and *einaba2* mutants showed no apparent root hair formation. It is notable also that all of the genotypes tested in these analyses revealed short roots following treatment with ACC and that *ctr1aba2* had more pronounced hairy root formation compared with its parental mutant *ctr1* (Fig. 3B). However, the mechanisms underlying the enhanced hairy root formation in *ctr1aba2* remain unknown. In addition to above data, *aba2* and each of the double mutants displayed a shorter primary root length on average than the wild-type and corresponding single mutant plants (Fig. 3A).

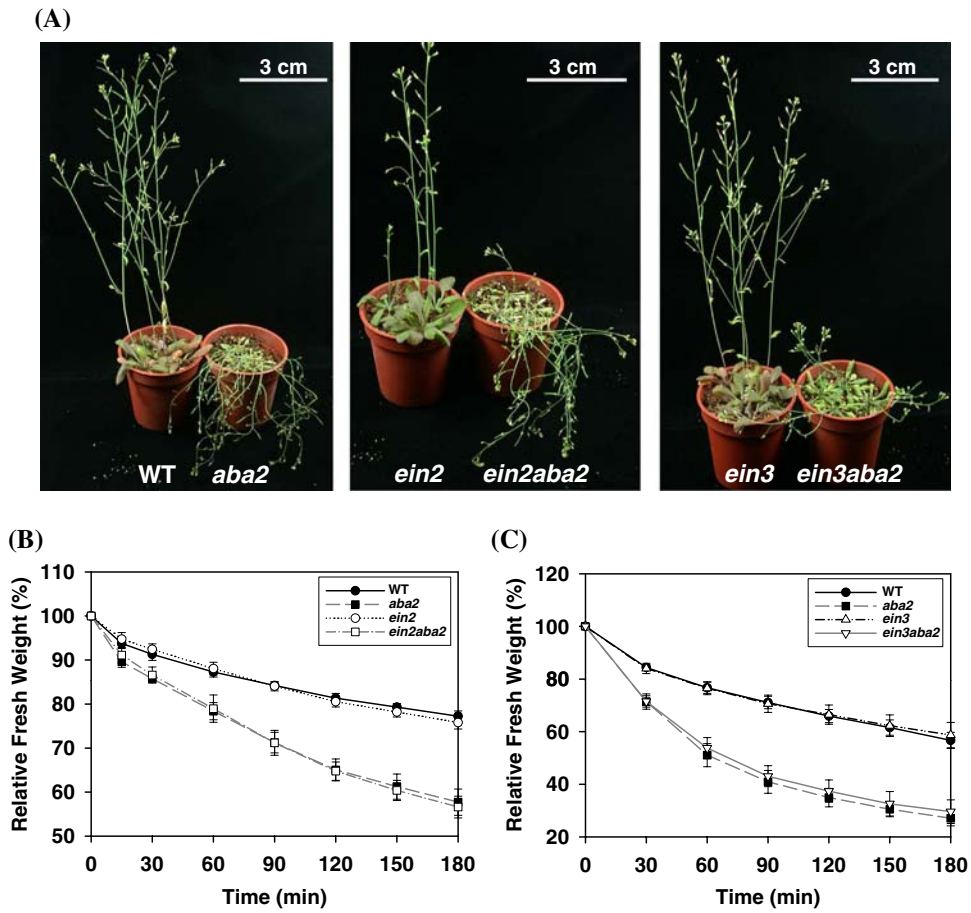

Fig. 2 Comparison of the effects of drought and water loss upon single and double ABA/ethylene mutants. **A** Drought treatment. Seeds were grown in soil for 28 days, and the resulting plants did not receive water for 4 additional days. Two independent experiments were performed, each with plants ($n \geq 9$), and gave consistent results. **B** and **C** Water loss test. Seeds were grown in soil for 28 days, and then the aerial portions were removed and subjected to a water loss test in an electronic dryer. The results shown are the means ± SD of triplicate analyses in one experiment, and each repeat was with two to five plants. Two independent experiments were performed and gave consistent results

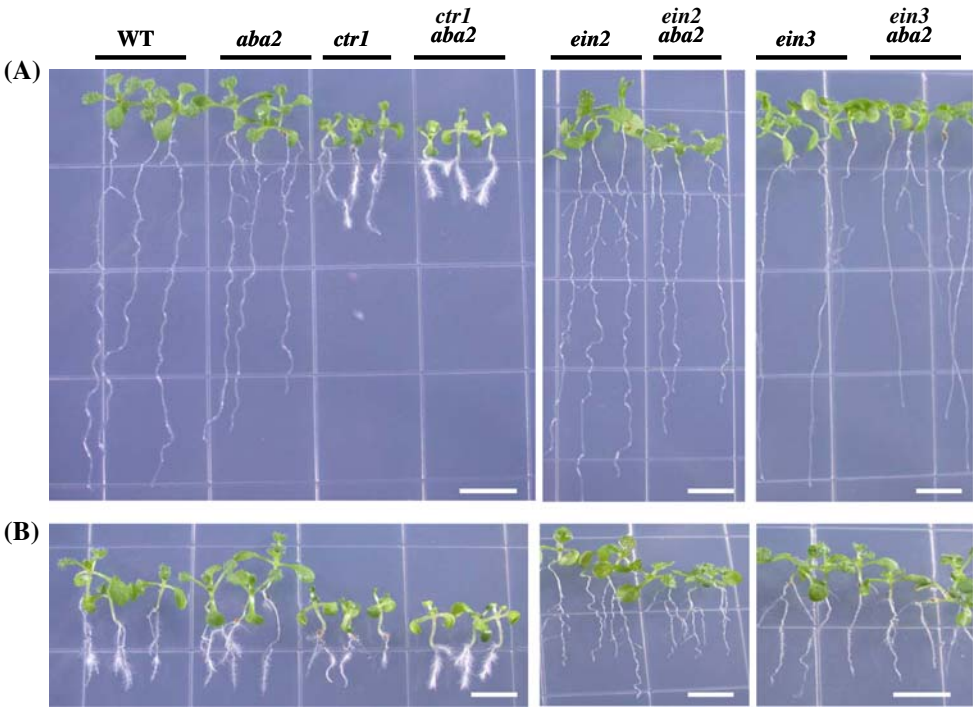

Fig. 3 Ethylene induction of root hair formation. Seeds were grown on 1% sucrose agar plates for 4 days, and the seedlings were transferred to the same fresh medium without ACC (**A**) or with 10 μM ACC (**B**) and grown vertically for another 5 days. Three independent experiments were performed, giving consistent results; each experiment had seedlings ($n \geq 6$). Scale bars represent 6 mm

As the *ctr1* and *ein* mutants were isolated according to their ethylene triple response under dark conditions, we tested the triple response of the *ctr1aba2* and *einaba2* double mutants following exposure to ACC in the dark. Our results showed that the wild type and *aba2* were ACC sensitive and had a primary root length that manifested ~87 and 84% reductions, respectively, relative to the wild type plants grown on the medium without ACC (Fig. 4B). The primary root in the *aba2* mutant was 28% shorter than that of the wild-type plant in the absence of ACC (Fig. 4B). The *ctr1* and *ctr1aba2* mutants had a constitutive ethylene triple response when grown on the media with or without ACC. However, *ctr1aba2* plants had only 43% of the primary root length of the *ctr1* mutant without ACC treatment. Although the *ctr1-1* allele is very strongly associated with the constitutive triple response to ethylene, its primary root showed a ~37% reduction in length in the presence of ACC, reflecting the fact that *ctr1* plants are still weakly sensitive to this agent. The *ctr1aba2* mutants also had a 27% reduction in their primary root elongation in the presence of ACC, which was slightly shorter than that in the *ctr1* single mutant under the same growth conditions. Each of the *ein* mutants exhibited an ACC insensitive phenotype (Fig. 3A, B) relative to the wild type in the presence of ACC. Similarly, the *einaba2* mutants also exhibited this phenotype when grown on the media with or without ACC. Notably, the double mutants all had shorter primary root lengths than their corresponding *ein* single mutants under the same growth conditions. The reduction in the primary root length in the double mutants, *ein2aba2* and *ein3aba2*, was about 31 and 23% shorter, respectively, than that of the corresponding *ein* single mutants when grown on the medium without ACC. The primary root lengths in the *ein6* and *ein6aba2* plants were more sensitive to ACC and showed a 50% reduction compared to plants grown in the absence of ACC.

