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# Ethylene Responses Are Negatively Regulated by a Receptor Gene Family in *Arabidopsis thaliana*

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## Summary

**A family of genes including *ETR1, ETR2, EIN4, ERS1,* and *ERS2* is implicated in ethylene perception in *Arabidopsis thaliana.* As only dominant mutations were previously available for these genes, it was unclear whether all of them are components in the ethylene signaling pathway and whether they code for positive or negative regulators of ethylene responses. In this study, we have isolated loss-of-function mutations of four of these genes (*ETR1, ETR2, EIN4*, and *ERS2*) and identified an ethylene-independent role of *ETR1* in promoting cell elongation. Quadruple mutants had constitutive ethylene responses, revealing that these proteins negatively regulate ethylene responses and that the induction of ethylene response in *Arabidopsis* is through inactivation rather than activation of these proteins.**

## Introduction

The biological role of ethylene was first discovered by Neljubow, who found that ethylene was the active component of illuminating gas that caused the horizontal growth of etiolated pea seedlings (Neljubow, 1901). Numerous physiological effects of ethylene on a variety of plant species have been discovered since then (Abeles, 1992), and among the best known is promotion of fruit ripening. Other ethylene effects include promotion of seed germination, inhibition or promotion of cell elongation and cell division, induction of leaf epinasty (downward-curled morphology), and induction of senescence and abscission of leaves and flowers. That ethylene is a plant hormone was established when it was found to be produced by the plants and to affect plant growth and development at very low concentrations (Abeles, 1992). Ethylene has also been identified as a mediator of adaptation responses to stress and pathogen infection (Abeles, 1992; O'Donnell et al., 1996; Penninckx et al., 1996) and a regulator of cell fate determination in root epidermis (Tanimoto et al., 1995). It is implicated in the regulation of morphogenesis in plant tissue culture as well (Kumar et al., 1998).

The action of ethylene was thought to be through a receptor, and a metal ion has been proposed to be involved in the binding (Burg and Burg, 1967). Attempts to isolate ethylene receptors through biochemical approaches were not successful. In the last decade, the application of molecular and genetic studies to *Arabidopsis thaliana* has allowed identification of the ethylene receptor(s). Several ethylene response mutants have been isolated (Ecker, 1995), and the genes that code for some of the components in the ethylene signal transduction pathway have been cloned. ETR1 has been demonstrated to be an ethylene receptor based on the following observations. *etr1* mutants are dominant and insensitive to ethylene in all the ethylene responses analyzed (Bleecker et al., 1988), and the *ETR1* gene acts upstream of the rest of the ethylene signal transduction pathway (Kieber et al., 1993). The ETR1 protein contains a novel amino-terminal domain that possesses ethylene-binding activity (Schaller and Bleecker, 1995). The carboxyl-terminal domain of ETR1 exhibits sequence homology to the bacterial two-component regulators (Chang et al., 1993). The two-component regulators are predominantly sensors and signal transducers of environmental stimuli in a variety of adaptation responses in bacteria (Parkinson and Kofoid, 1992). Recently, they have been found to act in osmolarity sensing in yeast (Maeda et al., 1994), hyphal growth in *Neurospora* (Alex et al., 1996), osmolarity sensing and development of *Dictyostelium* (Schuster et al., 1996; Wang et al., 1996), and possibly cytokinin signal transduction in *Arabidopsis* (Kakimoto, 1996).

Four *ETR1*-related genes have been identified in *Arabidopsis*: *ERS1, ETR2, EIN4,* and *ERS2* (Hua et al., 1995, 1998; Sakai et al., 1998). The deduced proteins of the five members of this gene family share 57%–79% sequence similarity to each other. The amino-terminal domain is the most similar region among all the family members, suggesting a possible conserved activity in ethylene binding. ETR1, ETR2, and EIN4 have both a putative histidine protein kinase domain and a receiver domain in the carboxyl-terminal region, whereas ERS1 and ERS2 lack the receiver domain. The involvement of *ERS1, ETR2, EIN4*, and *ERS2* in ethylene sensing has been suggested by their sequence similarity to the *ETR1* gene and by their mutant phenotypes. *etr2* and *ein4* mutants identified in genetic screens were all dominant and insensitive to ethylene (Roman et al., 1995; Sakai et al., 1998). Similar mutations in the *ERS1* and *ERS2* genes have not been isolated from chemical mutagenesis; however, transgenic plants with in vitro-mutated *ERS1* or *ERS2* genes exhibited dominant ethylene-insensitive phenotypes (Hua et al., 1995, 1998). The dominant mutant phenotypes of these four genes are very similar to those of *etr1* mutants in a number of responses. There are some slight differences between mutant alleles, which may result from the strength of the alleles or from the expression levels of these genes.

*CTR1* is a negative regulator of the ethylene responses, as its loss-of-function mutants have constitutive ethylene responses. It encodes a serine/threonine kinase that is closely related to RAF kinases (Kieber et al., 1993). Double mutant analysis indicates that *CTR1* acts downstream of the family of *ETR1*-like genes. A further downstream gene, *EIN3*, encodes a nuclear-localized protein (Chao et al., 1997). *ein3* loss-of-function mutants have an ethylene-insensitive phenotype,

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and overexpression of *EIN3* confers constitutive ethylene responses, indicating that it is necessary and sufficient to activate ethylene responses.

The functions of the family of *ETR1*-related genes are not well understood, since only dominant mutants have been available for study. As the dominance of these mutants may result from either loss of function or gain of function, it is unknown if all of these genes are involved in ethylene signaling. It is possible that some of them may not normally be involved in ethylene sensing at all, having ethylene effects only in gain-of-function mutant states. Moreover, whether these proteins are positive or negative regulators of ethylene responses cannot be inferred even if they are components of the ethylene signal transduction pathway.

It is thus crucial to analyze the loss-of-function mutant phenotypes of these genes to elucidate their roles in ethylene signal transduction. In this study, we used two approaches to isolate loss-of-function alleles of four members of this gene family. We also constructed double, triple, and quadruple mutants and characterized their phenotypes.

## Results

### Isolation of *etr1* Loss-of-Function Mutants

All of the *etr1* mutants (*etr1-1* to *etr1-4*) identified from previous screens are dominant ethylene insensitive, and they have missense mutations in the amino-terminal ethylene-binding domain (Chang et al., 1993). The lack of recessive alleles may have arisen from any of the following three scenarios. The *ETR1* gene may be essential, so that the loss-of-function mutants are lethal. *ETR1* may have redundant functions with other genes, so that the loss-of-function alleles have wild-type ethylene responses. Alternatively, *ETR1* may not normally be involved in ethylene sensing, so that the loss-of-function alleles do not have ethylene response defects. In the last two scenarios, the ethylene-insensitive mutants will revert to the wild-type ethylene response phenotype when the dominant mutant proteins are eliminated. It might thus be possible to isolate loss-of-function alleles of *ETR1* by looking for intragenic suppressors of *etr1* dominant mutants.

