

The Arabidopsis *eer1* Mutant Has Enhanced Ethylene Responses in the Hypocotyl and Stem¹

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By screening for *enhanced ethylene-response (eer)* mutants in Arabidopsis, we isolated a novel recessive mutant, *eer1*, which displays increased ethylene sensitivity in the hypocotyl and stem. Dark-grown *eer1* seedlings have short and thick hypocotyls even in the absence of added ethylene. This phenotype is suppressed, however, by the ethylene biosynthesis inhibitor 1-aminoethoxyvinyl-glycine. Following ethylene treatment, the dark-grown *eer1* hypocotyl response is greatly exaggerated in comparison with the wild type, indicating that the *eer1* phenotype is not simply due to ethylene overproduction. *eer1* seedlings have significantly elevated levels of *basic-chitinase* expression, suggesting that *eer1* may be highly sensitive to low levels of endogenous ethylene. Adult *eer1* plants display exaggerated ethylene-dependent stem thickening, which is an ethylene response previously unreported in Arabidopsis. *eer1* also has enhanced responsiveness to the ethylene agonists propylene and 2,5-norbornadiene. The *eer1* phenotype is completely suppressed by the ethylene-insensitive mutation *etr1-1*, and is additive with the constitutive ethylene-response mutation *ctr1-3*. Our findings suggest that the wild-type *EER1* product acts to oppose ethylene responses in the hypocotyl and stem.

Ethylene is a simple gaseous hydrocarbon that has profound effects on plant growth and development. Considered as one of the five classic plant hormones, ethylene controls many physiological processes ranging from seed germination to tissue senescence (Abel et al., 1992; Johnson and Ecker, 1998). Our current understanding of the molecular mechanisms of ethylene signaling has largely resulted from the isolation of ethylene-response mutants in Arabidopsis (Kieber, 1997; Johnson and Ecker, 1998; Chang and Shockey, 1999). These mutants have been isolated on the basis of defects in the ethylene-mediated triple response of dark-grown seedlings, which consists of shortening and thickening of the hypocotyl and root and exaggeration of apical hook curvature and proliferation of root hairs (Bleecker et al., 1988). Mutants lacking the triple response in the presence of ethylene are known as *ethylene-response (etr)* (Bleecker et al., 1988), *ethylene-insensitive (ein)* (Guzmán and Ecker, 1990; Roman et al., 1995), or *ACC-insensitive (ain)* (Van Der Straeten et al., 1992). Those that display the triple response even in the absence of exogenous ethylene are either *constitutive ethylene-response (ctr)* and *ran* mutants (Kieber et al., 1993; Hirayama et al., 1999; Woeste and Kieber, 2000) or *ethylene overpro-*

ducer (eto) mutants (Guzmán et al., 1990). Genetic epistasis analyses and isolation of the corresponding genes have provided molecular insight into the ethylene signal transduction pathway in Arabidopsis.

Ethylene action is initiated by a family of five ethylene receptors, which have significant similarity to His protein kinase receptors of two-component regulatory systems (Bleecker, 1999). ETR1 and ERS1 possess all of the required motifs for His kinase activity (Chang et al., 1993; Hua et al., 1995), and ETR1 was shown to have His autokinase activity in vitro (Gamble et al., 1998). Both ETR1 and ERS1 bind ethylene when expressed in yeast cells (Schaller and Bleecker, 1995; Rodriguez et al., 1999; Hall et al., 2000). Ethylene binding requires a copper cofactor as part of the functional receptor (Rodríguez et al., 1999), and a copper transporter protein, RAN1, is thought to provide copper ions to the receptors (Hirayama et al., 1999; Woeste and Kieber, 2000). The receptor genes *ETR1*, *ETR2*, and *EIN4* were identified as dominant gain-of-function mutations that confer ethylene insensitivity (Bleecker et al., 1988; Hua et al., 1998; Sakai et al., 1998). Introduction of the identical missense mutations into *ERS1* and *ERS2* results in similar ethylene insensitivity (Hua et al., 1995, 1998). The loss of function of three or more of the receptors results in a constitutive ethylene-response phenotype, indicating that the receptors act as negative regulators of the ethylene-signaling pathway (presumably maintaining activation of the negative regulator CTR1 in the absence of ethylene) (Hua and Meyerowitz, 1998).

CTR1, a negative regulator of ethylene responses, was identified based on loss-of-function mutations that result in pleiotropic constitutive ethylene responses independent of ethylene (Kieber et al., 1993).

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CTR1 has sequence similarity to the Raf family of MAPKKs, suggesting the involvement of a MAP kinase cascade in ethylene signaling (Kieber et al., 1993). Epistasis analysis demonstrated that CTR1 acts downstream of all identified ethylene receptors (Kieber et al., 1993; Roman et al., 1995; Hua et al., 1995, 1998; Sakai et al., 1998). The regulatory domain of CTR1 physically interacts with the presumed cytoplasmic domains of ETR1 and ERS1, implicating a direct role for the receptors in regulating CTR1 activity (Clark et al., 1998).

Additional components in the pathway acting downstream of CTR1 were identified through ethylene-insensitive mutations (Roman et al., 1995). The EIN2 protein has an N-terminal integral membrane domain, similar to the Nramp family of metal-ion transporters, and a C-terminal domain of unknown function (Alonso et al., 1999). Loss-of-function mutations in *ein2* result in ethylene insensitivity (Guzmán et al., 1990; Roman et al., 1995), however the function of EIN2 at the biochemical level has yet to be determined. Several positive regulators of a transcriptional cascade have also been uncovered. EIN3 and the related EIN3-like proteins are novel transcriptional activators of primary ethylene-response genes (Chao et al., 1997; Solano et al., 1998). A target of EIN3 is the *ERF1* gene, which encodes a protein that directly binds to the ethylene response element found in the promoters of many ethylene-inducible genes, including several pathogen-response genes such as *basic chitinase* (Solano et al., 1998).

The isolation of ethylene-response mutants in *Arabidopsis* has led to substantial insight regarding the components and mechanisms of ethylene signal transduction. The response pathway contains an intricate series of positive and negative regulators of ethylene responses, beginning with ethylene binding and leading to gene regulation. To further build upon this framework, the identification and analysis of additional components is required. Most of the ethylene mutant screens performed in the past have used saturating levels of ethylene or no added ethylene. The mutants obtained, consequently, have been generally limited to one of three classes: *ethylene insensitive*, *constitutive ethylene response*, or *ethylene over-producer*. Alternative mutant screens will probably be necessary to uncover additional components, including those that are redundant or tissue specific. With this in mind, we sought to isolate a new class of ethylene response mutants, which we designated *enhanced ethylene response (eer)*. To identify such mutants, we screened for the induction of triple response phenotypes using sub-threshold levels of ethylene. This approach resulted in the isolation of a novel ethylene-dependent mutant, *eer1*, described in this paper. Analysis of the *eer1* mutant suggests that the *EER1* product acts to oppose ethylene responses primarily in the hypocotyl and stem.