In the case of hypocotyls, the wild-type and *aba2* plants showed similar lengths in both the presence and absence of ACC. In the presence of ACC, the wild-type and *aba2* hypocotyl lengths were reduced by 52 and 48%, respectively, relative to the lengths measured in the absence of ACC. The *ctr1* and *ctr1aba2* mutants with a constitutive ethylene triple response phenotype showed similar hypocotyl lengths with a 34% reduction relative to wild type in the absence of ACC. Upon ACC treatment, both *ctr1* and *ctr1aba2* also showed similar hypocotyl lengths with slight reductions compared to their growth without ACC. The *ein* and *einaba2* mutants revealed ACC insensitive phenotype. The double mutants, *ein2aba2* and *ein3aba2*, had a slight reduction in their hypocotyl lengths compared with the corresponding single *ein* mutants when grown on media with or without ACC. However, the *ein6aba2* plants had hypocotyl lengths similar to the *ein6* single mutant in both the presence and absence of ACC. As *ein6* is in the L*er* background, whereas *aba2* is in the Col background, we speculated that the *ein6aba2* double mutant might possess a certain degree of hybrid vigor. We thus further analyzed only the *ctr1aba2*, *ein2aba2*, and *ein3aba2* plants,

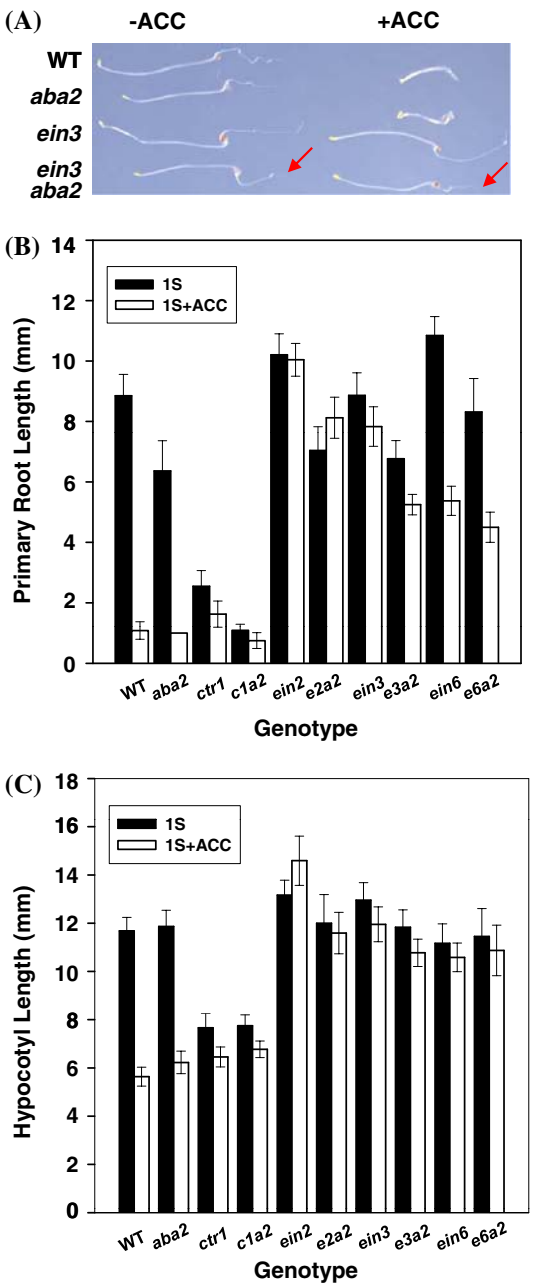

Fig. 4 Comparison of the ethylene response between single and double ABA/ethylene mutants. **A–C** Seeds were grown on 1% sucrose agar plates with or without ACC (10 μM) treatment for 4 days in the dark. A set of representative phenotypes is shown in (**A**); the *arrows* indicate the root tips. The etiolated seedlings were measured for primary root length (**B**) or hypocotyl length (**C**), and the data represent the means ± SD, $n = 8$–10, in one experiment. Three independent experiments were carried out and gave consistent results. *c1a2*, *ctr1aba2*; *e2a2*, *ein2aba2*; *e3a2*, *ein3aba2*; *e6a2*, *ein6aba2*

particularly in the latter two mutants. The data we obtained from the double mutants tested in this study revealed phenotypes that were similar to their respective ethylene single mutants (the constitutive triple response of *ctr1* and insensitive to ethylene response of *ein*). Hence, the double mutants conferred both the *aba2* phenotype and *ctr1* or *ein* phenotype. This indicates that based on their epistatic interaction, the ABA and ethylene signal transduction pathways act in parallel.

Ethylene sensitivity of root growth between the single and double ABA/ethylene mutants

To address the effects of ethylene upon primary root elongation, our current panel of single and double mutants was tested for their sensitivity to ethylene. As shown in Fig. 5A, the primary roots of the *aba2* mutant were sensitive to ACC and displayed a primary root elongation pattern that paralleled the wild type when the ACC concentrations were below 0.25 μM. Above this concentration of ACC, the *aba2* primary root length showed no difference from the wild type, indicating that 0.25 μM ACC is a saturating concentration that is sufficient to inhibit further primary root elongation between *aba2* and wild-type plants. Similarly, the double mutants *ein2aba2* and *ein3aba2* exhibited ACC insensitivity and have short but parallel primary root elongation to their corresponding *ein* single mutants, i.e., *ein2* and *ein3*, respectively, at all ACC concentrations tested.

Although the *aba2* mutant displayed a shorter primary root than that of the wild type plant, the relative reduction of primary root length in this mutant increased with the ACC concentration up to 0.5 μM, and this pattern very closely resembled the wild type response (Fig. 5B). These data indicate that the *aba2* mutant and wild-type plants have a near equal sensitivity to ACC, and hence the short primary root observed in the *aba2* mutant does not correlate with ACC sensitivity. In contrast to the above data, the relative reduction in primary root elongation in the *ein2aba2* double mutant was less altered and resembled the single mutant *ein2*. The double mutant *ein3aba2* exhibited a very slight increase in its relative reduction of primary root elongation following exposure to ACC and displayed a pattern that paralleled the *ein3* single mutant. These data lend further support to our contention that the double mutants are insensitive to ACC and suggest their shortened primary roots relative to the corresponding single mutants not due to a higher ACC sensitivity. In the case of