We carried out independent mutageneses of two *etr1* dominant alleles: *etr1-2* and *etr1-1*. Approximately 15,000 seeds homozygous for each allele were mutagenized by ethylmethane sulfonate (EMS), giving a 40% lethality of the M1 plants. Nearly 80,000 etiolated seedlings of the M2 generation for each allele were screened after they were grown in the presence of ethylene for 3 days. Ethylene induces the characteristic triple response in wild-type etiolated seedlings (Guzman and Ecker, 1990), including short hypocotyls and roots. *etr1-1* and *etr1-2* mutants are ethylene insensitive and when grown in the presence of ethylene have longer hypocotyls and roots than the wild type. Seedlings with short hypocotyls and roots, which mimicked the wild-type ethylene responses, were selected as potential suppressors. These lines were rescreened in the M3 generation. The confirmed suppressors were crossed to the parental *etr1-2* or *etr1-1* mutants, and all the F1 plants had an ethylene-insensitive phenotype, indicating that the suppressor mutations are recessive. These suppressors were also crossed to wild-type *Arabidopsis*. Most of the lines had ethylene-insensitive F1 progeny, suggesting that these suppressor mutations were extragenic. The progeny of one suppressor of *etr1-2* and three suppressors of *etr1-1* had wild-type ethylene responses, suggesting that these four lines had intragenic suppressors. They were designated *etr1-5* (from the *etr1-2* screen) and *etr1-6* to *etr1-8* (from the *etr1-1* screen) (Figure 1A).

To identify the molecular lesions of these suppressors, we amplified the coding regions of the *ETR1* gene by polymerase chain reaction (PCR), and the PCR products were directly sequenced. In each potential intragenic suppressor line, we identified a G-to-A transition (Figure 1B), which resulted in a stop codon at Trp-563 in both *etr1-5* and *etr1-8* and a stop codon at Trp-74 in *etr1-7.* In *etr1-6*, the transition occurred at the first nucleotide G in the second intron, which presumably destroyed the splicing consensus sequence. Reverse transcription-PCR experiment confirmed that this intron was not spliced in *etr1-6.* The deduced protein made from this unspliced transcript ends shortly after the mutation.

These suppressor alleles are loss-of-function or reduction-of-function alleles of *ETR1*. The predicted ETR1 protein in *etr1-7* only has the first 73 amino acids, and no ETR1 protein signal was detected in *etr1-7* protein extracts on Western blots (G. E. Schaller, personal communication). Western blot analysis did not detect any ETR1 protein signals from *etr1-5* and *etr1-6* protein extracts either (data not shown; G. E. Schaller, personal communication). Presumably, these truncated proteins are unstable.

### Isolation of Loss-of-Function Mutants of *etr2* and *ein4*

Both *etr2-1* and *ein4-1* mutants have ethylene-insensitive phenotypes similar to those of *etr1-1* (Roman et al., 1995; Sakai et al., 1998). Like *etr1* dominant mutations, mutations in *etr2-1* and *ein4-1* are missense mutations in the hydrophobic segments of the amino-terminal domains of the proteins (Hua et al., 1998; Sakai et al., 1998). Thus, the loss-of-function mutants of *ETR2* and *EIN4* might be identified by an approach similar to that applied to *ETR1.* We therefore independently mutagenized approximately 15,000 *etr2-1* and *ein4-1* seeds and screened more than 150,000 M2s for each mutant, using the criteria described above.

We isolated three potential intragenic suppressors of *etr2-1*, designated *etr2-2*, *etr2-3*, and *etr2-4. etr2-2* was a weak suppressor and still retained residual ethylene insensitivity in the triple response assay. Sequencing the *ETR2* gene in these lines revealed that *etr2-2* had a G-to-A transition that resides at the 5′ splicing site of the intron residing in the coding region of the putative histidine protein kinase domain of ETR2 (Figure 1C). This residual ethylene insensitivity might be due to the correct splicing of a very small amount of RNA or to weak dominant activity of the truncated *etr2-2* protein. *etr2-3* and *etr2-4*, two independent isolates, had identical molecular lesions in the *ETR2* gene: a G-to-A substitution producing a stop codon at Trp-312 (Figure 1C) at

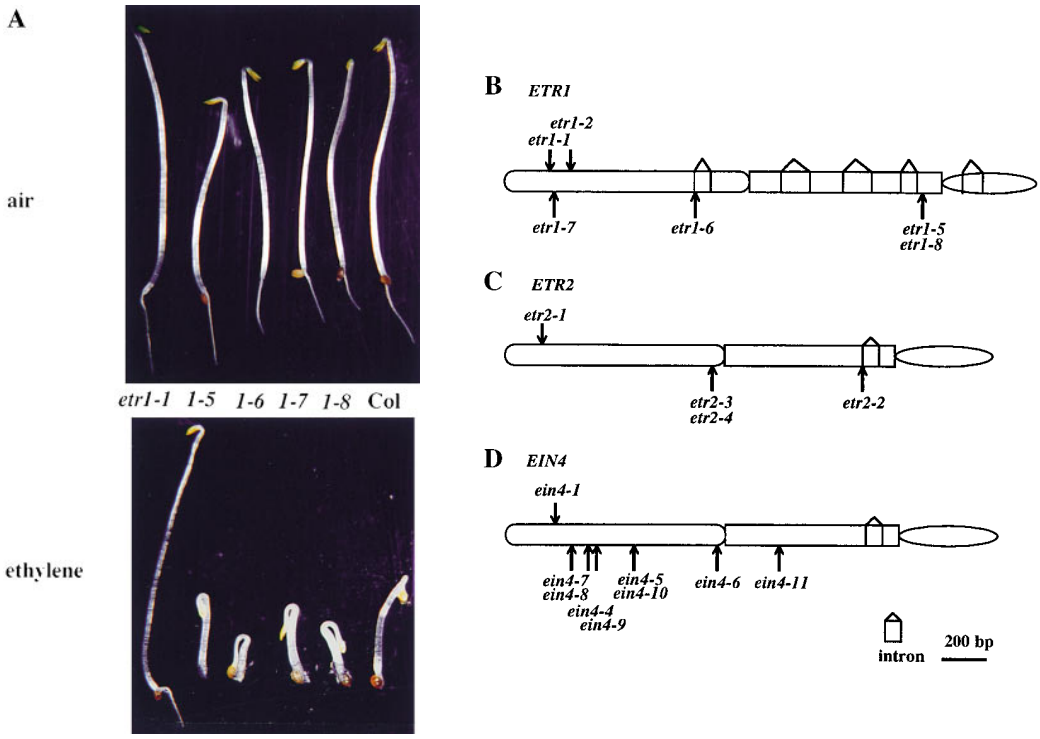


Figure 1. Suppressor Screens of the Ethylene-Insensitive Mutants

(A) Ethylene responses of four intragenic suppressors of *etr1-2* and *etr1-1. etr1-1* is ethylene insensitive, and the suppressor lines (*etr1-5* to *etr1-8*) have close to wild-type triple responses. Col, wild-type *Arabidopsis* ecotype Columbia.

