EIN4 and ERS2 Are Members of the Putative Ethylene Receptor Gene Family in Arabidopsis

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The Arabidopsis ethylene receptor gene ETR1 and two related genes, ERS1 and ETR2, were identified previously. These three genes encode proteins homologous to the two-component regulators that are widely used for environment sensing in bacteria. Mutations in these genes confer ethylene insensitivity to wild-type plants. Here, we identified two Arabidopsis genes, EIN4 and ERS2, by cross-hybridizing them with ETR2. Sequence analysis showed that they are more closely related to ETR2 than they are to ETR1 or ERS1. EIN4 previously was isolated as a dominant ethylene-insensitive mutant. ERS2 also conferred dominant ethylene insensitivity when certain mutations were introduced into it. Double mutant analysis indicated that ERS2, similar to ETR1, ETR2, ERS1, and EIN4, acts upstream of CTR1. Therefore, EIN4 and ERS2, along with ETR1, ETR2, and ERS1, are members of the ethylene receptor-related gene family of Arabidopsis. RNA expression patterns of members of this gene family suggest that they might have distinct as well as redundant functions in ethylene perception.

INTRODUCTION

The simple gas ethylene acts as an endogenous regulator of growth and development as well as a mediator of stress responses in higher plants. It is involved in many developmental processes, including seed germination, leaf and flower senescence, and fruit ripening (Abeles, 1992). It regulates basic cellular processes such as cell elongation (Abeles, 1992) and root hair formation (Tanimoto et al., 1995). It also acts as a signal in pathogen defense, wound responses, and nodule formation (Abeles, 1992; O’Donnell et al., 1996; Penninckx et al., 1996; Pennmetsa and Cook, 1997). The diverse roles that ethylene plays and the specificity of its action suggest complexity in the regulation of its synthesis and in its signal transduction pathway. This complexity has been observed in the regulation of ethylene production. The biosynthetic pathway of ethylene has been elucidated (Yang and Hoffman, 1984). The precursor S-adenosyl-L-methionine is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. Ethylene is produced from ACC by the action of ACC oxidase. These two key enzymes, ACC synthase and ACC oxidase, each encoded by a multigene family, are differentially expressed in response to developmental, environmental, and hormonal factors (Kende and Zeevaart, 1997).

Recently, progress has been made in understanding the signal transduction pathway of ethylene. This has been achieved largely by molecular and genetic studies that use the model plant Arabidopsis. More than a dozen ethylene response mutants have been identified by screening for alterations in the triple response (Ecker, 1995). Triples refers to the morphological changes of etiolated seedlings in response to ethylene, including short and thick hypocotyls, short roots, and exaggerated apical hooks (Bleecker et al., 1988; Guzmán and Ecker, 1990). The response mutants can be classified into two categories: ethylene-insensitive mutants and constitutive response mutants. The ethylene-insensitive mutants do not exhibit a triple response in ethylene, or they exhibit only a weak response. Such mutants include etr1 (for ethylene response), etr2, ein2 (for ethylene insensitive), ein3, ein4, ein5, ein6, ein7, and ain1 (for ACC insensitive) (Ecker, 1995; Sakai et al., 1998). The constitutive ethylene response mutants, including eto1 (for ethylene overproducer), eto2, eto3, and ctr1 (for constitutive triple response), exhibit a triple response even when grown in air. CTR1 acts in the ethylene response pathway, whereas ETO genes are regulators of ethylene synthesis because eto mutants produce more ethylene than does the wild type (Kieber et al., 1993). Double mutant analyses have placed these genes in a genetic pathway (Ecker, 1995), and cloning of some of the genes has shed light on the biochemical properties of the response process.

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The emerging picture is that ethylene signal transduction is performed by a phosphorylation cascade that might modulate the activity of transcription factors regulating gene expression. The ethylene signal appears to be perceived by a family of ethylene receptors. Three members of the putative ethylene receptor gene family, ETR1, ERS (for ETHYLENE RESPONSE SENSOR), and ETR2, have been cloned (Chang et al., 1993; Hua et al., 1995; Sakai et al., 1998). The C-terminal domains of these proteins show homology to the bacterial two-component regulators that are widely involved in adaptive responses in bacteria (Pankinson and Kofoid, 1992). The N-terminal domains of these proteins are very similar, and this domain of ETR1 is capable of ethylene binding when expressed in yeast (Schaller and Bleecker, 1995).