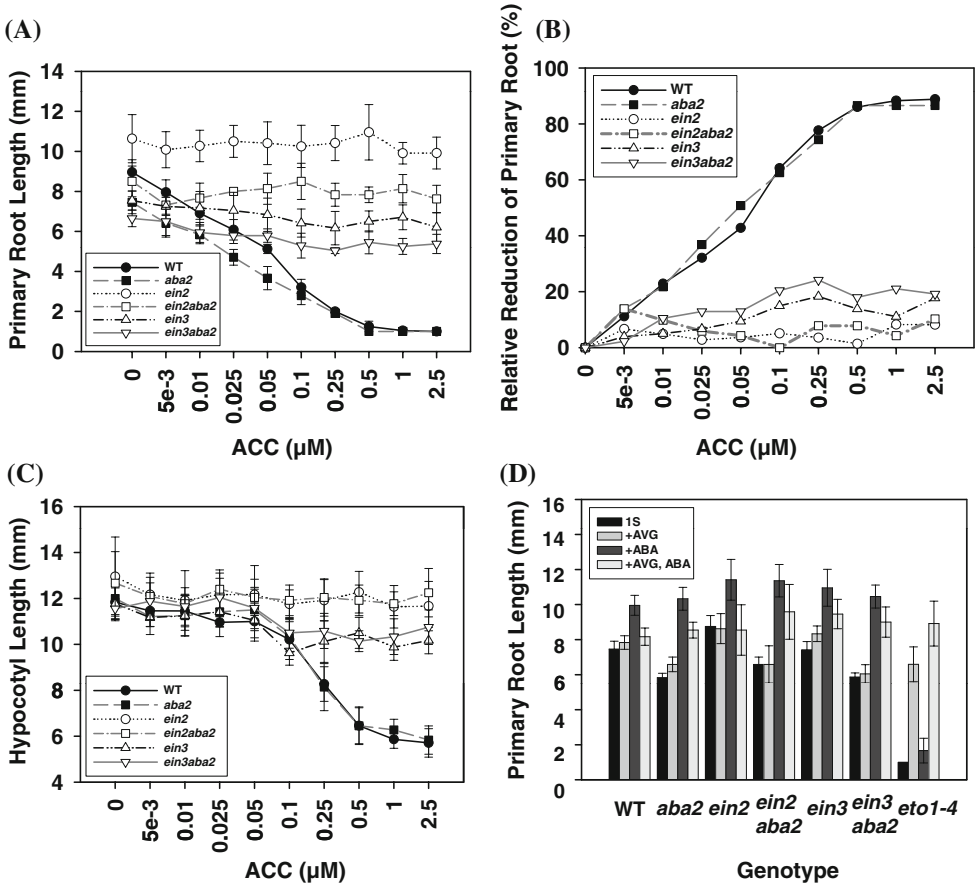

Fig. 5 Analysis of ethylene sensitivity between single and double ABA/ethylene mutants. **A–C** Seeds were grown on 1% sucrose agar plates supplemented with a series of different concentrations of ACC for 4 days in the dark. The resulting etiolated seedlings were then subjected to length measurements of their primary roots (**A**) or hypocotyls (**C**). In (**B**), the primary roots of ethylene-treated seedlings were normalized to a control without ACC treatment. **D** Seeds were grown on 1% sucrose agar plates supplemented with AVG, ABA, or AVG and ABA for 4 days under dark conditions. Subsequently, the etiolated seedlings were subjected to measurements of their primary root length. The data shown represent the means ± SD, $n = 8$–10. Three independent experiments were carried out and gave consistent results. WT, wild type; ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, L-α-(2-aminoethoxyvinyl)glycine hydrochloride

hypocotyl lengths, both the *aba2* and wild-type plants had shorter hypocotyls when treated with an ACC concentration over 0.1 μM, and both displayed a similar ACC sensitivity pattern. However, the *ein2* versus *ein2aba2* and *ein3* versus *ein3aba2* comparisons revealed a very similar and near parallel pattern of relative hypocotyl lengths for all ACC concentrations tested (Fig. 5C), again reflecting the nature of ACC insensitivity in *ein* and *einaba2* mutants.

In our previous study, we demonstrated that the short roots in the *aba2* mutant are due to the ABA deficit when grown in light (Lin et. al. 2007). It is thus likely that the short roots observed in the etiolated *aba2* and double mutants are due to the lack of ABA. To answer this question, the *aba2*, *ein2aba2*, and *ein3aba2* mutants were cotreated with ABA and AVG (aminoethxyvinylglycine), an ethylene biosynthesis inhibitor. As shown in Fig. 5D, the *aba2* mutant developed shorter primary roots than the wild type, and similar results were also observed for the *ein2aba2* and *ein3aba2* double mutants relative to their corresponding *ein2* and *ein3* single mutants. The *ethylene overproduction 1-4* (*eto1-4*) mutant displayed appreciably high levels of ethylene and a pronounced ethylene triple response when grown in dark conditions (Kieber et al. 1993). Upon the exogenous application of the ethylene biosynthesis inhibitor AVG, the wild-type, *aba2*, *ein2*, *ein2aba2*, *ein3*, and *ein3aba2* plants did not showed much change in their primary root lengths. However, the *eto1-4* mutant appeared to have restored lengths to wild-type levels (Fig. 5D). These data indicated that the short primary root observed in the *aba2*, *ein2aba2*, and *ein3aba2* mutants does not correlate with the endogenous ethylene contents, whereas the short primary root in the *eto1-4* mutant is mainly due to the high levels of ethylene. Moreover, the application of ABA to the growth medium promoted the primary root elongation of the *aba2*, *ein2aba2*, and *ein3aba2* mutants and caused a slight lengthening of the primary root compared to the plants with no ABA treatment. However, the primary root of the *eto1-4* mutant, in spite of the slight induction in its elongation following ABA treatment, remained very short relative to the wild type. The restoration of primary root elongation to normal levels was also observed following the addition of both AVG and ABA into the growth medium, although a slight reduction of primary root elongation was still observed compared with the addition of ABA alone. This suggests that the presence of ethylene at low endogenous concentrations is required for ABA to function in promoting root elongation. However, the *eto1-4* mutant grown in the presence of AVG and ABA displayed a longer primary root elongation than with the AVG treatment only. Presumably, the *eto1-4* plants might have a residual amount of ethylene production in the presence of AVG, which causes the enhancement of primary root elongation by ABA. Taken together, these data demonstrated that the shorter roots observed in the *aba2*, *ein2aba2*, and *ein3aba2* mutants are the result of an ABA deficit, whereas the short roots in the *eto1-4* mutant are mainly due to the overproduction of ethylene.

Effects of ABA and ethylene on seed germination and postgermination seedling growth in *Arabidopsis*

As shown in Fig. 6A, when grown on 2% glucose agar plates the *ctr1* and *aba2* mutants, respectively, possessing a constitutive ethylene triple response and an ABA deficiency, displayed earlier seed germination than wild-type plants within the first 3 days of germination. In the comparison between *ctr1* and *aba2*, the germination in the *aba2* mutant was earlier, and in the case of the *ctr1aba2* double mutant there was a slightly earlier germination than that of the *aba2* mutant. These data suggest that the combination of the *aba2* and *ctr1* alleles reveals no additive effect upon seed germination. The *ein2* mutant with a high ABA content (Ghassemian et al. 2000; Wang et al. 2007) displayed a significant germination delay relative to the wild type. Thus, the removal of ABA, as observed in the *ein2aba2* double mutant, considerably promoted seed germination in a manner that resembled the *ctr1* mutant. The *ein3* mutant, corresponding to a weak allele in terms of ethylene insensitivity, had a seed germination profile that was similar to the wild type. The block upon ABA biosynthesis in the *ein3aba2* double mutant enhanced the seed germination pattern so that it more closely resembled that of *aba2*. When grown on 4% glucose agar plates, the onset of seed germination of all genotypes was greatly delayed, particularly in the *ein2*, *ein3*, and wild-type plants (Fig. 6B). In general, the germination pattern on 4% glucose was found to be similar to that on 2% glucose with only a slight variation. Seed germination was also examined for two other ethylene-related mutants, *etr1-1* and *eto1-4*, because of the nature of their ethylene insensitivity and overproduction, respectively. The results showed that both *etr1-1* and *ein2* had a seed germination delay, but this was enhanced in the *eto1-4* and *ctr1* mutants (Fig. 6C). These data lend further support to the promotion of seed germination by an increase of ethylene signaling and/or production. Similar results were also observed when the seeds were grown on 4% glucose (Fig. 6D).

To examine the effects of ABA and ethylene upon early seedling growth, our panel of single and double ABA/ethylene mutants was grown on glucose media with/or without ABA and ACC added. The use of the glucose assay in this study made it easy to see the interactions between endogenous ABA and ethylene because ABA biosynthetic genes are induced, and its biosynthesis is increased in the presence of glucose (Cheng et al. 2002). As shown in