(B) Mutation sites in the *etr1-1* and *etr1-2* intragenic suppressor lines.

(C) Mutation sites in the *etr2-1* intragenic suppressor lines.

(D) Mutation sites in the *ein4-1* intragenic suppressor lines.

the end of the amino-terminal domain. These two alleles are thus likely to be loss-of-function mutants.

From the *ein4-1* suppressor screen, we identified nine intragenic suppressor lines, designated *ein4-4* to *ein4-12. ein4-6, ein4-11*, and *ein4-12* had C-to-T transitions, and the others had G-to-A transitions (Figure 1D). These mutations produced stop codons at the following positions: W138, W205, R356, W109, W109, E142, W205, Q442, and Q442 (*ein4-4* to *ein4-12*, respectively). Therefore, severely truncated proteins are expected to be made in these mutants, and these alleles are presumably loss of function.

### Isolation of an *ers2* Loss-of-Function Mutant

We took a different approach to identify *ers2* loss-of-function mutants, as only transgenic *ers2* mutants were available (Hua et al., 1998). We screened DNA pools representing 7000 T-DNA inserted lines (obtained from the Arabidopsis Biological Resources Center) to identify T-DNA insertions in the *ERS2* gene. Four *ERS2*-specific primers were used in combination with primers specific to the left border and the right border of the T-DNA to PCR-amplify the DNA pools (Figure 2). The PCR products were size fractionated, blotted, and probed with an *ERS2* genomic fragment. One DNA pool of 1000 lines gave positive signals from two PCR reactions, and we traced the signals to one single T-DNA line. Further PCR and sequence analysis showed that the T-DNA insertion is located in the nucleotides coding for Ala-424 at the beginning of the putative histidine protein kinase domain (Figure 2). This insertion likely has more than one T-DNA or has complicated configurations, as both ends of the insertion were of left borders of the T-DNA. This T-DNA line is thus likely to be a loss-of-function mutant of the *ERS2* gene due to insertion of a large piece of foreign DNA into the coding region.

### *etr1* Loss-of-Function Mutants Have Defects in Cell Elongation

The *etr1* loss-of-function mutants had close to wild-type triple responses. To analyze their ethylene responses quantitatively, we performed dose–response assays on *etr1-6, etr1-7,* and *etr1-8*. Etiolated seedlings were

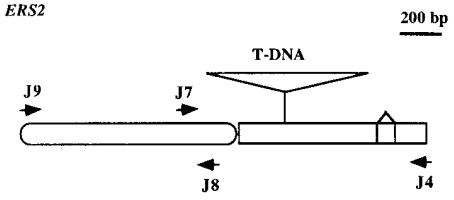


Figure 2. Screen for T-DNA Insertions in the *ERS2* Gene

*ERS2*-specific primers used for PCR screening are indicated. The *ers2-3* mutant has a T-DNA insertion in the *ERS2* coding region of the putative histidine kinase domain.

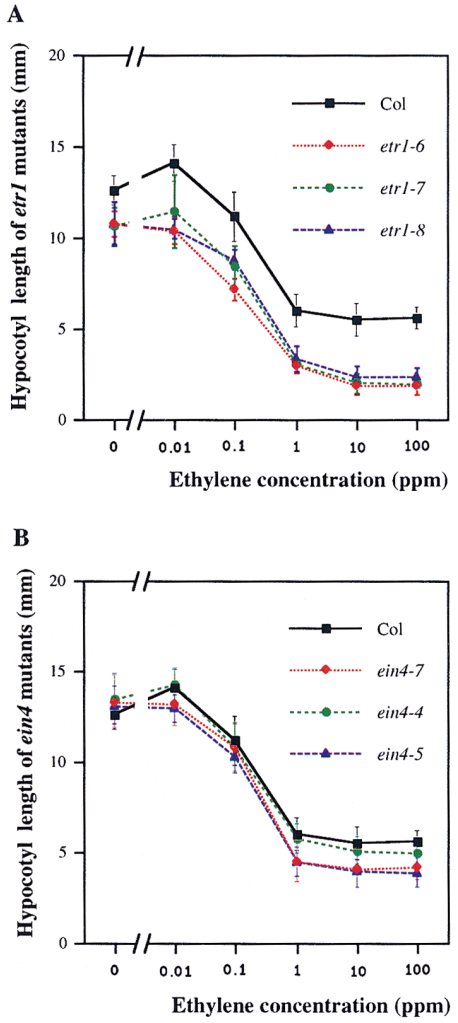


Figure 3. Ethylene Dose-Response Analysis of the Single Mutants

(A) Hypocotyl lengths of etiolated seedlings in wild type (Col); *etr1-6*, *etr1-7*, and *etr1-8* at various ethylene concentrations.

(B) Hypocotyl lengths of etiolated seedlings in wild type (Col); *ein4-4, ein4-5*, and *ein4-7* at various ethylene concentrations.

grown at different concentrations of ethylene (0, 0.01, 0.1, 1, 10, and 100 ppm) for 3 days, and their hypocotyl lengths were measured. The wild-type seedlings had increasingly reduced hypocotyl lengths at ethylene concentrations from 0.01 ppm to 10 ppm, and the half-maximum response was obtained at a concentration between 0.1 ppm and 1 ppm (Figure 3A). The *etr1* loss-of-function mutants exhibited ethylene sensitivity similar to that of wild type; that is, they responded to ethylene within a similar concentration range and with similar kinetics (Figure 3A). However, the hypocotyls of these mutants were shorter than those of wild type at the same ethylene concentrations (Figure 3A). For instance, at saturating concentrations of ethylene (greater than 10 ppm), the hypocotyls of the *etr1* mutants were only about half the length of the wild type. Microscopic analysis indicated that the reduction in the hypocotyl length was due to a reduction in cell elongation (data not shown). Similar defects were also observed in the roots of the *etr1* loss-of-function mutants (data not shown). The *etr1* mutants are thus defective in cell elongation in etiolated seedlings, and this defect does not seem to be ethylene dependent. The defect in hypocotyl and root elongation of the *etr1* mutant was also observed in a *ctr1* mutant background. *ctr1* mutants have constitutive ethylene responses, expressed in the etiolated seedlings as the constitutive triple response, including short hypocotyls and roots. Etiolated seedlings of *etr1-7; ctr1-1* had even shorter hypocotyls and roots than *ctr1-1* seedlings, indicating an additive phenotype in the double mutants.