By analogy to the bacterial histidine kinases, the binding of ethylene to the N-terminal domain of ETR1 could modulate the activity of the C-terminal histidine kinase, which in turn regulates downstream components. CTR1 acts downstream of these three genes, as indicated by epistasis analysis. It is a negative regulator of the ethylene response and encodes a serine/threonine protein kinase related to RAF kinases (Kieber et al., 1993). The signal from the receptor(s) could be transduced to CTR1 via mediators such as cognate response regulators, which are signal recipients of histidine kinases in both the bacterial two-component systems and the yeast osmolarity sensing pathway (Maeda et al., 1994). Alternatively, the ethylene receptors could interact directly with CTR1 (Clark et al., 1998). EIN3 acts downstream of ETR1 and CTR1. It encodes a novel nuclear-localized protein and probably is involved in transcriptional regulation (Chao et al., 1997). Because the level of EIN3 mRNA is not affected by ethylene treatment, the activity of EIN3 is likely to be regulated post-transcriptionally—perhaps by phosphorylation. Overexpression of the two EIN3 homologs, EIL1 or EIL2, complemented the ein3 mutation, suggesting that they participate in the ethylene signal transduction pathway as well (Chao et al., 1997).

We have gained insight into ethylene perception by identifying the ethylene receptor gene ETR1 and the two putative ethylene receptor genes ETR2 and ERS. However, additional components may be involved in this process, as suggested by the following observations. First, the genetic screens for ethylene response mutants are not saturated, as indicated by the availability of only one allele at some loci and by the failure to find ers mutants. Second, mutants with similar ethylene-insensitive phenotypes have been identified, but their molecular characteristics have not been determined. One such mutant is ein4, which had a dominant ethylene-insensitive phenotype in the triple response assay. Like the members in the putative ethylene receptor gene family, EIN4 acts upstream of CTR1. Therefore, we attempted to isolate more members of the putative ethylene receptor family in Arabidopsis through sequence homology. Here, we describe the cloning of two ETR2 homologous genes and show that one of them is the EIN4 gene. The other, ERS2, is also involved in ethylene perception, as indicated by transgenic mutant analysis. We analyzed the RNA expression patterns of members of this gene family and the regulation of their expression levels by ethylene. The structure and function of this gene family are discussed.

RESULTS

Isolation of Two Homologs of the ETR2 Gene

A genomic DNA library (Sakai et al., 1998) was screened with an ETR2 genomic DNA fragment at reduced stringency. The library also was screened with ETR2, ETR1, and ERS DNA fragments at high stringency. λ clones that hybridized with ETR2 at reduced stringency but did not hybridize with ETR1, ERS, and ETR2 at high stringency were chosen for further analysis. Ten positive clones were isolated. Cross-hybridization between these clones indicated that they belong to two groups. Sequence analysis revealed that each group represented an ETR2-related gene. cDNA clones of these two genes subsequently were isolated from a cDNA library.

Identification of One Homolog as the EIN4 Gene

One homolog was mapped to chromosome 3 by restriction fragment length polymorphism (RFLP) mapping. It is between molecular markers CITd39 and pCITy31, ~9.3 centimorgans from the top of chromosome 3. The EIN4 gene has been mapped to the top of chromosome 3 between markers GAPC and nga126 (Roman et al., 1995), and this region overlaps with the map region of this ETR2 homolog. It has been suggested that EIN4 may have a function similar to ETR1 and may encode an ETR1 homolog (Roman et al., 1995), because ein4 mutants have dominant ethylene-insensitive phenotypes similar to those of etr1, etr2, and ers mutants. Thus, we pursued the possibility that this homolog is the EIN4 gene.

We isolated two new ein4 alleles (designated as ein4-2 and ein4-3) from screens for ethylene-insensitive mutants. They both exhibited dominant ethylene-insensitive phenotypes in the triple response assay (Figure 1A). ein4-2 was obtained from diepoxybutane mutagenesis. When crossed to ein4-1, it failed to segregate any wild-type seedlings in 679 F2 progeny. ein4-3 was isolated from an ethylmethane sulfate–mutagenized population. Co-segregation of ein4-3 and ein4-1 was not tested, but this mutant is likely to be an ein4 allele, because it maps close to EIN4 (between nga172 and nga162). We identified a missense mutation in each ein4 allele (see below). On the basis of its map position, its sequence homology to the putative ethylene receptor genes, and the mutations in this gene in all three ein4 alleles, we concluded that this homolog is the EIN4 gene.
Approximately 3200 bp of the genomic DNA of EIN4 and its longest cDNA (~2990 bp) were sequenced. The size of the longest cDNA was approximately the same as the size of the corresponding RNA transcript on an RNA gel blot (data not shown), indicating that this cDNA is full length, or nearly so. Comparing the genomic DNA and cDNA sequences revealed that EIN4 contains one intron. The predicted protein has 766 amino acids with a predicted molecular mass of 86 kD. It has an overall 53% identity and 74% similarity to ETR2 in protein sequences and 62 and 60% similarity to ETR1 and ERS, respectively. That EIN4 is more related to ETR2 than to ETR1 or ERS was also evident when individual domains were compared. The N-terminal domain (positions 1 to 347) of EIN4 has 61% sequence identity and 78% similarity to that of ETR2. Like ETR2, it has an extra hydrophobic stretch of amino acids at the very N terminus when aligned to ETR1 and ERS (Figure 2A). The middle portion of the EIN4 protein (positions 348 to 631) is a putative histidine protein kinase domain. Similar to the corresponding domain of ETR2, it is more diverged than those of ETR1 and ERS from the canonical sequences of the bacterial protein histidine kinases (Figure 2B). It is 69% similar to the corresponding portion of ETR2 and 53% similar to those of ETR1 and ERS. However, unlike ETR2, the putative autophosphorylation site histidine residue (H-377) is present at the predicted site. The C-terminal domain has sequence similarity to the receiver domains of ETR2 (70% similarity) and ETR1 (66% similarity; Figure 2C). The two aspartates (D-648 and D-694) and a lysine residue (K-746) conserved in the bacterial two-component regulators are present at the predicted positions in EIN4.