RESULTS

Isolation of the *eer1* Mutant

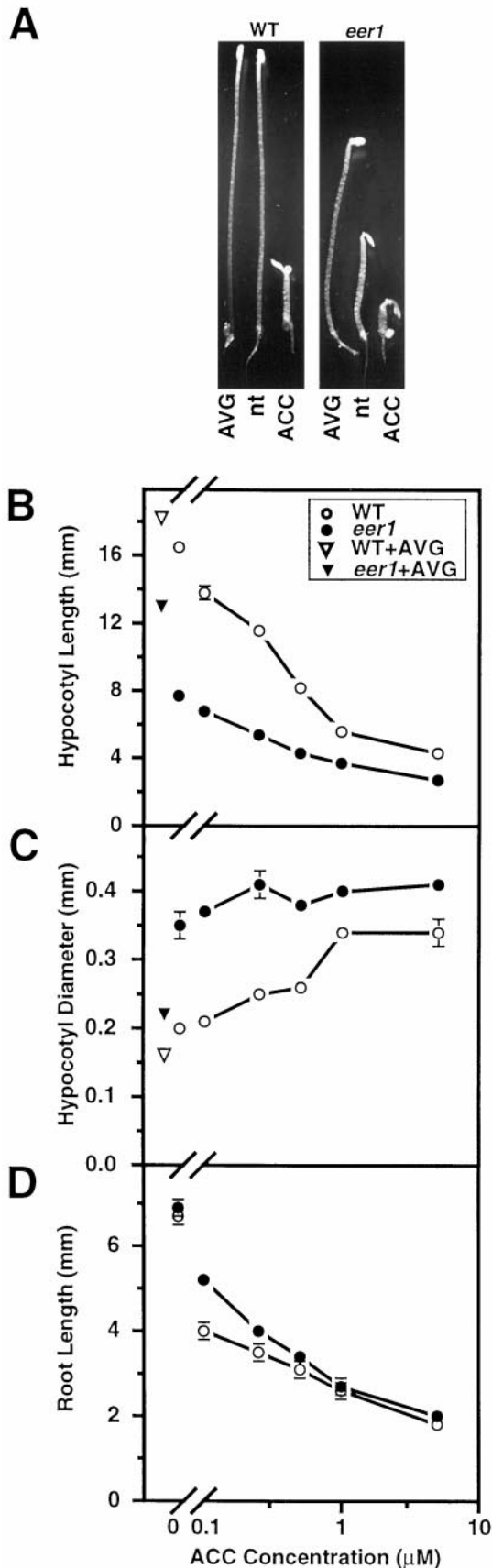
An *enhanced ethylene response (eer1)* mutant was isolated by screening T-DNA- and ethyl methanesulfonate (EMS)-mutagenized *Arabidopsis* for dark grown seedlings that exhibited facets of the ethylene-mediated triple response in the presence of sub-threshold levels of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is the immediate precursor to ethylene and is converted to ethylene via ACC oxidase (Yang and Hoffman, 1984). As shown in Figure 1A, characteristics of the seedling triple response include inhibition of hypocotyl and root elongation, radial swelling of the hypocotyl and root, increased curvature of the apical hook, and proliferation of root hairs (Bleecker et al., 1988). Putative *eer* mutants exhibited one or more of these phenotypes when grown on nutrient agar containing a low concentration of ACC (0.1 μ M), which causes only a slight manifestation of the triple response in the wild type (Fig. 1). The *eer1* mutant was identified on the basis of severe inhibition of hypocotyl elongation in conjunction with an increase in hypocotyl thickness at this concentration of ACC (Fig. 1).

The *eer1* phenotype results from a recessive mutation at a single locus. The F₁ progeny of *eer1* backcrossed to the wild type showed wild-type ethylene responses. The F₂ progeny from self-fertilized F₁ plants segregated 3:1 (141 wild type to 48 *eer1*; $\chi^2 = 0.0018$, $P > 0.95$). Although *eer1* was isolated from a T-DNA-mutagenized population, *eer1* was not kanamycin resistant (data not shown), suggesting that the mutation was not due to a T-DNA insertion. No EMS-derived alleles of *eer1* were identified.

Enhanced Ethylene Response in the *eer1* Hypocotyl

In dark-grown wild-type seedlings, the hypocotyl response to ethylene involves a re-orientation of cell expansion, resulting in the inhibition of hypocotyl elongation with a concomitant increase in radial thickness. The hypocotyl of *eer1* was significantly shorter and thicker than that of the wild type over a broad range of ACC concentrations (Fig. 1, B and C). This phenotype was restricted to the basal portion of the hypocotyl; the apical portion of the hypocotyl was not thicker, and there was no exaggerated hook formation. Roots of *eer1* seedlings were somewhat less sensitive than the wild type to low concentrations of ACC (Fig. 1D).

It should be noted that the hypocotyl of *eer1* was shorter and thicker than that of the wild type even in the absence of exogenously supplied ACC. This phenotype was substantially alleviated by treatment with 1-aminoethoxyvinyl-Gly (AVG), which inhibits ethylene biosynthesis by blocking ACC synthase activity (Yang and Hoffman, 1984), thus indicating that the *eer1* mutant phenotype is ethylene-dependent



rather than a product of constitutive ethylene signaling or a growth defect unrelated to ethylene (Fig. 1, A–C). The addition of 10 μM AVG to the growth medium caused a 70% increase in hypocotyl length for *eer1* compared with a 15% increase for the wild type; concomitantly, there was a 37% reduction in hypocotyl thickness for *eer1* compared with a 20% reduction for the wild type. The effect of AVG on roots could not be assessed because AVG treatment severely inhibited wild-type root growth. The AVG treatment did not fully revert *eer1* to wild type, perhaps due to incomplete inhibition of ethylene biosynthesis. However, as described later, the *eer1* phenotype was completely suppressed by the *etr1-1* ethylene receptor mutation, which blocks ethylene perception.

Having established the ethylene dependence of the *eer1* phenotype, we next assessed whether the phenotype could result from increased ethylene biosynthesis. Rates of ethylene biosynthesis were determined for dark-grown seedlings of the wild type and *eer1*. Ethylene was collected after a period of 12 h from 3-d-old seedlings enclosed in airtight vials. As shown in Table 1, *eer1* seedlings produced 2.8-fold more ethylene than the wild type (based on fresh weight).

Despite the observed increase in ethylene synthesis by *eer1*, it was determined that ethylene overproduction cannot be the primary defect causing the *eer1* phenotype. This conclusion is based on the response of *eer1* to treatment with ethylene gas as shown in Figure 2. All of the ethylene treatments were performed in the presence of 10 μM AVG, which was added to inhibit ethylene biosynthesis. AVG treatment effectively removed the ethylene overproduction aspect of *eer1*, so that we could measure the ethylene response of *eer1*. As in Figure 1, the *eer1* hypocotyl phenotype was substantially alleviated by AVG in the absence of added ethylene (Fig. 2). When

Figure 1. Dark-grown *eer1* seedlings have an enhanced ethylene response when exposed to the ethylene precursor ACC. A, Dark-grown wild-type and *eer1* seedlings treated with either 10 μM AVG, 0 μM ACC (nt, no treatment), or 10 μM ACC for 3.5 d. B, ACC dose response curves for hypocotyl length of 3.5-d-old dark-grown wild-type and *eer1* seedlings. Control treatments included no ACC and 10 μM AVG. Mean ± SE values were determined from 25 to 30 seedlings. C, ACC dose response curves for hypocotyl diameter of 3.5-d-old dark-grown wild-type and *eer1* seedlings as in B. D, ACC dose response curves for root length of 3.5-d-old dark-grown wild-type and *eer1* seedlings as in B.