The *etr1* loss-of-function mutants had almost normal growth and development under light except that they had slightly smaller leaves than the wild type. This feature became more obvious when plants were grown under suboptimal conditions, such as growth on MS medium without sucrose for more than 10 days. Similar to the defect in hypocotyl elongation, this defect of *etr1* mutants in leaf growth was additive with that of *ctr1-1*. It remains to be determined by dose–response analysis whether this function of *ETR1* is ethylene independent.

### Single *etr2, ein4,* and *ers2* Loss-of-Function Mutants Do Not Appear to Have Ethylene Response Defects

The *etr2, ein4,* and *ers2* single loss-of-function mutants did not exhibit obvious abnormal phenotypes when grown under light. To analyze the ethylene responses of these mutants grown in the dark, we performed dose–response analyses on their hypocotyl elongation. All of the *ein4* loss-of-function alleles tested (including *ein4-4, ein4-5,* and *ein4-7*) had essentially the same dose–response curves as the wild type (Figure 3B), indicating wild-type ethylene responses in these *ein4* mutants.

The *etr2-3, etr2-4*, and *ers2-3* mutants also had close to wild type dose–response curves, although a small reduction of hypocotyl elongation was observed at lower concentrations of ethylene (less than 0.1 ppm) when compared to wild type (data not shown). This deviation is likely to be a result of an overall later germination of the seeds used for analyses.

Taking these data together, it appears that the single loss-of-function mutants of *ETR1, ETR2, EIN4*, and *ERS2* are viable and have wild-type ethylene responses, explaining the failure to find such mutants in previous genetic screens.

### *etr1; ein4* Double Loss-of-Function Mutants Have an Ethylene Response-like Phenotype in Air

To reveal functions of these homologous genes that might be masked by genetic redundancy, we constructed double mutants between *etr1, etr2,* and *ein4* loss-of-function alleles. The *etr1* and *etr2* double mutants (both *etr1-6; etr2-3* and *etr1-7; etr2-3*) and *etr2* and *ein4* double mutants (*etr2-3; ein4-4*) did not appear to have a more severe phenotype than the single mutants when grown either in the light (Figure 4A) or in the dark (data not shown).

We observed abnormal phenotypes in the double mutants lacking functional *ETR1* and *EIN4* (both *etr1-6; ein4-4* and *etr1-7; ein4-4*). The double mutants grown under light had reduced leaf size compared to wild type

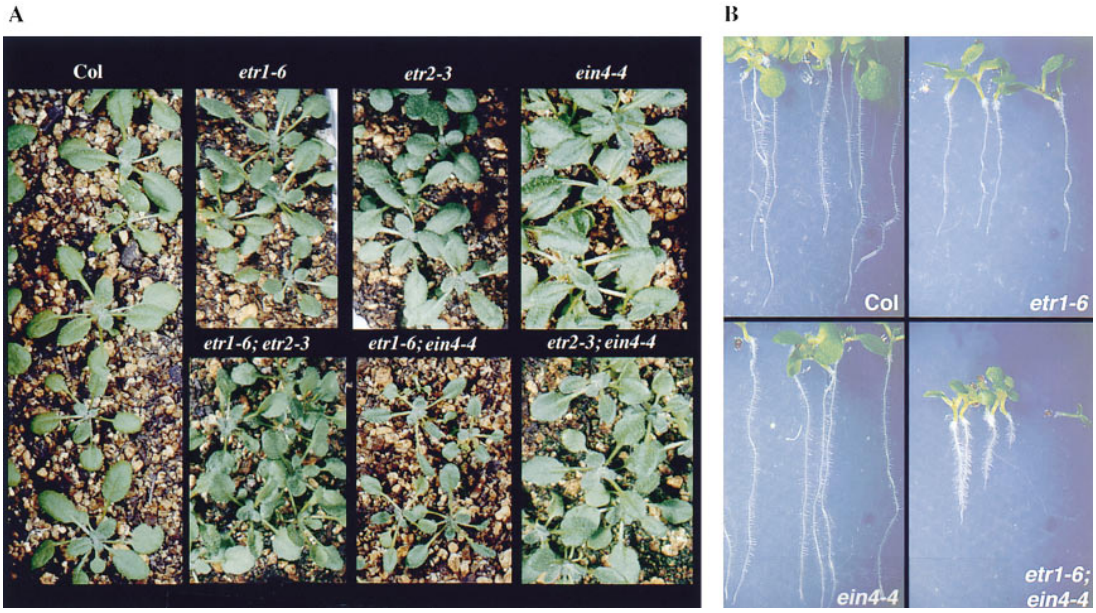


Figure 4. Phenotypes of the *etr1; ein4* Double Mutants

(A) Three-week-old light-grown seedlings of wild type, three single loss-of-function mutants, and three double loss-of-function mutants grown on soil.

(B) One-week-old light-grown seedlings of wild type, *etr1-6, ein4-4,* and *etr1-6; ein4-4* mutants grown on MS medium.

or to the single mutants (Figure 4A). The leaf area was less than half that of the wild type. Microscopic analysis of the leaf epidermal cells indicated that the reduction of leaf area was mostly due to a reduction in cell size (data not shown). The roots of *etr1; ein4* were much shorter and appeared to have more root hairs than the wild type (Figure 4B). Ethylene has been shown to greatly reduce leaf expansion and root elongation and induce root hair formation in *Arabidopsis* (Kieber et al., 1993; Tanimoto et al., 1995). The phenotypes of the *etr1; ein4* double loss-of-function mutants suggest that these mutants grown in air have weak responses similar to those elicited by exposure to ethylene.

Dose–response analysis was performed on the hypocotyl elongation of the *etr1; ein4* etiolated seedlings. They behaved essentially like the *etr1* single mutants (data not shown). It thus appears that *etr1* and *ein4* have synergistic effects on the development of light-grown plants but not on hypocotyl elongation in etiolated seedlings.

### *etr1, etr2,* and *ein4* Triple Mutants Display Strong Ethylene Response Phenotypes in Air

Triple mutants were constructed between *etr1, etr2,* and *ein4* loss-of-function mutants, and a dramatic phenotype was observed in these mutants (both *etr1-6; etr2-3; ein4-4* and *etr1-7; etr2-3; ein4-4*). Shortly after germination under light, the cotyledons and the first few true leaves of the triple mutants were very much reduced in size compared to wild type or to *etr1; ein4* double mutants, and they had a downwardly curved morphology that was reminiscent of ethylene-induced epinasty (Figure 5A). This epinastic phenotype was observed in *ctr1* mutants (Figure 5A) throughout their lives. The triple mutants consistently had smaller leaves and shorter stems than *etr1; ein4* double mutants, but their epinastic leaf phenotypes were much alleviated later in development. They grew slightly larger and were apparently healthier than the *ctr1* mutants.