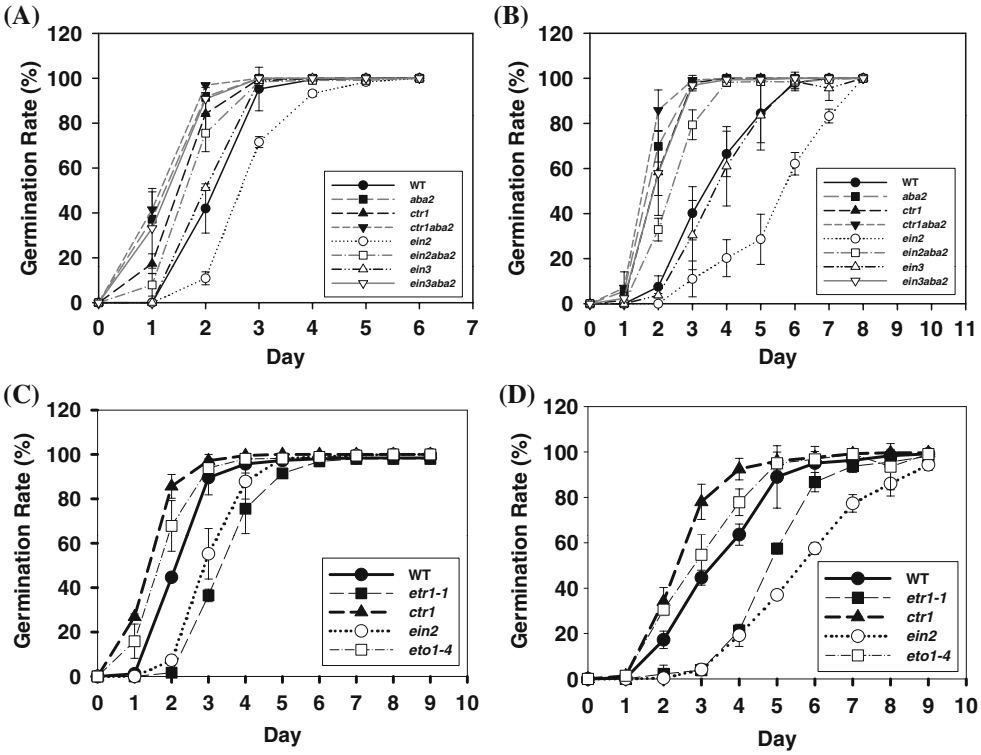

Fig. 6 Antagonistic effects of ABA and ethylene upon seed germination. **A**–**D** Seeds were grown on 2% (**A** and **C**) or 4% (**B** and **D**) glucose for the periods indicated. Radicle emergence over 1 mm is referred to as germination. Germination rates were counted as germinated seeds/total germinated seeds at day 10. The results shown are the means ± SD of three independent experiments using different seed batches and giving consistent results, each with 100–150 (**A** and **B**) or 100 (**C** and **D**) seeds

Fig. 7, wild-type plants displayed postgermination developmental arrest when grown on 6% glucose agar plate for 10 days, whereas the *aba2* mutant grew with expanded cotyledons and greening leaves, a typical glucose insensitive (gin) phenotype. *ctr1* also showed a gin phenotype despite having a smaller plant size than *aba2*. A gin phenotype was also observed in the *ctr1aba2* double mutant. In contrast, *ein2* showed developmental arrest on 4% glucose, which is a glucose oversensitive (glo) phenotype. The *ein3* mutant had a weak glo phenotype and a smaller plant size than the wild-type plants on 4% glucose concentration. The glo phenotype observed in the *ein2* and *ein3* plants reverted to a gin phenotype in the *ein2aba2* and *ein3aba2* double mutants because of the presence of the *aba2* locus and thus an ABA deficit in these plants. In the presence of ACC (10 μM), the developmental arrest observed in wild-type and *ein3* seedlings grown on 6% glucose disappeared, and a gin phenotype was evident. However, the *ein2* mutant

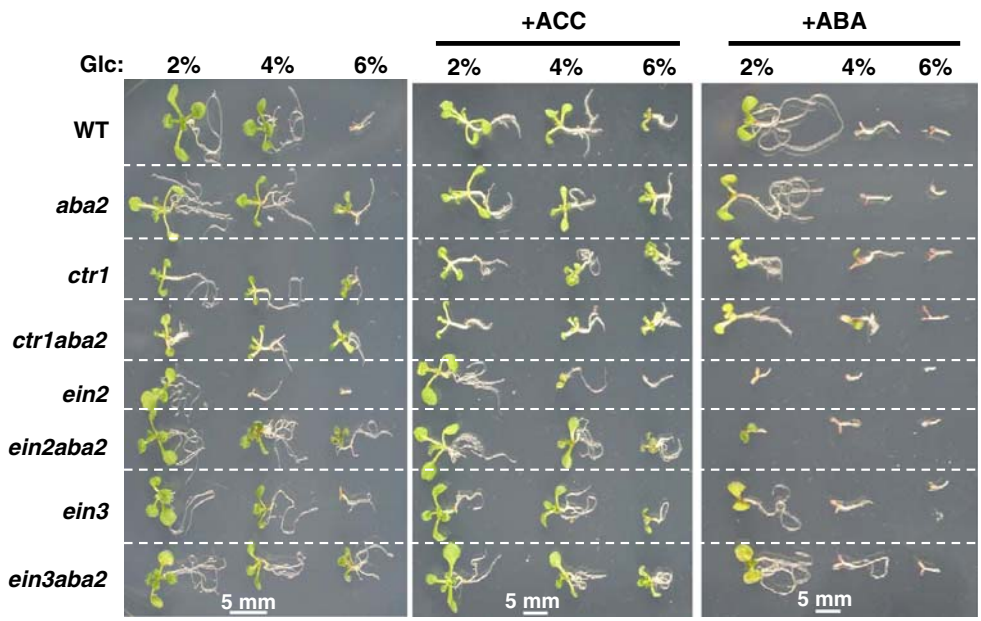

Fig. 7 Effects of ABA and ethylene upon early seedling growth. Seeds were grown on 2, 4, or 6% glucose agar plates supplemented with or without ACC (10 μM) or ABA (100 nM) for 10 days in light. Three independent experiments were performed, each with ≥30 seedlings and gave consistent results

still displayed a developmental arrest on 6% glucose. In contrast, the developmental arrest in the wild type first appeared at a 4% glucose concentration in the presence of ABA (0.1 μM). Under such growth conditions, the ABA-deficient mutant *aba2* reverted to a wild-type phenotype and also showed developmental arrest at 4% glucose. Similarly, the *ein2*, *ein3*, *ein2aba2*, and *ein3aba2* seedlings also exhibited developmental arrest at 4% glucose in the presence of ABA. It is noteworthy that the *ein2* mutant started to show arrested seedlings at 2% glucose. *ctr1* and *ctr1aba2* retained a gin phenotype at 4% glucose in the presence of ABA, but not at 6% glucose. Collectively, these data suggest that ethylene promotes seed germination and postgermination seedling development, whereas ABA inhibits these processes.

Analysis of the gene expression profile of the *aba2*, *ein2*, and *etr1-1* mutants

To further study the components that might be involved in the antagonistic interaction between ABA and ethylene, we analyzed the gene expression profile of the *aba2*, *ein2*, and *etr1-1* mutants using Affymetrix ATH1 GeneChip analysis. These mutant plants were grown on 1% sucrose agar plates for 12–14 days. Thus, in this study the gene expression profile is mediated by the endogenous ABA and ethylene interaction without exogenous application of hormones or stress. Our results showed that in the *aba2* mutant, there were 323 genes (188 up and 135 downregulated genes) with at least a 2-fold change in expression compared with the wild type (Table 1 and Supplementary Table S1; raw

Table 1 ABA- and ethylene-related gene expression in the *aba2*, *etr1*, and *ein2* mutants

Locus ID	Description[a]	Signal fold change[b]
Significant changes of gene expression in the *aba2* mutant		
At1g12010	Similar to 1-aminocyclopropane-1-carboxylate oxidase	2.60
At2g20880	Ethylene-responsive transcription factor ERF053 (ERF053)	10.2
At1g52340	**ABA deficient 2, ABA2; glucose insensitive 1, GIN1**	**0.48**
At5g59220	**Protein phosphatase 2C, putative; response to ABA stimulus**	**0.33**
At5g15960	**Cold and ABA inducible protein KIN1**	**0.31**
At2g29090	**CYP707A2: cytochrome P450, family 707, subfamily A, polypeptide 2**	**0.21**
Significant changes of gene expression in the *etr1* mutant		
At4g37770	ACS8, 1-Amino-cyclopropane-1-carboxylate synthase 8	7.85
At2g40940	ERS1, Ethylene response sensor 1	0.46
At1g77330	Similar to 1-Aminocyclopropane-1-carboxylate oxidase	0.44
At1g04310	ERS2, Ethylene response sensor 2	0.25
At3g16770	Ethylene-responsive element binding protein, ERF72	0.19
At5g25350	EBF2, EIN3-binding F box protein 2	0.22
At4g26080	**ABI1, Abscisic acid insensitive 1**	**0.41**
At2g29090	**CYP707A2: cytochrome P450, family 707, subfamily A, polypeptide 2**	**0.18**
Significant changes of gene expression in the *ein2* mutant		
At4g37770	ACS8, 1-Amino-cyclopropane-1-carboxylate synthase 8	3.87
At3g23150	ETR2, Ethylene response 2	0.35
At2g40940	ERS1, Ethylene response sensor 1	0.35
At5g03280	EIN2, Ethylene insensitive 2	0.23
At1g04310	ERS2, Ethylene response sensor 2	0.14
At5g25350	EBF2, EIN3-binding F box protein 2	0.10
At5g67030*	**ABA1, Abscisic acid deficient 1**	**0.95**
At3g14440	**NCED3, Nine-cis-epoxycarotenoid dioxygenase3**	**2.38**
At5g15960	**Cold and ABA inducible protein KIN1**	**2.45**
At3g02480	**ABA-responsive protein-related**	**3.81**