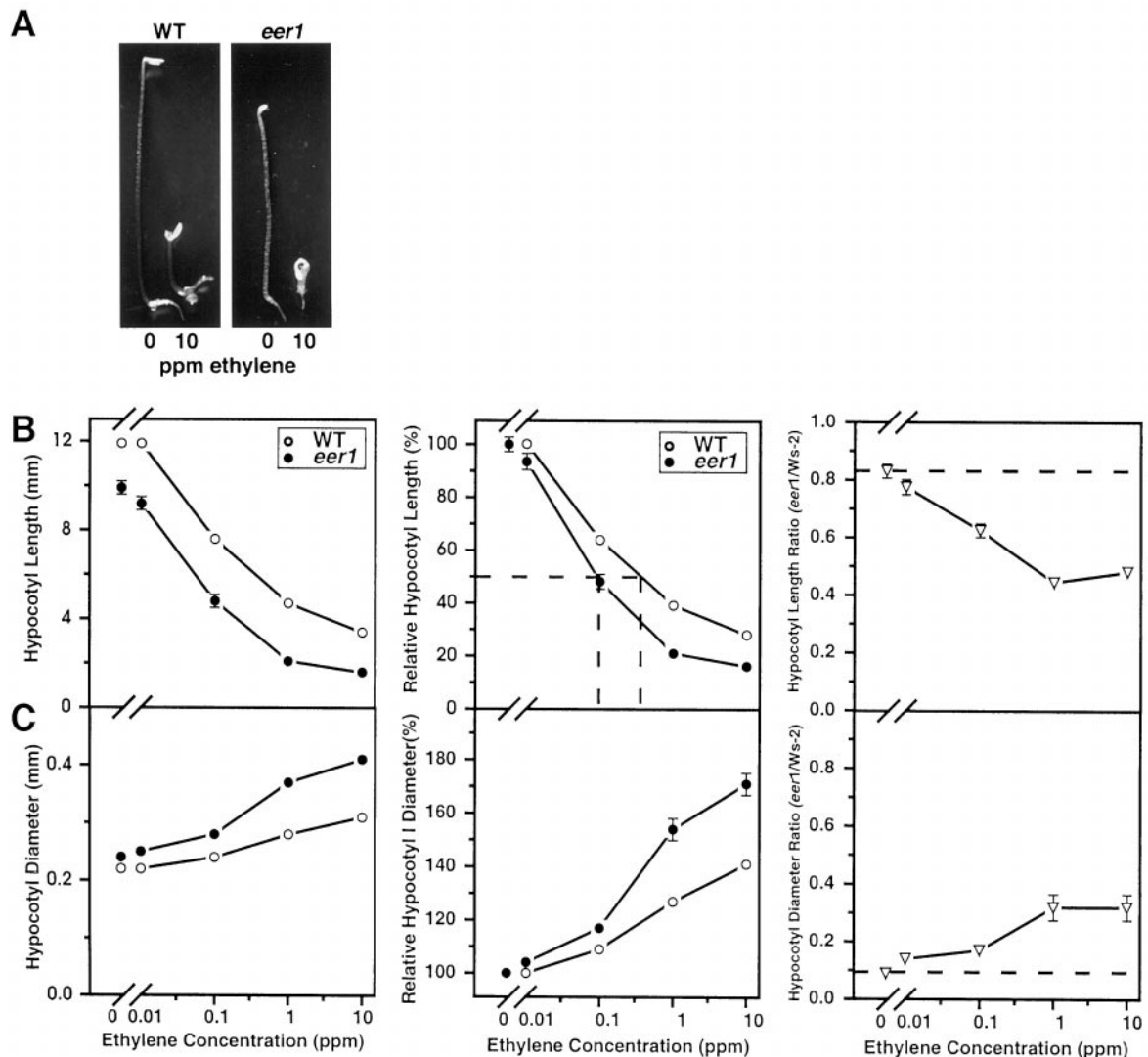


Figure 2. Ethylene treatment causes an exaggerated response in dark-grown *eer1* seedlings. A, Dark-grown wild-type and *eer1* 3.5-d-old seedlings grown in air or $10 \mu\text{L L}^{-1}$ ethylene in the presence of $10 \mu\text{M}$ AVG. B, Ethylene dose response curves for hypocotyl length of 3.5-d-old dark-grown wild-type and *eer1* seedlings grown in the presence of $10 \mu\text{M}$ AVG. Left, Actual hypocotyl length. Center, Relative inhibition of hypocotyl length (length/length at $0 \mu\text{L L}^{-1}$ ethylene) with the concentration of ethylene that gives 50% inhibition denoted by (- - -). Right, The ratio of *eer1* hypocotyl length over wild-type hypocotyl length for each ethylene concentration with (- - -) denoting the predicted ratio if the *eer1* mutant were not ethylene responsive. Mean \pm SE values were determined from 25 to 30 seedlings. C, Ethylene dose response curves for hypocotyl diameter of dark-grown wild-type and *eer1* seedlings treated as in B. Left, Actual hypocotyl diameter. Middle, The relative increase in hypocotyl diameter (diameter/diameter at $0 \mu\text{L L}^{-1}$ ethylene). Right, The ratio of *eer1* hypocotyl diameter over wild-type hypocotyl diameter for each ethylene concentration, with (- - -) denoting the predicted ratio if the *eer1* mutant were not ethylene-responsive.

treated, however, with a range of ethylene gas concentrations in the presence of AVG, the *eer1* hypocotyl was consistently shorter and thicker than that of the wild type (Fig. 2, A–C). For example, the hypocotyl length of *eer1* was inhibited 50% at an ethylene concentration of $0.1 \mu\text{L L}^{-1}$, whereas the wild type required greater than $0.3 \mu\text{L L}^{-1}$ ethylene to achieve the same level of inhibition (Fig. 2B). These results suggest an increased sensitivity to ethylene in the *eer1* hypocotyl. If the *eer1* phenotype resulted from only a

general growth defect, then this differential could be expected to remain constant (Fig. 2, B and C).

At $10 \mu\text{L L}^{-1}$ ethylene, the severity of the *eer1* phenotype reached a level that has not been reported for the wild type (e.g. Bleecker et al., 1988). If the *eer1* phenotype was the result of ethylene overproduction, then the ethylene dose response curves of the wild type and *eer1* would be expected to converge at a compensation point at which the effects of exogenously added ethylene mimic the effects of endog-

enously produced ethylene. The apparent increase in sensitivity coupled with the severity of the *eer1* phenotypic response indicate that ethylene overproduction is not the primary defect of *eer1*.

Because the extreme phenotype achieved by *eer1* is not reproduced by ethylene-treated wild-type seedlings, we were interested to see if *eer1* could be phenocopied by ethylene treatment of *ctr1* mutants. *ctr1* loss-of-function mutants display a constitutive ethylene-response phenotype less severe than the phenotype of ethylene-treated *eer1*. Although ethylene responsiveness of *ctr1* mutants had not been previously reported, all three of the *ctr1* mutant lines that we tested (including a newly isolated allele *ctr1-15* [Larsen and Chang, unpublished data]) were capable of responding to ethylene both in terms of hypocotyl shortening and increased radial thickness. This result, shown in Figure 3, argues for the existence of an alternative ethylene signaling pathway in Arabidopsis that bypasses *CTR1*. Despite being ethylene-responsive, *ctr1* (or wild-type) seedlings treated with ethylene did not produce the exaggerated phenotype of *eer1*, perhaps due to the presence of functional EER1. As described later, when *eer1* is combined with *ctr1*, the double mutant has a more severe phenotype than *ctr1* alone, supporting the idea that wild-type EER1 limits the hypocotyl response in both ethylene-treated wild type and *ctr1*.

Exaggerated Cell Expansion in the *eer1* Hypocotyl

Confocal microscopy revealed that the ethylene-dependent radial thickening of the *eer1* hypocotyl is due to exaggerated cell expansion as shown in Figure 4. Dark-grown seedlings were exposed to either 10 μM AVG or a high level of exogenous ethylene (10 $\mu\text{L L}^{-1}$). AVG treatment resulted in etiolated hypocotyls with no pronounced apical hook formation for both the wild type and *eer1* (Fig. 4). Etiolation was correlated with cell elongation parallel to the vertical plane of the hypocotyl with cell shape in the wild type and *eer1* being indistinguishable. In contrast, high concentrations of ethylene produced dramatic differences in the morphologies of the wild type and *eer1* hypocotyl (Fig. 4). In the ethylene-treated wild type, there was pronounced apical hook formation along with a re-orientation of cell expansion that resulted in compact, less elongated cells compared with untreated hypocotyls. In ethylene-treated *eer1* seedlings, there was no pronounced apical hook formation, perhaps due to constraints applied by the structure of the lower hypocotyl. In contrast, cell expansion in *eer1* was severely exaggerated in the basal portion of the hypocotyl correlating with a dramatic increase in radial thickness. It is interesting that this phenotype was strictly localized as there was a sharp transition in cell size between the apical hook region and the basal portion of the hypocotyl (Fig. 4).