Etiolated seedlings of the *etr1; etr2; ein4* triple mutants also had a phenotype not observed in the double mutants. Air-grown seedlings of the triple mutants had very short hypocotyls and roots, and exaggerated apical hooks were often formed (Figure 5B). This triple response morphology of air-grown etiolated seedlings indicates a strong ethylene response in the triple mutants. To analyze this phenotype in detail, we compared their ethylene responses in hypocotyl elongation with those of wild type, *etr1-6,* and *ctr1-1* (Figure 5C). In the absence of exogenous ethylene, hypocotyls of the triple mutants (*etr1-6; etr2-3; ein4-4* and *etr1-7; etr2-3; ein4-4*) were much shorter than those of the wild type and *etr1-6* and were about the same as or slightly longer than those of *ctr1* mutants. As ethylene concentration increased, the triple mutants, but not the *ctr1* mutants, displayed reduced hypocotyl elongation. This ethylene response of the triple mutants became saturated at 0.1 ppm of ethylene, which was less than found for wild type or the *etr1* single mutants. This phenotype indicated that the triple mutants had a strong ethylene response when grown in the absence of exogenous ethylene yet were still able to respond to applied ethylene.

The strong ethylene response displayed by the air-grown triple mutants may be due to a constitutive ethylene response, an increased sensitivity to endogenous ethylene, or an overproduction of ethylene. To distinguish between these possibilities, we compared the growth of the triple mutants on media with or without silver ion or aminoethoxyvinylglycine (AVG). Silver ion is an inhibitor of ethylene binding (Beyer, 1976), and

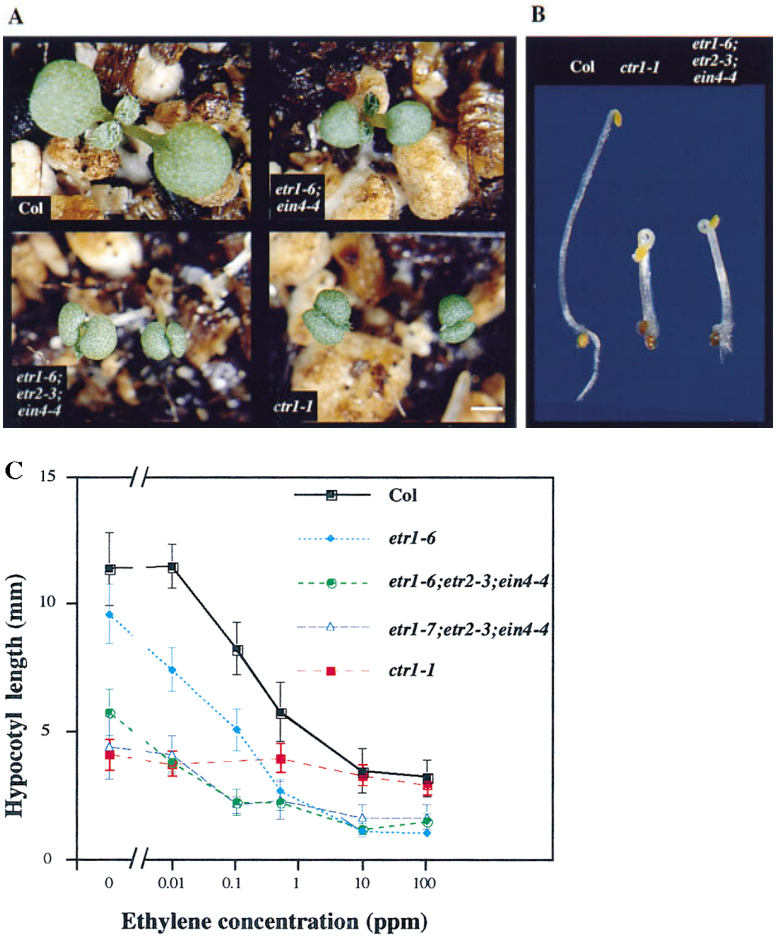


Figure 5. Phenotypes of the Triple Mutants

(A) Light-grown *etr1-6; etr2-3; ein4-4* seedlings have compact cotyledons like *ctr1* mutants.

(B) The etiolated seedling of *etr1-6; etr2-3; ein4-4* triple mutant has a triple response in air similar to the *ctr1-1* seedling.

(C) Dose–response analysis of hypocotyl elongation of etiolated seedlings in *etr1; etr2; ein4* and *ctr1*.

AVG is an inhibitor of ethylene biosynthesis (Yang and Hoffman, 1984). These two substances would be expected to block the effects of endogenous ethylene. Compared to their growth on the regular medium, the triple mutants had approximately a 20% or 50% increase in hypocotyl length when grown on media with silver ion or AVG, respectively. The hypocotyl lengths of these triple mutants, however, were still less than half that of the wild type. Similar hypocotyl length increases were also observed in the wild type and the *ctr1* mutants when grown on these two types of media. This indicated that the strong ethylene response phenotype displayed by the *etr1; etr2; ein4* mutants was not due to enhanced ethylene production or sensitivity, but rather to an activation of ethylene responses at a step later than ethylene perception.

### *etr1; etr2; ein4; ers2* Quadruple Loss-of-Function Mutants Have Constitutive Ethylene Responses

The quadruple loss-of-function mutants (*etr1-6; etr2-3; ein4-4; ers2-3*) were obtained from PCR-genotyped progeny of a quadruply heterozygous mutant. The homozygous quadruple mutants had a more severe phenotype than any of the triple mutants. The leaves were persistently compact and epinastic (Figure 6A). The quadruple mutant plants had greatly reduced stature, even more so than *ctr1* mutants (Figure 6B). The miniature morphology was apparently due to a reduction in cell expansion, as suggested by the greatly reduced leaf epidermal cell size in the quadruple mutant (Figure 6C). More than half of the quadruple mutant plants wilted and died before bolting, although their triple mutant siblings grown in the same pot were healthy. The quadruple plants may have been sicker, more susceptible to pathogen infections, or both. The surviving quadruple mutants were delayed in bolting when compared to the triple mutants. They had shorter inflorescences and fewer and smaller flowers than the triple mutants or the *ctr1* mutants (Figure 6B). No seeds were formed in the quadruple mutants, unlike *ctr1* mutants that are largely fertile (Kieber et al., 1993).