[a] The bold type stands for genes related to ABA function, whereas the other represents the ethylene-related genes

[b] Signal fold changes in the mutants are the means of two biological experiments and normalized to its corresponding wild type. Genes are referred to have significant change with the following criteria: the expression signal over 100 at either wild type or mutants, each experiment with signal fold change over 1.92 and at least 2-fold changes on average of two biological experiments. The raw data are available in GEO database with an accession no. GSE 12715

* Indicates no significant signal fold change

data with a GEO accession no. GSE12715). Of these genes, only one identified transcript, *ACC oxidase* (*ACC*, At1g12010), is involved in ethylene biosynthesis (Table 1). Hence, none of the genes involved in the primary ethylene signal transduction pathway were found to be significantly regulated in this analysis. Another ethylene response gene, *ETHYLENE RESPONSE FACTOR* (*ERF053*; At2g20880), was also found to be upregulated. The *ABA2* transcript levels in the *aba2* mutant were downregulated by 2.1-fold. Two ABA-inducible genes, *protein phosphatase 2C* (At5g59220) and *KIN1* (At5g15960), were downregulated by ~3-folds. *CYP707A2*, encoding an ABA 8′-hydroxylase, was shown to be reduced by ~4.8-fold (Table 1), presumably due to the ABA substrate deficiency in this mutant. In addition to ABA- and ethylene-related genes listed in Table 1, genes that had an altered expression in the *aba2* mutant and participated in hormonal biosynthesis or signaling, and stress and ubiquitination regulation are listed in Supplementary Table S1. For example, the expression of *gibberellin 20 oxidase* (*GA20ox*, At5g51810), an important enzyme in the biosynthesis of the bioactive GA compound GA_4 in *Arabidopsis* (for review, see Hedden and Phillips 2000), was increased by ~4.7-fold. As ABA is a stress hormone, various genes in the *aba2* mutant were found to be abiotic or biotic stress-related. Also, genes likely involved in protein modification (ubiquitination) were also observed in *aba2* (Supplementary Table S1).

In the *ein2* mutant, a total of 229 genes had altered expression, with 113 up and 116 downregulated genes (Table 1 and Supplementary Table S1). Of these, several genes involved in ethylene biosynthesis, signaling, and response, such as *ACS8*, *ETR2*, *ERS1*, *ERS2*, *EIN2*, and *EBF2*, were significantly regulated. *EIN2* transcripts in this mutant were downregulated by ~4.3 fold. However, only one ABA biosynthesis gene, *NCED3*, was appreciably enhanced by 2.4 fold (Table 1). Two other ABA-responsive genes, *KIN1* and *At3g02480*, were also induced in the *ein2* mutant. Several genes with altered expression were involved in hormonal biosynthesis or signaling of gibberellin, cytokinin, jasmonic acid, and salicylic acid. It is noteworthy that three JA-responsive marker genes, *vegetative storage protein1* (*VSP1*, At5g24780), *lipoxygenase* (*LOX2*, At3g45140), and *thionin* (*THI2.1*, AT1g72660), which are involved in the wounding response, were upregulated in the *ein2* mutant, but not in *etr1*. Altered genes involved in stress (abiotic and biotic) response and protein ubiquitination were also observed in *ein2* (Supplementary Table S1).

To then examine whether the increased expression of *NCED3* in the *ein2* mutant is a common feature of ethylene-signaling defective mutants, the *etr1-1* mutant was analyzed in the same way. The results showed that in the *etr1-1* mutant, 191 genes (94 up and 97 downregulated) were differentially expressed compared to the wild type (Table 1 and supplementary Table S1). Like *ein2*, the *etr1* mutant had several ethylene biosynthesis, signaling, and response genes that showed significant changes in their expression profiles (Table 1), such as *ACS8*, *ACO*-like (At1g77330), *ERS1*, *ERS2*, *ERF72*, and *EBF2*. *etr1-1* is a point mutation showing no conceivable change in its transcript. However, in the case of ABA-related genes, only two genes were significantly changed; *ABI1*, involved in ABA signaling, was downregulated by ~2.4 fold, and *CYP707A2* was reduced by 5.6 fold. In addition, many other biosynthesis or signal genes of hormones, such as auxin, cytokinin, gibberellin, jasomonate, and brassinosteroid, were also changed in their expression in the *etr1* mutant. It is noteworthy that at least five auxin responsive and jasmonate biosynthesis or signaling genes were upregulated in *etr1*. Similarly, several altered genes in this mutant were regulated by abiotic and biotic stress and ubiquitination (Supplementary Table S1). Genes with altered expression listed in Table 1 were further validated by semiquantitative RT-PCR, and the results were generally in agreement with the microarray data, except for a few genes showing variation (Fig. 8A); several genes listed in Table S1 were also verified and are shown in Fig. S2.

Measurement of ABA and ethylene contents in the ABA/ethylene mutants

As *ACO* (At1g12010) expression is upregulated in the *aba2* mutant, it is conceivable that this mutant might have higher ethylene content than the wild type, as evidenced by LeNoble et al. (2004). To confirm whether the higher ethylene level in *aba2* is because of the upregulation of this *ACO*, we thus measured ethylene in these plants using HP-GC and found that the *aba2* mutant had an ~82% higher ethylene content than the wild type (Fig. 8B) and that the *acoaba2* double mutant had ~65% higher levels of ethylene. The *aco* mutant only contained ~85% of the wild-type ethylene level. These results suggest that the upregulation of *ACO* in the *aba2* mutants only contributes to the partial induction of ethylene. The other *ACS* or *ACO* genes may contribute to the increase in the ethylene levels in the *aba2* mutant, but they might be regulated at the translational or post-translational levels, and thus not be detectable by microarray. Interestingly, and despite the higher levels of endogenous ABA described previously (Ghassemian et al. 2000; Wang et al. 2007), the *ein2* mutant also was found to accumulate a 4.2-fold higher ethylene content than the wild type. The *etr1-1* mutant had a 20% increased ethylene content compared with the wild type. The *ctr1* mutant only contained ~53% of the wild-type ethylene level, whereas the *eto1-4* mutant accumulated ethylene at a ~14.2-fold higher level than the wild type (Fig. 8B).