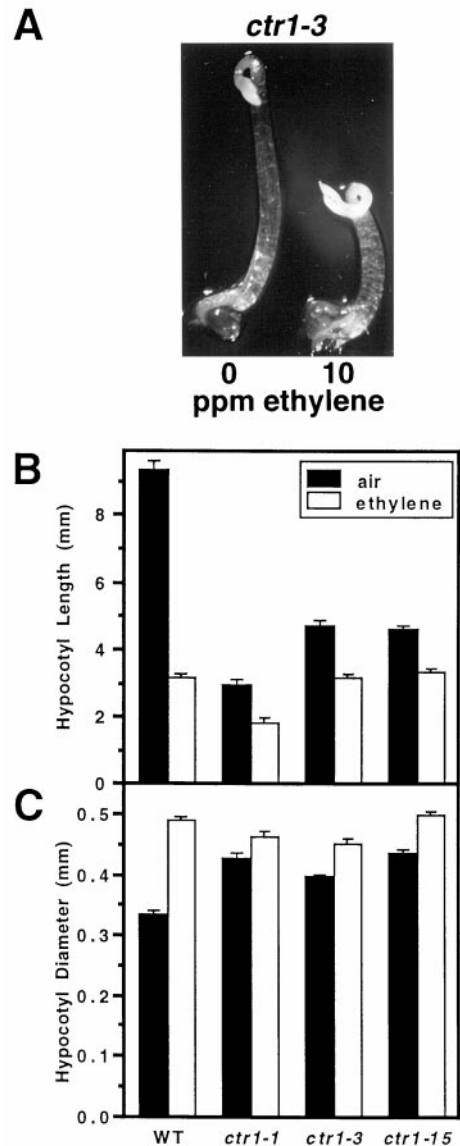


Figure 3. Dark-grown *ctr1* seedlings respond to ethylene treatment. A, Dark-grown *ctr1-3* seedlings grown in air or 10 $\mu\text{L L}^{-1}$ ethylene for 3.5 d. B, Hypocotyl length of the wild type (Col-0) and three different *ctr1* mutant alleles grown in the dark for 3.5 d either in the presence or absence of 10 $\mu\text{L L}^{-1}$ ethylene gas. The *ctr1-1* missense mutation results in an amino acid substitution of a highly conserved residue within the kinase domain. *ctr1-3* carries a stop codon in the N-terminal regulatory domain. The *ctr1-15* mutation has not yet been identified. Mean \pm SE values were determined from 25 to 30 seedlings. C, Hypocotyl diameter of the wild type (Col-0) and the three *ctr1* mutant alleles treated as in B.

Response of *eer1* to Ethylene Agonists

Since the *eer1* phenotype was dependent upon the ethylene signal, we tested whether other compounds that are known to bind to the ethylene receptors could elicit a similar response in *eer1* hypocotyls. Both propylene, which is known to elicit ethylene responses when applied at concentrations 100-fold higher than ethylene (Abeles et al., 1992), and 2,5-

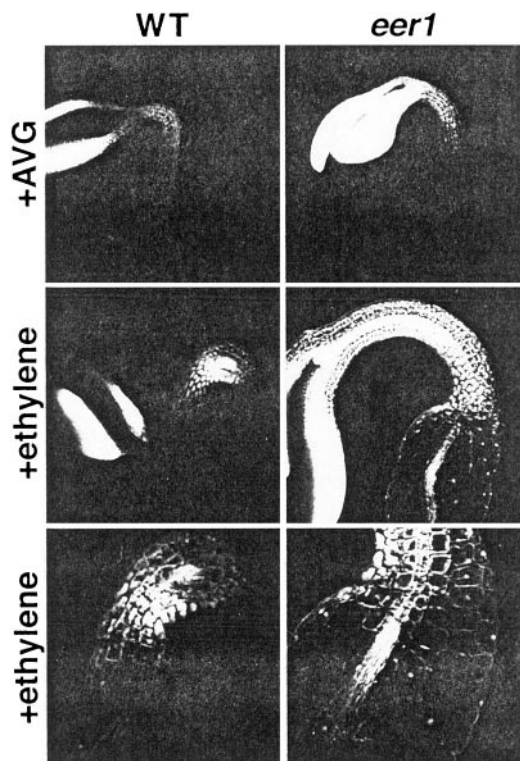


Figure 4. The enhanced ethylene response of *eer1* is restricted to the basal portion of the hypocotyl. Confocal microscopy of dark-grown wild-type and *eer1* seedlings treated with either $10\ \mu\text{M}$ AVG or $10\ \mu\text{L L}^{-1}$ ethylene for 3.5 d. Top two sets, The cotyledons, apical hook, and apical portion of the hypocotyl ($10\times$ magnification). Bottom, The transition zone between the apical hook region and basal portion of the hypocotyl ($20\times$ magnification).

norbornadiene (NBD) (Sisler, 1991), which is a competitive inhibitor of ethylene action, were tested for their effects on *eer1* hypocotyls.

Dark-grown wild-type and *eer1* seedlings were exposed to a range of propylene concentrations ($10\ \mu\text{L L}^{-1}$ – $10\ \text{mL L}^{-1}$) in the presence of $10\ \mu\text{M}$ AVG to limit endogenous ethylene production. As with the ethylene dose response curve, *eer1* hypocotyls were both more sensitive and more responsive to propylene (Fig. 5A). This enhanced response included an increase in sensitivity in comparison with the wild type, as demonstrated by the lower level of propylene required for 50% inhibition of hypocotyl length. In addition, *eer1* hypocotyls demonstrated the same increase in level of response as seen with ethylene treatment, again indicating that the *eer1* phenotype results from both an increase in sensitivity and in the amplitude of response.

The effect of NBD on *eer1* was also tested. Dark-grown wild-type and *eer1* seedlings were exposed to either $229\ \mu\text{L L}^{-1}$ NBD, air, or $1\ \mu\text{L L}^{-1}$ ethylene, each in the presence of $10\ \mu\text{M}$ AVG to limit ethylene production. As shown in Figure 5B, instead of reverting *eer1* to wild type, NBD actually elicited a partial ethylene response; *eer1* seedlings displayed a 25%

decrease in hypocotyl length, whereas no response was detected in the wild type. Since olefin compounds other than ethylene are capable of eliciting limited ethylene responses (Sisler, 1991; Abeles et al., 1992), binding of NBD to the ethylene receptors possibly triggers the signaling pathway but at a level that is imperceptible in the wild type. It is conceivable that *eer1* is sensitized to even the low level of signaling that might be initiated by NBD.

Basic-Chitinase Expression in *eer1*

Basic-chitinase is an ethylene-regulated pathogenesis-related gene (Solano et al., 1998). Northern analysis was performed using *basic-chitinase* as an indicator of whether the *eer1* mutation results in changes in ethylene-regulated gene expression. Total RNA was isolated from wild-type and *eer1* dark-grown seedlings treated with air, 0.1, or $1\ \mu\text{L L}^{-1}$ ethylene either in the presence or absence of $10\ \mu\text{M}$ AVG. In both the wild type and *eer1*, AVG had little effect on the levels of *basic-chitinase* expression. As shown in Figure 6, the *basic-chitinase* transcript was slightly induced in wild-type seedlings at $0.1\ \mu\text{L L}^{-1}$ ethylene and was reduced to the level of the air-treated control at $1\ \mu\text{L L}^{-1}$ ethylene. In contrast, *eer1* seedlings had high levels of *basic-chitinase* expression in the absence of exogenous ethylene, and expression was reduced to nearly undetectable levels with ethylene treatment. It is notable that the level of *basic chitinase* expression in untreated *eer1* seedlings was substantially higher than ever detected in the wild type. This is consistent with the exaggerated morphological responses displayed by *eer1* in the absence of ACC or ethylene. The altered pattern of *basic-chitinase* expression further suggests that the *eer1* mutation results in both a shift in ethylene sensitivity and a dramatic increase in the amplitude of response.