To characterize the quadruple mutant at the molecular level, we analyzed the RNA expression of an ethylene-regulated gene in various mutants. Total RNAs were isolated from leaves of air-grown plants, and RNA blots were probed with the *Chitinase B (CHIB)* gene, which has been shown to be up-regulated by ethylene in leaves (Kieber et al., 1993). No *CHIB* expression was detected in wild type, *etr1* single mutant, or *etr1; ein4* double mutant leaves (Figure 6D). Low expression was seen in the *etr1; etr2; ein4* triple mutant. In the quadruple mutant, the *CHIB* gene was very highly expressed, and the expression level of *CHIB* was comparable to or even higher than that of *ctr1-1* (Figure 6D). This indicates

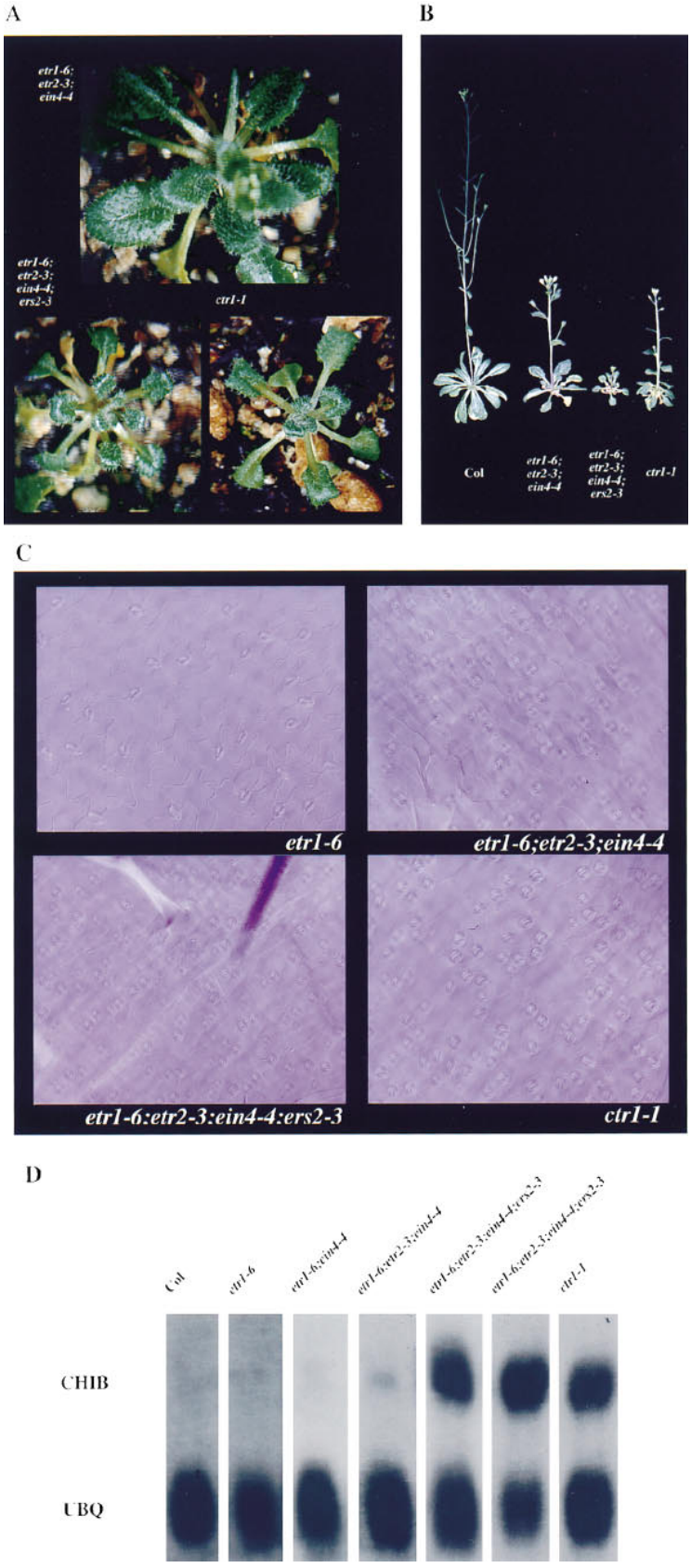


Figure 6. Phenotypes of *etr1; etr2; ein4; ers2* Quadruple Mutants

(A) Leaves of 1-month-old plants.

(B) Whole-plant morphology of various mutants.

(C) Differential interference contrast microscopy of the leaf upper epidermal cells of various mutants.

(D) *CHIB* expression in various mutant lines. Total RNAs were prepared from leaves of 1-month-old plants. *UBQ* is a constitutively expressed ubiquitin extension (*Ubiquitin 5*) gene (Callis et al., 1990).

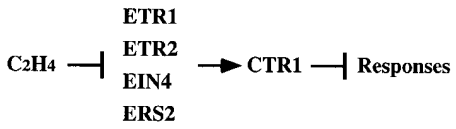


Figure 7. Ethylene Signaling Pathway in *Arabidopsis*

Ethylene inhibits the signaling activities of members of the receptor gene family (ETR1, ETR2, EIN4, and ERS2), which are activators of CTR1.

that the ethylene-induced *CHIB* gene is constitutively expressed in the quadruple mutant. In addition, the expression level of the *CHIB* gene parallels the severity of the constitutive ethylene responses in these mutants.

## Discussion

### Ethylene Responses Are Negatively Regulated by a Receptor Gene Family

We have isolated recessive loss-of-function mutants of the *ETR1, ETR2, EIN4*, and *ERS2* genes, four members of a putative ethylene receptor gene family, using two approaches. One was to screen for intragenic suppressors of the previously identified dominant mutants, and the other to screen for T-DNA insertions in the gene of interest. Ethylene response defects were not detected in these loss-of-function mutants, which explains why only dominant alleles of these genes were identified in previous genetic screens. However, we observed a constitutive ethylene response in the quadruple mutant manifested by the triple response of etiolated seedlings grown in the absence of ethylene, a compact morphology of the adult plant, as a result of a reduction in cell expansion, and a constitutive expression of an ethylene-induced gene. Similar but weaker phenotypes to those of the quadruple mutant were observed in some double and triple mutant combinations.

These results demonstrate that *ETR1, ETR2, EIN4*, and *ERS2* have redundant functions in ethylene signaling (Figure 7). Ethylene is likely sensed by multiple receptors or receptor complexes in *Arabidopsis*. ETR1 is one ethylene receptor, as an ethylene-binding activity has been demonstrated for its amino-terminal domain. The amino-terminal domains of ETR2, EIN4, and ERS2 exhibit strong sequence homology to that of ETR1, suggesting that they may bind ethylene and act as ethylene receptors, like ETR1. Alternatively, these three proteins may not bind ethylene directly, but instead form receptor complexes with other ethylene-binding proteins.