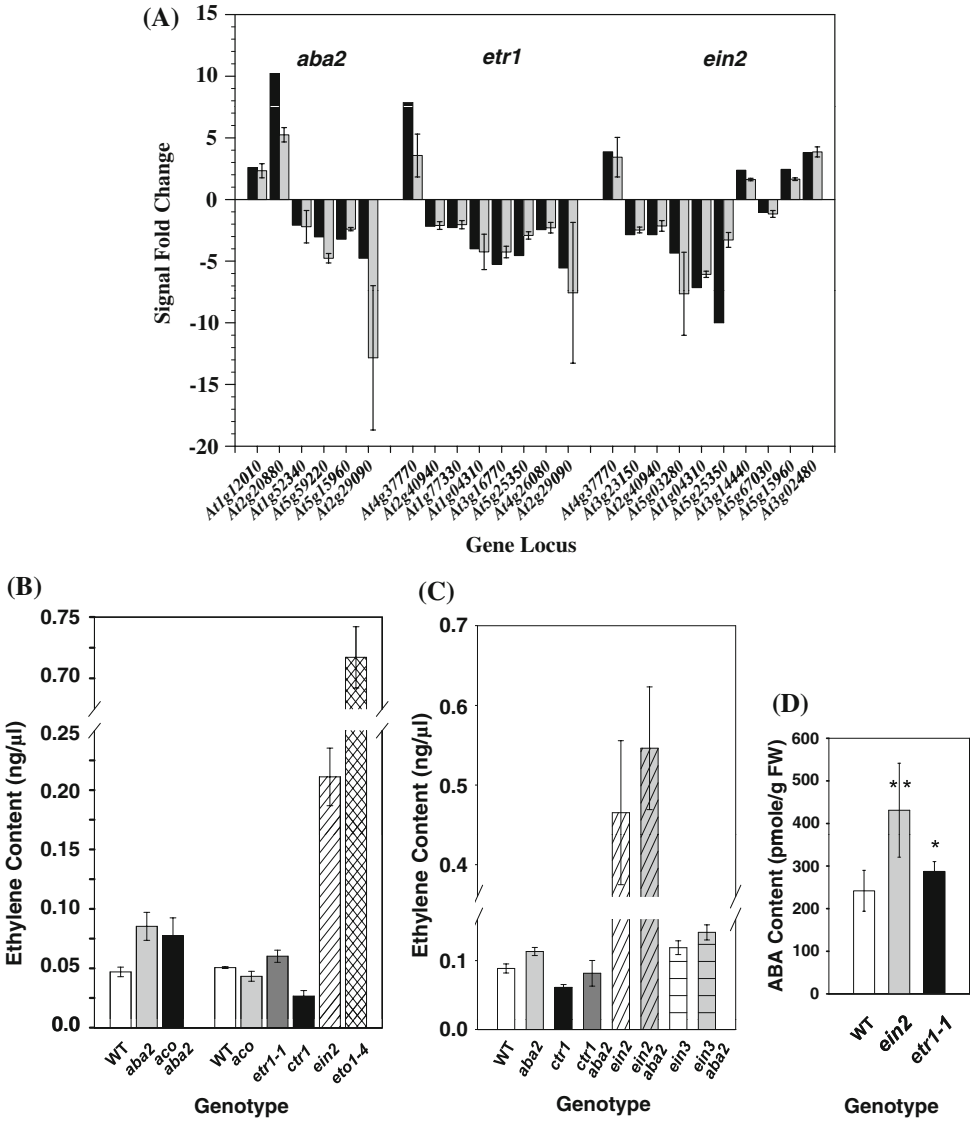

Fig. 8 Validation of ABA- and ethylene-related gene expression, and measurement of the ethylene and ABA contents in the ABA/ethylene mutants. **A** Validation of the GeneChip data by semiquantitative RT-PCR. *Black bars* represent means of normalized GeneChip signal of two independent experiments. *Grey bars* represent means ± SD of two independent experiments of semiquantitative RT-PCR. Each experiment was duplicated. The genes tested here were derived from Table 1. **B**, **C** Analysis of ethylene contents. Seeds were grown on 1% sucrose agar medium under dark conditions for 3 days (**B**) or 4 days (**C**). The etiolated seedlings were subsequently measured for ethylene content. The data shown are the means ± SD of two experiments, each with a triplicate and giving the consistent results. **D** ABA contents. Seeds were grown on 1% sucrose agar plates for 14 days. Then, the seedlings were harvested and subjected to the ABA assay. The values are the means ± SD of three independent experiments, each with a duplicate and giving consistent results. * $P < 0.05$ ($P = 0.049$), Student's *t* test. ** $P < 0.01$ ($P = 0.005$), Student's *t* test. *ACO*, *ACC oxidase* (*At1g12010*); *UBQ* ubiquitin, WT wild type

Comparison of ethylene levels among these ABA/ethylene mutants revealed that the double mutants *ctr1aba2*, *ein2aba2*, and *ein3aba2* had slightly higher ethylene levels than their corresponding single ethylene mutants *ctr1*, *ein2*, or *ein3* (Fig. 8C). The higher ethylene contents in these double mutants were attributable to the *aba2* mutant allele showing higher ethylene production. For ABA contents, the *aba2* mutant ordinarily contains ~20–30% of wild-type ABA levels (Lin et al. 2007). The *ein2* mutant was found to have a ~1.8-fold higher level than the wild type; the *etr1-1* had a ~20% higher level of ABA than that of the wild type (Fig. 8D). These data indicate that *ABA2* is a negative regulator of ethylene biosynthesis. Similarly, *EIN2* was revealed as a negative regulator controlling ABA biosynthesis, whereas *ETR1-1* was found to be a positive regulator controlling *ABI1* and *CYP707A2* expression. The antagonism between ABA and ethylene and their possible interaction in seed germination and early seedling growth is illustrated schematically in Fig. 9.

Discussion

Parallel signaling between ABA and ethylene and their effects on shoot and root growth

There is now compelling evidence that ABA and ethylene are very closely involved in various aspects of plant growth, development, and stress response. For instance, these two hormones have contrasting effects on stomatal aperture (Tanaka et al. 2005), hyponastic growth (Benschop et al. 2007), seed germination (Beaudoin et al. 2000; Ghassemian et al. 2000), postgermination seedling growth (Zhou et al. 1998), defense gene expression, and disease

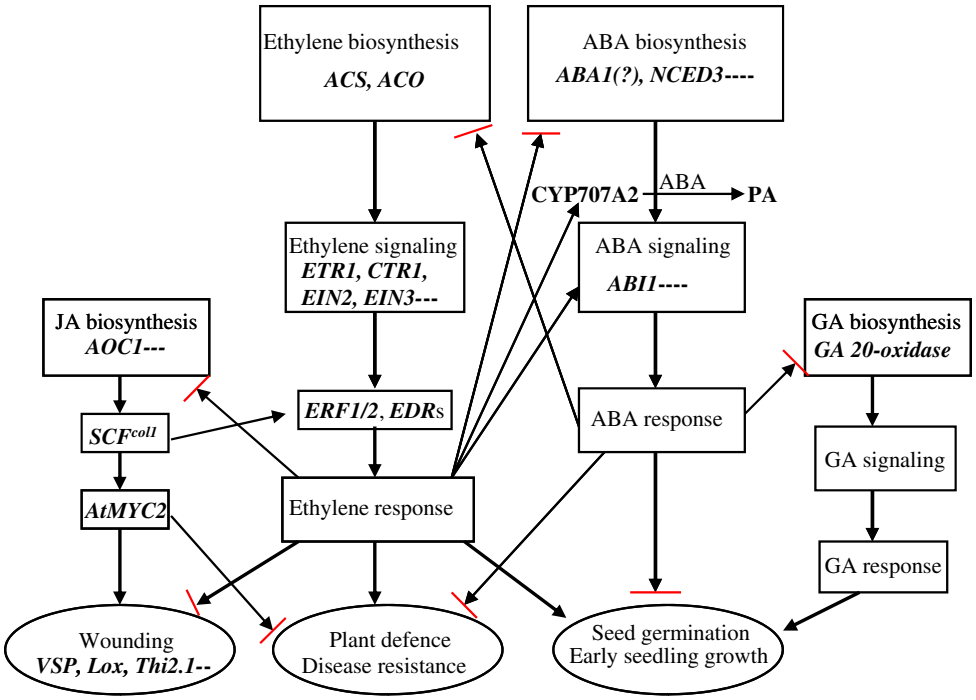

Fig. 9 Schematic representation of a possible mechanism underlying the antagonistic interaction between the ABA and ethylene-signaling pathways. The model depicted is based on genetic epistasis analysis, microarray data, and the measurement of the ethylene and ABA contents of the mutants under study. In general, the ABA and ethylene signal transduction pathways appear to act in parallel with their antagonistic effects upon seed germination and early seedling growth. Moreover, ABA and ethylene in the *aba2*, *ein2*, and *etr1* mutant seedlings may control the hormonal biosynthesis, catabolism, or signaling of each other to enhance their antagonistic roles. It is noteworthy that ABA signal pathway is parallel to the primary (or early) ethylene pathway upstream of EIN3, whereas it crosstalks with the secondary (or late) stages of ethylene-signaling pathway downstream of EIN3. CYP707A2 is an ABA 8′-hydroxylase that catalyzes the breakdown of ABA into phaseic acid (PA). The JA and ethylene interaction was partially adopted from Adie et al. (2007)