Ethylene-Dependent Stem Thickening

Adult *eer1* plants showed no obvious mutant phenotypes with respect to leaf or rosette size, height, flower morphology, inflorescence development, or flowering time. The examination of senescence phenotypes was inconclusive (data not shown). In contrast, the stems of *eer1* were noticeably thicker than those of the wild type. *ctr1* mutants, which have constitutive ethylene responses throughout their life cycle, also have thicker stems (data not shown). These observations led us to examine whether stem thickening represents an ethylene response in Arabidopsis, and to determine whether stem thickening in *eer1* is an enhanced ethylene response. Adult wild-type and *eer1* plants (3 weeks old), which were just beginning to bolt, were exposed to either $229\ \mu\text{L L}^{-1}$ NBD, air, or $1\ \mu\text{L L}^{-1}$ ethylene for 8 d. The thickness of the stem was measured immediately following this treatment. As shown in Figure 7, wild-type stems

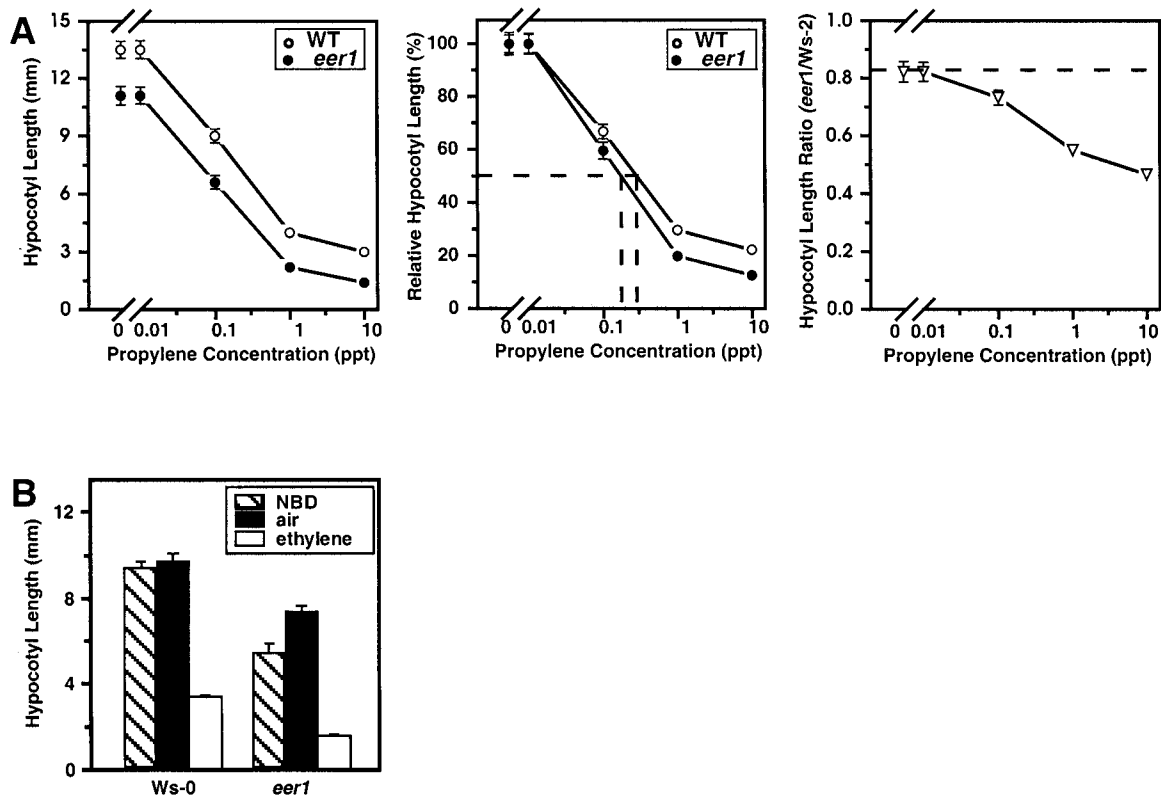


Figure 5. *eer1* hypocotyls have an enhanced response to ethylene agonists. A, Propylene dose response curves for hypocotyl length of 3.5-d-old dark-grown wild-type and *eer1* seedlings grown in the presence of 10 μM AVG. Left, Actual hypocotyl length. Middle, Relative inhibition of hypocotyl length (length/length at 0 $\mu\text{L L}^{-1}$ ethylene) with the concentration of propylene causing 50% inhibition denoted by (- - -). Right, The ratio of *eer1* hypocotyl length over wild-type hypocotyl length for each propylene concentration with (- - -) denoting the predicted ratio if the *eer1* mutant were not propylene responsive. Mean \pm SE values were determined from 25 to 30 seedlings. B, Hypocotyl length was measured for dark-grown wild-type and *eer1* seedlings exposed to either 229 $\mu\text{L L}^{-1}$ NBD, air, or 1 $\mu\text{L L}^{-1}$ ethylene for 3.5 d. Mean \pm SE values were determined from 25 to 30 seedlings.

were thicker with exposure to ethylene, suggesting that increased stem thickness is an ethylene-response phenotype in Arabidopsis. *eer1* stems that developed in the presence of high levels of ethylene were nearly 2-fold thicker than *eer1* stems in air (Fig. 7). As with the hypocotyl response, the extent of this phenotype was not achieved in the wild type treated with ethylene. Furthermore, we found that *eer1* stems were responsive to NBD as seen for the *eer1* hypocotyl; stems of NBD-treated *eer1* plants were significantly thicker than the air-treated controls, whereas no response to NBD was detected in the wild type (Fig. 7). These findings indicated that the *eer1* mutant possesses enhanced ethylene response in the adult stem and that the thicker stems in the absence of ethylene treatment were likely due to increased sensitivity to endogenous ethylene.

Double Mutant Analysis

Double mutant analysis was performed with *eer1* and three mutants: the dominant ethylene-insensitive mutant *etr1-1*, the recessive ethylene-insensitive mu-

tant *2-1*; and the recessive constitutive ethylene-response mutant *ctr1-3*. Dark-grown seedlings of selfed *eer1/etr1-1/+* individuals segregated approximately 3:1 (79 *etr1* to 23 *eer1*, $\chi^2 = 0.21$, $P > 0.6$) on 10 μM ACC. This high concentration of ACC induces a severe triple response phenotype in *eer1* but does not elicit a response in *etr1-1*. As shown in Table II, the hypocotyl length of the *eer1 etr1-1* double mutant was indistinguishable from that of *etr1-1* alone. The same was found for the *eer1/etr1-1/+* mutant (data not shown). Moreover, the *eer1* phenotype was completely suppressed by the *ein2-1* mutation in ACC-treated dark-grown seedlings of the *eer1 ein2-1* double mutant (data not shown). The finding that both *etr1-1* and *ein2* completely masked the *eer1* phenotype indicated that *eer1* acts in (or upon) the ethylene-response pathway.