Furthermore, our work reveals that the ethylene receptors or receptor complexes act in an opposite fashion to most known animal hormone or growth factor receptors. Hormones or growth factors, when binding to their receptors, usually induce responses by activating the receptors. For instance, steroid and thyroid hormones bind to nuclear receptors and enable the receptors to bind DNA and regulate transcription (Evans, 1988). Human growth hormone induces dimerization of the receptors, which activates a tyrosine kinase noncovalently linked to the receptors (Wells, 1994). The lack of the receptors for hormones and growth factors usually leads to the same or similar phenotype as the lack of the ligands (Kastner et al., 1995; Conover and Yancopoulos, 1997). Our findings show that knocking out the ethylene receptor(s) does not cause an absence of ethylene response (ethylene-insensitive phenotype), but rather leads to constitutive ethylene responses. Thus, in air, the ethylene responses are actively repressed by the receptors or receptor complexes (Figure 7). It is conceivable that the wild type receptor–related proteins achieve ethylene sensing by having two states. When ethylene is absent, they are in a signaling active state. When ethylene is present, they switch to a signaling inactive state. Nullifying these proteins in the quadruple mutants abolishes the repression, and the characteristic ethylene response results. The phenotype of the loss-of-function mutants of these four genes are opposite to the phenotype of their dominant alleles, establishing that these dominant mutants are gain-of-function and that the mutant proteins are in the signaling active state.

As many ethylene responses are stress responses, it may be advantageous for the plants to have multiple receptors, repressing them so that a stress response is not easily induced unless the repression activities of most of the receptors are substantially relieved. Both positive and negative regulators of hormonal responses for other plant hormones have been identified. BRI1, a positive regulator of brassinosteroid signal transduction pathway, may itself be a receptor for brassinosteroid (Li and Chory, 1997). GAI is a negative regulator of gibberellin signal transduction (Peng et al., 1997), but it is not known whether it is a receptor for gibberellin or not. Identifying receptors of other plant hormones should reveal how prevalently negative regulation at the receptor level is used in plant hormone perception.

Members of the receptor gene family all act through CTR1 because a *ctr1* loss-of-function mutation is epistatic to their dominant alleles. Loss-of-function mutants of the four receptor-related genes and that of the *CTR1* gene both have constitutive ethylene responses, indicating that the receptor-related proteins are positive regulators of CTR1 (Figure 7). The mechanism of the activation is not known. However, it has been shown that ETR1 and CTR1 can physically interact (Clark et al., 1998). The activation of CTR1 could be via a direct phosphorylation or dephosphorylation by the receptor-related proteins or through localization of CTR1 by physical attachment to the proteins.

The quadruple mutant of the four members of the ethylene receptor gene family, however, had a stronger phenotype than the *ctr1* mutant. We also noted that double mutants between the dominant alleles of each of these four genes and the *ctr1* mutation are slightly bigger and healthier than a *ctr1* single mutant, although they largely resemble the single *ctr1* mutant (data not shown). Therefore, this receptor gene family may regulate an additional ethylene response pathway that is independent of the CTR1 pathway. This is consistent with the observation that ethylene responses may not be fully activated in *ctr1* mutants (Kieber et al., 1993). In addition, some of the receptors might have functions that are not related to regulating ethylene response. *ETR1* appears to have an ethylene-independent function in promoting cell elongation. The compact leaves of the quadruple mutants may result from a constitutive

ethylene response combined with the cell expansion defects associated with the *etr1* mutation.

### Genetic Redundancy between Members of the Ethylene Receptor Gene Family

Multiple *ETR1*-like genes have also been isolated in other species, such as tomato (Zhou et al., 1996, and H. Klee, personal communication). It appears unlikely that multiple ethylene receptor–related genes arose through recent gene duplication in *Arabidopsis*. The questions then arise as to why there are several ethylene receptors or receptor complexes in *Arabidopsis* and how they are maintained during evolution. Our molecular and genetic studies of these receptor-related genes suggest that they might have been selected for their emergent functions as well as their divergent functions, as defined by Thomas (1993).

Emergent function refers to a property that depends on the similar functions of several genes but cannot be accomplished by individual genes. One emergent function of the ethylene receptors or receptor complexes might be to enable the plants to sense ethylene over a wide range of concentrations. Each of them may have different ethylene affinity. The one with the highest affinity may be responsible for response at low concentration of ethylene, and the one with the weakest affinity may contribute to response at high concentration of ethylene. The response of multiple receptors may thus be extended over the response range of all the receptors.

Members of the ethylene receptor gene family are differentially regulated by ethylene, at least at the transcriptional level. The RNA levels of *ETR1* and *EIN4* are not appreciably regulated by ethylene, while those of *ERS1, ERS2*, and *ETR2* are up-regulated (Hua et al., 1998). This up-regulation might be a mechanism for adaptation in ethylene responses. Ethylene induces specific responses by relieving the repression activity of the ethylene receptor gene family, and at the same time, it induces the expression of some members of this family. The induction of these genes may lead to the production of more proteins that can repress the ethylene response. By this up-regulation, the system may be progressively desensitized, as higher and higher ethylene concentrations may be needed to maintain the same level of response.

Yet another emergent function of the receptor-related genes may be to obtain different ethylene sensitivities in different tissues at different developmental stages. This can be accomplished by differentially expressing members of the receptor gene family. This view is suggested by RNA in situ hybridization data, which indicates that these genes are differentially expressed in some tissues (Hua et al., 1998). It is also supported by the different levels of severity in phenotype exhibited by the same mutant in different tissues. For instance, the hypocotyl elongation of *etr1; ein4* etiolated seedlings is the same as that of *etr1*, while the double mutants have much more pronounced defects in leaf and root development in light-grown plants than those seen in *etr1* single mutants. Therefore, *ETR1* and *EIN4* together seem to contribute more toward repression of the ethylene response in light-grown plants than in etiolated seedlings, perhaps due to a different expression level compared with the other three genes in these two processes.

In addition, multiple ethylene receptor–related genes might also have been maintained by their unique functions. This has been observed for the *ETR1* gene, which has a role in regulating cell elongation independent of its activity in ethylene perception. The above possibilities are not exclusive, and other possibilities also exist. For instance, the ethylene receptor–related proteins may not act independently, but rather may form heterocomplexes or phosphorelays to sense ethylene.

In summary, ethylene perception in *Arabidopsis* is carried out by a receptor gene family whose members have overlapping functions, and the perception is through a derepression process rather than an activation process. Future molecular and biochemical studies should allow the comparisons of ethylene-binding activities and signaling activities of the ethylene receptors or receptor complexes. The loss-of-function mutants of these receptor-related genes will provide good genetic backgrounds to test the biochemical activities of individual receptors or receptor complexes and to analyze the ethylene-sensing properties of modified ethylene receptors or receptor complexes.