resistance (Anderson et al. 2004; De Paepe et al. 2004). They also confer synergistic inhibition of root growth (Beaudoin et al. 2000; Ghassemian et al. 2000). Despite the physiological significance of ABA and ethylene, it was unknown whether there were any convergent points between the corresponding hormone signal transduction pathways or if they functioned only in parallel. To address this issue, we generated double mutants by crossing ethylene mutants (*ctr1*, *ein2*, *ein3*, and *ein6*) with the *aba2* (or *gin1-3*) mutant. The genetic hierarchy of the components of the ethylene signaling pathway has been well established by epistasis analysis of ethylene mutants (Roman et al. 1995). The essential components, ETR1, CTR1, EIN2, and EIN3, form a largely linear pathway in the early stages of ethylene signal transduction in plants (Bleecker and Kende 2000; Guo and Ecker 2004). Although the precise relationship between EIN6 and other components of the ethylene-signaling pathway has remained obscure, biochemical data suggested that EIN6 acts upstream of EIN3 (Guo and Ecker 2003). ABA has multiple sites of perception and a complex signaling pathway. In addition, the *ABI* genes identified to date only respond to parts, but not all, of ABA or stress-signaling pathways. Hence, the crosstalk between ABA and ethylene might also be diverse. To simplify the study of the ABA–ethylene interaction, we used the *aba2* mutant as it has an ABA deficiency and subsequently influences all ABA-signaling pathways. The null mutant *aba2* has a wilty phenotype and small plant size and is ideal for being able to isolate double mutants and classify the interaction relationship between the ABA and ethylene signal transduction pathways. From previous reports and also the data presented in this study, several lines of evidence now indicate that ABA and ethylene signalings act in parallel. First, previous studies have demonstrated that the *etr1aba2* double mutant exhibits phenotypes that closely resemble *aba2* (Zhou et al. 1998; LeNoble et al. 2004). In addition, this double mutant also displayed ethylene insensitivity with no induction of root hairs in the presence of ACC, further suggesting that ABA and ethylene act largely independently of each other in these phenotypes (Zhou et al. 1998). In our current study, we provided further evidence for the independent relationship between ABA and ethylene by analyzing the downstream ethylene signaling components, *ctr1*, *ein2*, and *ein3* (Figs. 1, 2, 3, 4). Second, our current double mutants displayed both wilty phenotype and water-loss kinetics

similar to *aba2* (Fig. 2). Third, in addition to light-grown conditions, the double mutants showed a constitutive ethylene triple response in *ctr1aba2* or ethylene insensitivity in *einaba2* mutants in the dark (Fig. 4). These findings indicate that the double mutants contain both *aba2* and ethylene mutant phenotypes, suggesting that ABA and ethylene function in parallel at least during primary (or early) signal transduction pathways.

In addition, ABA and ethylene have differential effects on plant growth and development. In general, a comparison of *aba2* with *ein* and *einaba2* revealed that ABA-deficient mutants *aba2* and *einaba2* had wiltiness and small plant size. Similar results regarding ABA and ethylene interaction and their effects on shoot growth have also been reported previously. In *aba2-1*, for instance, the shoot growth is substantially inhibited, and the plant ethylene content is higher than that of wild type. However, the *aba2-1etr1-1* double mutant showing ABA deficit and ethylene insensitivity substantially, but not completely, restores shoot growth to *etr1-1* (LeNoble et al. 2004). Similar inhibition of shoot growth through high ethylene production was also observed in the ABA-deficient *flacca* and *notabilis* mutants of tomato (Sharp et al. 2000). These data suggest that the impairment of shoot growth in the ABA-deficient mutants of *Arabidopsis* or tomato is at least partly attributable to the increased ethylene production (LeNoble et al. 2004). In addition to reduced shoot growth, the *aba2* mutant displayed a short root phenotype that was also observed in maize ABA-deficient mutant *vp5*. Short root in *vp5* can be restored by exogenous application of ABA, or substantially improved by ethylene biosynthesis or signaling inhibitors AVG or STS (silver thiosulfate) (Sharp 2002; Sharp and LeNoble 2002; Spollen et al. 2000). In *Arabidopsis*, although exogenous application of ABA can restore *aba2* short roots to normal root growth, the application of AVG in *aba2* only slightly improved its root growth (Fig. 5). One possibility for this discrepancy is that maize and *Arabidopsis* might have different ethylene sensitivity in roots. Collectively, these data demonstrate that normal or basal levels of ABA are essential to maintain root growth.

Interaction between ABA and ethylene in seed germination and early postgermination seedling growth

Previous studies demonstrated that transgenic plants overexpressing the *Arabidopsis* ABA biosynthesis genes, *NCED3*, and *ABA2*, cause ABA accumulation, an increase in dormancy, seed germination delay, and stress tolerance (Iuchi et al. 2001; Lin et al. 2007). Conversely, ABA-deficient mutants ordinarily display a decrease in dormancy, early seed germination, and stress sensitivity. Notably, in our current studies we tested the effect of endogenous ABA and ethylene on seed germination and early seedling growth, rather than by adding exogenous phytohormones as reported previously (Ghassemian et al. 2000; Beaudoin et al. 2000). In wild-type seeds, exogenous ethylene does not promote seed germination in the absence of ABA, whereas it enhances seed germination in the presence of ABA (Ghassemian et al. 2000). In planta, however, the overproduction of ethylene in *eto1-4* and the constitutive triple response in *ctr1* lead to early seed germination (Fig. 6C, D), particularly in the case of *ctr1*; both *eto1-4* and *ctr1* showed little change in ABA sensitivity as compared to the wild type (Supplemental Fig. S1). Although both *aba2* and *ctr1* promoted seed germination, the effects of an ABA deficit and ethylene constitutive signaling are not additive (Fig. 6). In contrast to *eto1-4* and *ctr1*, the ethylene-insensitive mutants *ein2* and *etr1* showed a seed germination delay and an increased ABA sensitivity (Figure S1; Ghassemian et al. 2000; Beaudoin et al. 2000), presumably due to the accumulation of ABA in these mutants (Fig. 8; Ghassemian et al. 2000; Chiwocha et al. 2005). However, this is not a common feature of ethylene-insensitive mutants, because *ein3* showed little change in seed germination relative to the wild type (Fig. 6). Taken together, our data generally demonstrated the presence of an antagonistic interaction between ABA and ethylene in terms of seed germination.

In addition to seed germination, ABA and ethylene also show antagonistic interactions in postgermination seedling growth (Zhou et al. 1998). Several ABA deficient and insensitive mutants (*aba1*, *aba2*, *aba3*, *abi4* and *abi5*) display a glucose insensitive (gin) phenotype in the presence of 6% glucose, a concentration that causes postgermination developmental arrest in wild-type plants, but not in these mutants (Zhou et al. 1998; Arenas-Huertero et al. 2000). Similarly, the ethylene mutants, *ctr1* and *eto1*, also have a gin phenotype (Zhou et al. 1998). In contrast, the ethylene-insensitive mutants, *etr1*, *ein2*, and *ein3*, exhibited the glucose over-sensitive (glo) phenotype at 4% glucose, a concentration in which wild-type plants can grow steadily, but in which these three mutants show postgermination developmental arrest (Zhou et al. 1998; Yanagisawa et al. 2003). In our current study, *ein3* only showed a weak glo phenotype with a small plant size relative to the wild type (Fig. 7), presumably due to differences in the growth conditions. In general, the *etr1* and *ein2* mutants showed a strong glo phenotype that correlated with their ABA accumulation and sensitivity, whereas the gin phenotype was found to be associated with ABA deficiency/or insensitivity and ethylene overproduction/or ethylene constitutive signaling. Thus, ABA inhibits seed germination and postgermination seedling growth, whereas ACC or ethylene promotes these processes.

Possible mechanism for the antagonistic interaction between the ABA and ethylene signaling cascades

As mentioned above, the ABA and ethylene signal transduction pathways function in parallel and have antagonistic interaction during seed germination and early seedling growth. Previous studies have shown the gene expression profiles mediated by exogenous application of plant hormones or stress (Van Zhong and Burns 2003; Anderson et al. 2004; De Paepe et al. 2004). However, in our current studies we focused on the role of endogenous ABA and ethylene in *Arabidopsis* physiological effects and hormone interaction without adding exogenous hormones or stress. Thus, the *aba2* mutant with its ABA deficiency and higher ethylene contents than the wild type, might further stimulate, at least in part, seed germination and early seedling establishment. However, the high level of ethylene in this mutant was not related to its short primary root, because the short root was the cause of ABA deficiency (Fig. 5). We speculated that the higher ethylene production in *aba2* was due to the upregulation of the *ACO* (At1g12010) and other ethylene biosynthetic genes at post-translational levels. As several lines of evidence have demonstrated that ethylene biosynthesis and signaling genes are regulated at post-translational levels in *Arabidopsis* or tomato (Wang et al. 2002; Guo and Ecker 2003; Potuschak et al. 2003; Yanagisawa et al. 2003; Wang et al. 2004; Kevany et al. 2007), these gene products would not be detectable by microarray. ABA-deficient mutants with higher levels of ethylene production have also been observed in tomato (Tal et al. 1979) and *Arabidopsis* (LeNoble et al. 2004). Collectively, the evidence to date suggests that ABA may regulate ethylene biosynthesis. It has been long believed that the ABA–GA balance plays a critical role in seed dormancy and germination (Koornneef et al. 1982; for review, see Finkelstein et al. 2008). Hence, the change of ABA levels will alter the GA contents as well. Most recent studies revealed that the mutation of genes involved in carotenoid biosynthesis causes ABA deficiency, but induces GA accumulation in rice (Fang et al. 2008). Supporting evidence was also observed in our current studies. For instance, the lack of ABA in the *aba2* mutant caused the upregulation of a GA biosynthetic gene, *GA20-oxidase* (Supplementary Table S1; Fig. S2). Hence, the early seed germination and seedling growth observed in *aba2* might predominantly be attributable to the change of the ABA–GA balance.