The *eer1 ctr1-3* double mutant displayed primarily additive phenotypes, suggesting that *CTR1* and *EER1* are both necessary to repress ethylene responses. Dark-grown seedlings of selfed *eer1/etr1-1/+* individuals segregated roughly 3:1 (108 *eer1* to 37 *eer1 ctr1*, $\chi^2 = 0.0023$, $P > 0.95$) in the absence of ACC. As

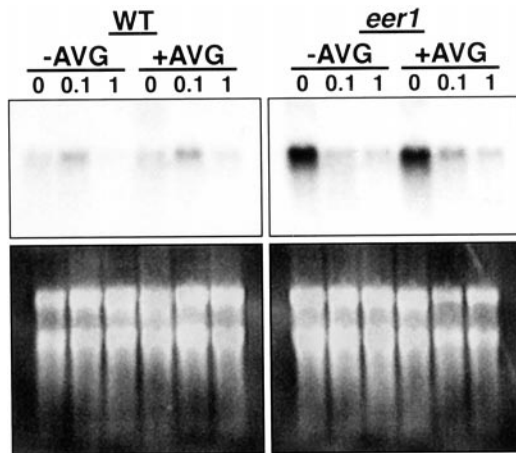


Figure 6. Ethylene-dependent regulation of *basic-chitinase* expression in dark-grown seedlings. Top, Autoradiograms of northern blots hybridized with a *basic-chitinase* gene probe. Dark-grown seedlings of the wild type and *eer1* were treated with either 0, 0.1, or 1 $\mu\text{L L}^{-1}$ ethylene, either in the presence or absence of 10 μM AVG for 3.5 d. Bottom, Ethidium bromide-stained rRNA bands in the corresponding gels prior to blotting.

shown in Table II and Figure 8, the hypocotyl of the dark-grown *eer1 ctr1-3* double mutant had a more severe phenotype than either untreated *eer1* or *ctr1*, resembling *eer1* treated with high levels of ethylene. In contrast, the root growth inhibition of *ctr1-3* seedlings was partially alleviated in the *eer1 ctr1-3* double mutant (Fig. 8), consistent with the earlier observation of decreased ethylene sensitivity in *eer1* roots (Fig. 1). At the adult stage, the *eer1 ctr1-3* double mutant exhibited phenotypes that were more severe than in either parent, including a reduction in rosette size that primarily resulted from reduced petiole length (data not shown). Stem thickening, which occurs independently in *eer1* and *ctr1-3*, was greatly exaggerated in the double mutant in both the primary and secondary stems (data not shown).

Map Location of the *EER1* Locus

EER1 was mapped to a position on the top arm of chromosome 1 using a cross between *eer1* (ecotype Wassilewskija) and wild-type Landsberg (data not shown). The *eer1* phenotype showed linkage with two cleaved amplified polymorphic sequence (CAPS) markers, placing the *EER1* locus approximately 4.7 cM south of CAPS marker m235 (position 34 cM) and 9.2 cM north of the CAPS marker UFO (unusual floral organs; position 49.6 cM). To our knowledge, no other mutants involved in ethylene signaling have been mapped to this location.

DISCUSSION

We have identified an *Arabidopsis* locus, *EER1*, which modulates the magnitude of ethylene responses specifically in the hypocotyl and stem. *EER1*

was identified on the basis of a recessive mutant that displays a short thick hypocotyl in response to either sub-threshold levels of ACC, ethylene, or the ethylene agonists propylene and NBD. The *eer1* mutation causes a profound enhancement in both the sensitivity and amplitude of response to ethylene, such that the mutant is sensitive to even low levels of endogenously produced ethylene. In dark-grown *eer1* seedlings, the mutant phenotype is restricted to the lower portion of the hypocotyl; there is no enhanced response in either the apical hook or root. In adult *eer1* plants, there is enhanced ethylene-dependent thickening of the stem. Stem thickening has not been previously reported as an ethylene response in *Arabidopsis*. This thickening, or swelling, might result from ethylene-induced changes in cell wall structure or synthesis, producing effects similar to those observed in the hypocotyl. How ethylene triggers these cell wall changes is not fully understood (Abeles et al., 1992). *EER1* action does not seem to be specific to cell expansion in that the *eer1* mutant also displays enhanced expression of *basic-chitinase*, which is an ethylene-regulated gene involved primarily in pathogen responses.

There are several lines of evidence indicating that the *eer1* mutant phenotype results from a defect in ethylene response. The alleviation of this phenotype

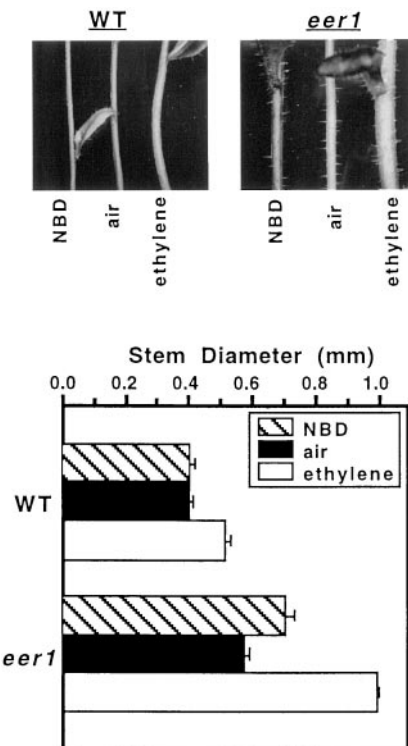


Figure 7. Ethylene treatment causes radial swelling of *Arabidopsis* stems. A, Stems of 3-week-old wild-type and *eer1* plants treated with either 229 $\mu\text{L L}^{-1}$ NBD, air, or 1 $\mu\text{L L}^{-1}$ ethylene for 8 d. B, Measurements of stem thickness immediately following the treatments described in A. Mean \pm SE values were determined from 10 plants.

Table II. Hypocotyl lengths of dark-grown ethylene-response mutants^a

Treatment ^b	Wild type ^c (Ws)	Wild type ^c (Col)	<i>eer1</i> ^c (Ws)	<i>etr1-1</i> ^c (Col)	<i>eer1 etr1-1</i> ^d	<i>ctr1-3</i> ^c (Col)	<i>eer1 ctr1-3</i> ^e
No ACC	10.6 ± 0.6	9.7 ± 0.2	6.9 ± 0.1	ND ^f	ND	5.0 ± 0.2	2.1 ± 0.1
10 μM ACC	2.8 ± 0.2	2.9 ± 0.3	1.4 ± 0.1	12.3 ± 0.4	13.3 ± 0.2	ND	ND

^a The unit of measure for hypocotyl length is mm. ^b Seedlings were grown for 4 d in the dark in either the presence or absence of ACC. ^c Mean ± SE values were determined from 15 individuals. ^d Mean ± SE values were determined from 102 individuals. ^e Mean ± SE values were determined from 37 individuals. ^f ND, Not determined in this experiment.

by the ethylene biosynthesis inhibitor AVG shows that *eer1* is ethylene-dependent. In the absence of AVG, ethylene levels in *eer1* seedlings are approximately 3-fold higher than in wild type, however ethylene overproduction is unlikely to be the primary defect, because *eer1* displays greater sensitivity to ethylene and has a more extreme ethylene-response phenotype than the maximal ethylene-response phenotype in the wild type. In addition, propylene and NBD, two ethylene agonists that bind to the ethylene receptors and trigger a signaling event, elicit the same enhanced responsiveness in *eer1* as seen with ethylene treatment. These results are consistent with *eer1* having a primary defect in ethylene response rather than in ethylene overproduction. The basis for the increase in ethylene production in *eer1* remains unclear; presumably, it is a downstream effect of the *eer1* mutation.

AVG did not fully restore the *eer1* phenotype to wild type. This was perhaps due to incomplete inhibition of ethylene biosynthesis. Although the incomplete rescue of the *eer1* phenotype by AVG raises the possibility that a portion of the *eer1* phenotype is due

to an ethylene-independent growth defect, the double mutant results with *etr1-1* or *ein2* argue against this. The finding that the *eer1* phenotype is completely suppressed by either of the ethylene-insensitive mutations *etr1-1* or *ein2-1* (which block ethylene signaling) indicates that the entire *eer1* phenotype is fully ethylene dependent. Therefore, the most plausible explanation for why the AVG treatment did not completely restore the *eer1* hypocotyl length to wild type is that suppression of ethylene biosynthesis by AVG was incomplete, and *eer1* responded to the low level of ethylene, which was still produced.