## Experimental Procedures

### Mutageneses and Genetic Screens

Mutant seeds were imbibed in 0.25% EMS with shaking for 12 hr before they were washed with several changes of water for 3 hr. They were then resuspended in 0.1% agarose and sowed on soil at about 80 seeds per pot. Approximately 60% of the M1 seeds germinated. M2 seeds from one pot were collected as a family, and 10–20 M2 seeds per M1 plant were screened. The M2 seeds were sterilized and plated on MS plates, cold-treated for 3 days, and exposed to light at room temperature for 6 hr. The plates were then placed in the dark and in an ethylene chamber for 3 days. Seedlings with shorter hypocotyls and roots were transferred to fresh plates and grown under light for about 1 week before being transplanted to soil.

*etr1-1, etr1-2, etr2-1,* and *ein4-1* are of Columbia ecotype. Intragenic suppressors (*etr1-6, etr1-7, etr1-8, etr2-3,* and *ein4-4*) were outcrossed to wild type (ecotype Columbia) at least once. *ers2-3* is of Wassilewskija ecotype.

### Sequence Analysis to Identify Mutations in the Intragenic Suppressor Lines

Genomic DNAs were prepared as described (Konieczny and Ausubel, 1993), except that the second ethanol precipitation was not performed. Respective receptor-related genes were PCR-amplified using gene-specific primers. PCR products separated on low–melting point agarose gels were excised for sequencing using Sequenase Version 2.0 (U. S. B.). Sequencing reactions from the wild type and the mutant were run side by side, and their sequences were compared directly on the autoradiograph films.

### Screen for T-DNA Insertions in the *ERS2* Gene

Four *ERS2* gene-specific primers were used. They are J9 (5′ ATTGT TACTCAGGGAAACAG), J7 (5′ GGTTCTCGATACCGATTAGTG), J8 (5′ GCTAGTCACTGAGGCAAGCAC), and J4 (5′ CTGACGTCAATGAT CAGTGG). Primers specific for the left border and the right border of the T-DNA are LB3 (5′ GACAAGTATCAAACGGATGTGAA) and RB1 (5′ GGTTTCTGACGTATGTGCTT). Eight total combinations of T-DNA-specific primers and *ERS2* gene-specific primers were used in the PCR-based screen. Conditions for amplifications were as follows: 30 s at 94°C, 30 s at 52°C, and 2 min at 72°C. The cycle was repeated 40 times, preceded by 3 min at 94°C, and followed by 5 min at 72°C. PCR products were size fractionated on 1% agarose gels and blotted onto nylon membrane. The *ERS2* genomic DNA fragment (PCR amplified using J9 and J4 primers) was labeled

by random primers (Boehringer Mannheim) to probe the blots. Positive signals were detected after 1 hr exposure to X-ray film at room temperature.

### Genotyping

PCR-based genotyping was designed for mutations of *etr1-1, etr2-1, ein4-1,* and *ers2-3*. Leaf tissues were prepared for PCR as described (Klimyuk et al., 1993). The amplification conditions were as follows: 94°C for 15 s, 48°C for 15 s, and 72°C for 30 s. The cycle was repeated 40 times, preceded by 3 min at 94°C, and followed by 5 min at 72°C. Mismatches (underlined) to the genomic sequences were introduced in the primers to incorporate restriction sites in the PCR products of one allele or to destabilize the primer annealing. For the *etr1-1* mutation, ETR1-1 ApaII (5′ AAGTTAATAAGATGAGTT GGTGCA) and ETR1 C (5′ AGAAATCAGCCGTGTTTCCG) were used in 20 μl of the PCR reaction. Four microliters of the reaction was used for ApaII digestion, and the digest was separated on 4% agarose gels. The wild-type allele gave two bands of 60 bp and 20 bp, while the *etr1-1* allele gave a band of 80 bp. For the *ein4-1* mutation, EIN4-1 MseI′ ( 5′ GAGTCATTCCACATAGGACAT) and EIN4 H25 (5′ GTGATCTCTTAATAGCCATTG) were used. Four microliters of the reaction was digested with MseI and separated on 4% gels. The wild-type allele yielded two fragments of 120 bp and 20 bp, while *ein4-1* gave one band of 140 bp. For the *etr2-1* mutation, ETR2-1w (5′ TAGCTTATTTCTCAATTCC) and ETR2-1m (5′ TAGCTTATTTCTC AATTCT) were combined with ETR2-41 (5′ CAGAGTAAGTCCAACC ATG), respectively, as primers for PCR. Positive signals from ETR2-1m and ETR2-41 reaction indicated the presence of the *etr2-1* mutation, and positive signals from ETR2-1w and ETR2-41 reaction indicated the presence of the wild-type allele. For the *ers2-3* mutation, primers J2 (5′ ACGACGTAGTCAGGCAATGCTC), J7, and LB3 were used in the same PCR reaction. The PCR products were fractionated on 2% agarose gels. The amplification of a 200 bp product (from J2 and LB3) indicated the presence of the *ers2-3* mutation, and the amplification of a 740 bp product (from J2 and J7) indicated the presence of the wild-type *ERS2* gene.

### Construction of Mutant Combinations

The double mutants were identified by PCR-based genotyping of the F2 progeny of the crosses between single mutants. *etr1; etr2; ein4* mutants were identified by genotyping the F2 progeny of crosses between *etr1; etr2* and *etr2; ein4* or *etr1; ein4* and *etr2; ein4.* The quadruple mutants were constructed first by crossing *ers2-3* to *etr1-6*/+; *ein4-4*/+; *etr2-3.* F1 plants were genotyped, and the quadruply heterozygous mutants were identified and selfed. Etiolated F2 seedlings were screened on MS medium in air, and plants with shorter hypocotyls were selected. After these selected plants produced several leaves in soil, they were PCR-genotyped to identify the quadruple mutants.

### RNA Analysis

Total RNA was prepared from leaves of 1-month-old plants according to the instruction of the RNeasy Plant Mini Kit (QIAGEN). RNA blots were prepared as described (Sambrook et al., 1989). The *CHIB* gene was PCR-amplified from the genomic DNA using primers as previously described (Chen and Bleecker, 1995).

### Dose–Response Analysis

Seeds were plated on petri dishes with MS medium, cold-treated for 3 days, and exposed to light at room temperature for 6 hr before they were placed in 3-liter jars in a dark room. The jars were flushed with air and injected with diluted ethylene to get the desired concentration once a day for 3 consecutive days. Zero parts per million of ethylene was obtained by placing ethylene-absorbent $KMnO_4$ in the jar. Ethylene concentrations were monitored twice a day by gas chromatography. Pictures were taken of the petri dishes after 3 days of incubation, and the lengths of the hypocotyl and the root were measured either by projecting the photos onto a screen or by scanning the images into a computer and using the NIH Image program.

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