ETR1, an ethylene receptor, plays a negative role in regulating the ethylene signal transduction pathway. The gain-of-function mutation of ETR1 in *etr1-1* causes insensitivity to ethylene, a seed germination delay, and an ABA oversensitivity. The *etr1-2* mutant also has been shown previously to have higher levels of ABA (~47%) than the wild type (Chiwocha et al. 2005). The level of ABA induction for the *etr1-1* mutant measured in our current study was increased by ~20% (Fig. 8C) relative to the wild type, but this is not sufficient to account for the severe germination delay and ABA oversensitivity, in contrast to the *ein2* mutant, which has ~2-fold higher ABA levels (Fig. 8C; Ghassemian et al. 2000; Wang et al. 2007). Our microarray data further revealed that the *ABI1* and *CYB707A2* (Fig. 8A; Table 1) genes were downregulated in the *etr1* mutant. As *ABI1* is a negative regulator of ABA signaling (Sheen 1998; Gosti et al. 1999), its downregulation might cause ABA oversensitivity in this mutant. Supporting evidence has been shown that the recessive loss-of-function alleles of *ABI1*, *abi1-2*, and *abi1-3*, enhance response to ABA both in seed and vegetative tissues of *Arabidopsis* (Saez et al. 2006). In addition, CYB707A2 is the key enzyme for ABA catabolism. Mutation of CYB707A2 in the *cyb707a2* mutant causes hyperdormancy and ABA accumulation in seeds (Kushiro et al. 2004). This is consistent with our current result that the reduction in the expression of the ABA catabolic enzyme CYB707A2 may give rise to ABA accumulation in the *etr1* mutant. Taken together, the downregulation of *ABI1* and *CYB707A2* in the *etr1* mutant resulted in increased both seed dormancy (or seed germination delay) and ABA sensitivity.

EIN2 plays a central role in mediating the ethylene signal transduction pathway and acts as a node that interacts with jasmonic acid, oxygen radicals, and other stress inducers (Alonso et al. 1999). Similar to the *etr1* mutant, the *ein2* mutant displays a germination delay and a high ABA sensitivity, presumably due to the accumulation of ABA (Fig. 8C; Ghassemian et al. 2000; Wang et al. 2007). The high ABA levels in this mutant are most likely due to the upregulation of *NCED3* (Fig. 8A; Table 1), a key enzyme in the ABA biosynthetic pathway. Previous studies have revealed that the high ABA levels in *ein2* are associated with the upregulation of *ABA1* (At5g67030) (Ghassemian et al. 2000; Wang et al. 2007), conflicting with our current results that showed no change of expression in *ABA1* (Fig. 8A; Table 1). One reason could be the difference in plant developmental stages, stress treatment, or growth conditions. *ein2* with high levels of ethylene production (Fig. 8B) was strongly insensitive to ethylene and showed little change in primary root elongation under saturated ACC treatment conditions (Fig. 4B). In contrast, high ethylene concentration in submerged *Rumex Palustris* and deepwater rice causes strong growth enhancement, with a rapid inhibition of endogenous ABA biosynthesis through the regulation of *NCED* expression (Kende et al. 1998; Benschop et al. 2005). These data thus reflect that a functional ethylene signal transduction pathway plays a negative role in mediating ABA biosynthesis through the

regulation of *NCED* expression. Our current data revealed that two ethylene-insensitive mutants, *etr1* and *ein2*, contained higher levels of ethylene, whereas the constitutive triple response mutant *ctr1* had a lower ethylene content, only ~53% of total wild-type levels. These finding are consistent with a negative feedback regulation of ethylene biosynthesis. The higher levels of ethylene in both *etr1* and *ein2* were most likely due to the upregulation of *ACS8* in these mutants. Furthermore, *ein2* had more ethylene production than *etr1* did, reflecting that the post-translational event might occur in ACS8 or other ethylene biosynthetic genes in these mutants.

In general, the microarray analysis revealed at least two common features in these mutants. First, ABA and ethylene signaling show a complex interaction with other hormones; hence, the change of one hormonal biosynthesis or signaling pathway alters other hormone-related gene expression, which has been reported previously (Chiwocha et al. 2005; for reviews, see Li and Guo 2007; Finkelstein et al. 2008). Second, ABA and ethylene are closely associated with abiotic and biotic stress. Comparison of microarray data also showed that the ABA-inducible gene *KIN1* was ABA-dependent, with its reduction in *aba2* but induction in *ein2* (Table 1; Fig. 8A). A similar result was also observed in a cold-regulated gene *COR15A* (At2g42540) (Table S1; Fig. S2). Two ethylene receptors, *ERS1* and *ERS2*, were both downregulated in *etr1* and *ein2*, whereas *ETR2* expression was only reduced in *ein2*, further consistent with previous report that these three ethylene receptors are ethylene-inducible (Hua et al. 1998). *EBF2*, encoding an EIN3-binding F box protein, also had an altered expression pattern in both *etr1* and *ein2* (Table 1; Fig. 8A). Taken together with the *ACS8* upregulation in these two mutants, ethylene may regulate genes involved its own biosynthesis and signal transduction pathway as also described previously (Van Zhong and Burns 2003). Ethylene and JA have well-characterized synergistic effects on plant defense and disease resistance, and an antagonistic interaction in wounding in *Arabidopsis*. Our current data provided evidence that both *etr1* and *ein2* promoted the expression of *allene oxide cyclase 1* (*AOC1*; At3g25760), which catalyzes an essential step in JA biosynthesis. Another gene *OPDA reductase 3* (*OPR3*, At2g06050) required for JA biosynthesis was also stimulated in *etr1*. These data support that ethylene signaling may negatively regulate JA biosynthesis, and its regulation is differential between *etr1* and *ein2*. As both *etr1* and *ein2* are strong ethylene-insensitive mutants, expression profiles of some genes are expected to be similar to some extent, as partially evidenced by the results described above. However, a substantial level of dissimilarity was observed, as shown in Table 1 and Table S1. For example, the three JA-mediated markers, *VSP1*, *LOX2*, and *THI2.1* (for review, see Adie et al. 2007), in response to wounding were upregulated in *ein2* but not in *etr1* (supplemental Table S1; Fig. S2), reflecting that these two mutants, at least in part, might have a different pathways in response to wounding.

The possible mechanisms underlying the antagonistic interaction between ABA and ethylene signaling are illustrated in Fig. 9. By epistasis analysis, we demonstrated parallel signaling between ABA and ethylene in the primary (or early) signal pathway. As the key components, ETR1, CTR1, EIN2, and EIN3, form a largely linear pathway in the early (or primary) stages of ethylene signal transduction pathway in plants. The activated EIN3 or EIN3-like (EIL) transcription factors may switch expression of ERF or EDF transcription factors, which in turn trigger transcription of secondary (or late) ethylene-response genes. These secondary ethylene-response genes are involved in specific branches of the ethylene response pathway downstream of EIN3 (Stepanova and Alonso 2005). Global expression analysis further confirms a wide range of ethylene-response genes involved in various metabolic, signal, developmental, and structural functions (Alonso et al. 2003). Thus, the antagonistic crosstalk in this model will occur via secondary (or late) signal transduction pathways. In addition, seed dormancy and germination are regulated by a combination of environmental and endogenous signals (for review, see Finkelstein et al. 2008). The latter includes the integration of plant hormones, such as ABA, GA, and ethylene, and the effect of other hormones or factors on seed dormancy and germination cannot be excluded by our current findings.

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This work is dedicated to Miss Pei-Chi Lin (1978–2007).

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