The *eer1* mutant displays a greater sensitivity and amplitude of response than the wild type, as indicated by both the lower ethylene concentration required to achieve 50% inhibition of hypocotyl length and the exaggerated phenotypes in the hypocotyl and stem. The enhanced responsiveness to propylene and NBD can also be interpreted as a heightened response to ethylene signaling. By binding to the ethylene receptor, NBD might initiate a low level of ethylene signaling as seen for other olefins (Sisler and Yang, 1984; Bleecker et al., 1987; Sisler, 1991; E. Sisler, personal communication). Stimulation of ethylene responses by NBD binding has been previously described for deep water rice, in which treatment with high concentrations of NBD causes an ethylene-like response independent of ethylene (Bleecker et al., 1987). In wild-type Arabidopsis, the low level of signaling that is presumably triggered by NBD may be incapable of producing an observable phenotypic response. The responsiveness of *eer1* to the predicted signaling initiated by NBD is consistent with *eer1* having enhanced ethylene sensitivity and an increased amplitude of response.

A potentially similar result was obtained with respect to gene expression. *Basic-chitinase*, which is commonly used as a reporter gene for ethylene response, was highly expressed in untreated *eer1* seedlings. In fact, the levels of *basic-chitinase* expression attained in untreated *eer1* seedlings were not observed in wild-type seedlings even following ethylene treatment. Wild-type seedlings exposed to 0.1 μL L⁻¹ ethylene displayed a mild induction of *basic-chitinase* expression, whereas identically treated *eer1* seedlings already showed a dramatic reduction of expression. It is unclear why AVG did not significantly reduce *basic-chitinase* expression in *eer1* seed-

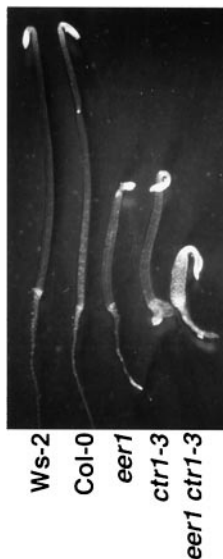


Figure 8. The *eer1 ctr1-3* double mutant phenotype is additive. Dark-grown 3.5-d-old seedlings of the wild type (Ws-2 and Col-0), *eer1*, *ctr1-3*, and the double mutant *eer1 ctr1-3* without ethylene treatment. Hypocotyl inhibition of the *eer1 ctr1-3* double mutant was greater than for either *eer1* or *ctr1-3* alone, indicating that the two mutations are additive.

lings, but it is possible that *eer1* seedlings are highly sensitive to even the low levels of ethylene production that may still occur during AVG treatment. The effect of ethylene on *basic-chitinase* in seedlings is apparently complex. The results do parallel, however, the morphological manifestations of the mutant phenotype and reflect two aspects of the mutant phenotype: (a) the kinetics of the *eer1* response are shifted toward higher sensitivity, e.g. low levels of ethylene cause the same degree of response in *eer1* as obtained by high levels of ethylene in wild type and (b) the magnitude of the *eer1* response, both in terms of morphology and gene expression, is much greater than ever observed for ethylene-treated wild type.

The masking of the *eer1* mutant phenotype by *etr1-1* and *ein2-1* indicates that *EER1* acts in or upon the ethylene-response pathway. The specific step at which *EER1* acts cannot be established, because the usual interpretations of epistasis do not apply to double mutants in which enhanced sensitivity is involved. For instance, if the ethylene signal is required for the enhanced response to be exhibited, then blocking of the signal with the *etr1-1* mutation will mask the phenotype of mutations acting downstream of *etr1-1*. Thus, one interpretation of the *eer1 etr1-1* and *eer1 ein2-1* double mutants is that ethylene signaling is required for the *eer1* mutant phenotype, and *EER1* acts at a point downstream of *ETR1* or *EIN2*. An alternate interpretation is that *EER1* acts at or upstream of the ethylene receptors, similar to *RAN1*. The *ran1* mutant shows responsiveness to the ethylene antagonist trans-cyclooctene. *ran1* mutants, however, do not display enhanced response to ethylene (Hirayama et al., 1999; Woeste and Kieber, 2000). Like *eer1*, *ran1* is suppressed by *etr1* and *ein2* (Hirayama et al., 1999). The *RAN1* protein is thought to provide the ethylene receptors with copper ions necessary for ethylene binding as well as for stability of the receptor complex (Hirayama et al., 1999; Woeste and Kieber, 2000). The latter idea is based on the observation that *ran1* loss-of-function mutants display responsiveness to trans-cyclooctene (Hirayama et al., 1999). It is speculated that a copper deficiency in the ethylene-binding site of the receptors may destabilize the receptor complex, making it easier for less potent ethylene antagonists to trigger a signaling event. *EER1* may similarly act at or upstream of the ethylene receptors and be involved in an aspect of regulating receptor function. Taking this view, the *eer1* phenotypes might result from tissue-specific modification of the ethylene receptors, which does not occur when the *etr1-1* or *ein2-1* alleles are present (as in the double mutants).

The additive phenotypes of the *eer1 ctr1-3* double mutant suggest that *EER1* acts in addition to *CTR1* to oppose ethylene responses. Wild-type *EER1* is likely to counter the ethylene-independent response generated in the *ctr1* loss-of-function mutant. This could explain why the *eer1 ctr1-3* double mutant phenotype

is both similar to that of ethylene-treated *eer1* and more severe than either of the single mutant phenotypes (in the absence of ethylene). We can also take into account the finding that *ctr1* loss-of-function mutations are capable of responding to ethylene. This result provides evidence that Arabidopsis seedlings possess an alternative ethylene-signaling pathway that bypasses the requirement for functional *CTR1*. The existence of such a pathway is also supported by the observation that *ctr1* mutations do not fully suppress mutations in the ethylene receptor genes (Hua and Meyerowitz, 1998; Hua et al., 1998). The alternative pathway perhaps involves a functionally redundant *CTR1* homolog. *EER1* might oppose both the alternative ethylene-response pathway as well as the primary pathway.

The *eer1* mutant has striking similarities to the previously described Arabidopsis mutant *sabre*. *sabre* was identified as having exaggerated radial swelling of cortical cells in the root (Aeschbacher et al., 1995). Although dose response analysis was not used to determine whether the *sabre* phenotype results from enhanced ethylene sensitivity, cell swelling in *sabre* is suppressed by *etr1-1* and can be partially rescued by an ethylene biosynthesis inhibitor. As in *eer1*, the ethylene-dependent cell enlargement in *sabre* is exaggerated in comparison with the wild type, and reflects a re-orientation of cell expansion perpendicular to the axis of growth. Dark-grown *sabre* and *eer1* also have similar hypocotyls and apical hooks. *eer1* produces only a partial apical hook, which is perhaps somehow constrained by the extreme thickness of the lower part of the hypocotyl. This same exaggerated hypocotyl thickening appears to occur in *sabre* along with a partial apical hook. In terms of *basic-chitinase* expression, *sabre* shows no increase in *basic-chitinase* message levels in leaves. However, expression levels of *basic-chitinase* are unknown in the root cortex, which is the tissue having the most dramatic ethylene-related phenotype. Thus we cannot rule out the possibility that *EER1* and *SABRE* are functionally analogous. *SABRE* has been cloned and codes for a hydrophilic protein of unknown function (Aeschbacher et al., 1995). It remains unclear how the *SABRE* protein opposes ethylene-regulated cell expansion.

It is worth noting that unlike the additive hypocotyl phenotype in the *eer1 ctr1-3* double mutant, *eer1* partially suppresses *ctr1*-dependent root growth inhibition and root hair formation. This is consistent with the insensitivity of *eer1* roots to low doses of the ethylene precursor ACC and suggests that *EER1* may have an opposite function in roots. Future isolation of the *EER1* gene will hopefully allow us to better understand its function and dissect the regulation of ethylene signaling. The *EER1* gene may also advance our understanding of how the ethylene hormone regulates processes such as cell expansion and gene expression.

MATERIALS AND METHODS

Plants and Growth Conditions

The Wassilewskija, Columbia, and Landsberg ecotypes of *Arabidopsis* were used in this study. T-DNA-mutagenized seeds of Wassilewskija (Ws-2) were obtained from the Arabidopsis Biological Resource Center (ABRC; Columbus, OH). In all experiments comparing *eer1* with the wild type, strain Ws-2 from the ABRC was used as the wild type and is referred to as Ws in the text. EMS-mutagenized seeds of ecotype Columbia (Col-0) were obtained from Lehle Seeds (Round Rock, TX). The Landsberg (La-0) ecotype (ABRC) was used for genetic mapping.

For seedling growth, seeds were surface-sterilized then cold-stratified at 4°C for 4 d in the dark to synchronize germination. Seeds were then suspended in 0.15% (w/v) agarose and sown in rows on plant nutrient agar medium with Suc (Lincoln et al., 1992) [2 mM KNO₃, 0.2 mM KH₂PO₄, 2 mM MgSO₄, 0.25 mM (NH₄)₂SO₄, 1 mM Ca(NO₃)₂, 1 μM MnSO₄, 5 μM H₃BO₃, 0.05 μM CuSO₄, 0.2 μM ZnSO₄, 0.02 μM NaMoO₄, 0.1 μM CaCl₂, 0.001 μM CoCl₂, 0.5% (w/v) Suc, and 0.8% (w/v) agar, pH 5.7]. The medium was supplemented with ACC (Sigma, St. Louis) or AVG (Sigma) as needed. The medium was contained in Petri dishes or vials as indicated. Seedlings were germinated for 3.5 d in the dark at 20°C. In experiments using ACC, the Petri dishes were oriented vertically for seedling growth.

All adult plants in this study were grown in soil under a 16-h-light/8-h-dark cycle at 20°C in plant growth chambers.

Mutant Screening

In all, approximately 6,500 T-DNA-mutagenized T4 seeds and approximately 30,000 EMS-mutagenized M2 (approximately 3,125 M1) seeds were screened. Seeds were sown on nutrient agar supplemented with 0.1 μM ACC, which is a sub-threshold level for the triple response in the wild type. Seedlings were germinated in the dark at 20°C with the Petri dishes vertically oriented. After 3.5 d, the seedlings were scored for individuals exhibiting one or more of the triple response phenotypes. Putative mutants were rescued to nutrient medium lacking ACC and subsequently transferred to soil. The self-progeny of putative mutants were rescreened using the same concentration of ACC, and the confirmed mutants were tested using the same conditions on nutrient agar supplemented with 10 μM AVG instead of ACC.

Treatment with Ethylene, Propylene, or NBD

Surface-sterilized seeds were sown on 60 × 15-mm Petri dishes (Fisher Scientific, Pittsburgh) containing 6 mL of nutrient agar (as described above) supplemented with 10 μM AVG [(2-aminoethoxyvinyl Gly), Sigma Chemical Company]. Petri dishes were then enclosed in wide mouth Mason jars sealed with a perforated lid containing a rubber syringe cap. For ethylene treatment, ethylene gas (Air

Products, Allentown, PA) was first diluted to 100 or 1,000 μL L⁻¹ in a sealed flask, and the resulting ethylene concentration was determined by gas chromatography. Ethylene was then injected into the Mason jars to specific concentrations using a syringe. Treatment with propylene (Scott's Specialty Gas, Plumsteadville, PA) was conducted in the same manner.

For NBD treatment, liquid NBD (Fluka, Milwaukee, WI) was first diluted to a concentration of 100 μL L⁻¹ then added to the Mason jars to a final liquid concentration of 1 μL L⁻¹, which is equivalent to 229 μL L⁻¹ NBD gas. The growth conditions were as described for the ACC treatment.

Adult plants were grown in air until the point of bolting (approximately 3 weeks), then exposed to either ethylene or NBD in an airtight chamber (Plas Labs, Lansing, MI) or air for 8 d. The chamber was flushed with air every 2 d. Immediately after this treatment, stem thickness was measured under a dissecting microscope fitted with an eyepiece micrometer.

Measurement of Ethylene Production

Surface-sterilized seeds were placed in 5-mL glass scintillation vials containing 0.5 mL of nutrient agar. The vials (uncapped) were placed into a sterile covered beaker and incubated in the dark for 72 h at 20°C. The vials were then sealed in the dark with a rubber syringe cap, allowing ethylene to collect in the vial. After 12 h of further incubation in the dark, 1 mL of headspace was sampled from each vial and the ethylene content was measured by gas chromatography as above. The total fresh weight of the tissue was measured for each sample.

Confocal Microscopy

Germinating seedlings were exposed to either 10 μM AVG or 10 μL L⁻¹ ethylene in the dark for 3.5 d as described above. The seedlings were fixed and stained according to Running et al. (1995) and were visualized by confocal microscopy (model MRC 1024; Bio-Rad Laboratories, Hercules, CA).

Northern Analysis

Germinating seedlings were treated with either 0.1 or 1.0 μL L⁻¹ ethylene gas, and/or 10 μM AVG in the dark for 4 d as described above. Total RNA was extracted from whole seedlings using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Total RNA (5 μg) from each sample was separated by electrophoresis in a 1% (w/v) denaturing agarose gel, and the gel was blotted to nylon membrane (Hybond N⁺, Amersham, Arlington Heights, IL). A ³²P-labeled *basic-chitinase* gene probe was generated using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis). Prehybridization, hybridization, and washes were carried out at 65°C following the manufacturer's instructions (Amersham), and the results were visualized by autoradiography.

Genetic Analysis

For backcrossing, *eer1* (male) was crossed to wild-type Ws-2 (female), and the F₁ and F₂ progeny were scored on plant nutrient agar medium with Suc containing 0.1 μM ACC.

Double mutants were created by crossing *eer1* (male) to *etr1-1*, *ein2-1*, and *ctr1-3* plants (females). Self-fertilization of the resulting F₁ produced the F₂ progeny. F₂ progeny of the cross with *etr1-1* were screened on 10 μM ACC to isolate ethylene-insensitive individuals (which were thus *etr1-1* homozygous or heterozygous). Among those, *eer1* homozygotes were identified using a CAPS marker tightly linked to the *EER1* locus. Double homozygotes were identified in the next generation after selfing. F₂ progeny of the cross with *ein2-1* were screened on 0.1 μM ACC and progeny demonstrating the *eer1* phenotype were selected. F₃ families from self-fertilized homozygous *eer1* F₂ lines were scored on 10 μM ACC for segregation of ethylene-insensitive individuals, which were the *eer1 ein2-1* double mutants. F₂ progeny of the cross with *ctr1-3* were screened in the absence of ACC, and those individuals displaying the *eer1* phenotype (homozygous for *eer1*), but not the *ctr1-3* phenotype, were isolated. F₃ families from F₂ self-fertilized lines were scored in the absence of ACC to observe segregation of potential *eer1 ctr1* double mutants.

For genetic mapping of the *EER1* locus, *eer1* (male) was crossed to wild-type La-0 (female). The mapping population consisted of 400 F₂ individuals (F₁ self-progeny), all of which were homozygous for *eer1*. The map location was determined by identifying simple sequence length polymorphism (Bell and Ecker, 1994) and CAPS (Konieczny and Ausubel, 1993) markers that cosegregated with the *eer1* phenotype. Genetic distance was determined using the Mapmaker II program (Lander et al., 1987).

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