We sequenced the EIN4 gene from the three ein4 mutants; in each allele, we observed a missense mutation (Figure 2A). Both ein4-1 and ein4-2 contained an A-to-T transversion, which results in a substitution of isoleucine at position 84 by phenylalanine in the putative transmembrane segment II. This change is the same as those in etr1-4 and ers-1 (Chang et al., 1993; Hua et al., 1995), ein4-3 contained a C-to-T transition, resulting in a threonine change to methionine at position 117 in segment III. All of the previously identified dominant mutations in this gene family reside in their corresponding segments I, II, and III (Figure 2A). We conclude that we indeed have identified the EIN4 gene (GenBank accession number AF048982). This conclusion was supported further by the identification of nonsense mutations in this gene in ein4-1 intragenic suppressor alleles (J. Hua and E.M. Meyerowitz, unpublished results).
ERS2 Is a New Member of the Putative Ethylene Receptor Family

Approximately 2.4 kb of the genomic DNA of the second homolog and its longest cDNA, which did not seem to be full length, were sequenced. A conceptual coding sequence was constructed based on the size of its RNA transcript as determined by RNA gel blot analysis, the longest cDNA sequence available, and the alignment of its genomic DNA sequence with the ETR2 and EIN4 sequences. One intron was found in this gene. The deduced protein has 645 amino acids with a predicted molecular mass of 72 kD. It shows sequence similarity to the four previously identified members (ETR1, ETR2, EIN4, and ERS) of the ethylene receptor-related

Figure 2. Amino Acid Sequence Alignment of the Putative Ethylene Receptor Gene Family (ETR1, ERS1, ETR2, EIN4, and ERS2).

(A) Alignment of the N-terminal domains. The four hydrophobic segments (I to IV) are underlined. The residues that are mutated in the dominant mutants are in boldface.

(B) Alignment of the putative histidine protein kinase domains. The five consensus motifs (H, N, G1, F, and G2) found in bacterial histidine kinases are indicated above the Arabidopsis sequences. Symbols are modified from Parkinson and Kofoid (1992). Open diamonds indicate nonpolar residues; filled diamonds indicate polar residues. The plus sign indicates basic residues; minus signs indicate acidic or amidic residues. Black dots indicate positions with ≥50% conservation in bacterial histidine kinases.

(C) Alignment of the receiver domains of ETR1, ETR2, and EIN4. The conserved aspartate and lysine residues (D and K) are indicated above the alignment.

In (A) to (C), dashes indicate the gaps added for the alignment. Similar amino acids are shaded.
gene family. No receiver domain like those found in ETR1, ETR2, and EIN4 is present in this gene. Because both ERS and this homolog only contain the sensor (histidine protein kinase) domain and lack the receiver domain, we renamed ERS as ERS1 and named this homolog ERS2 (GenBank accession number AF047976).

The predicted ERS2 protein is related more closely to ETR2 and EIN4 (73 and 68% similarity) than it is to ETR1 or ERS1 (58 and 57% similarity). The N-terminal domain (positions 1 to 362) has 81 and 75% similarity to those of ETR2 and EIN4, respectively. Like ETR2 and EIN4, it has a fourth putative transmembrane segment at the N-terminal end (Figure 2A). The three hydrophobic segments (I, II, and III), which are essential for ethylene binding in ETR1, are conserved in ERS2. The C-terminal domain of ERS2 exhibits 63, 58, 53, and 47% similarity to the putative histidine kinase domains of ETR2, EIN4, ETR1, and ERS1, respectively. It is the most divergent from the canonical form of the bacterial histidine protein kinases among the five proteins. Most of the signature motifs of histidine kinases are not easily recognizable in ERS2, and it lacks the histidine residue at the predicted autophosphorylation site. Also, unlike the other four proteins, ERS2 contains a unique stretch of six serines and five glutamic acids close to the end of the protein (Figure 2B).

The ERS2 gene was mapped to the top of chromosome 1 by RFLP mapping. The newly released sequences from the Arabidopsis genome project contain the gene F19P19.21, which appears to be the same as ERS2. The F19P19.21 annotated sequence has a 16-amino acid deletion from C-470 to R-485 and has an extra six amino acids between K-592 and Y-593 of the predicted ERS2 sequence. These discrepancies apparently are due to annotation errors.

No previously identified mutations reside close to the chromosomal region where the ERS2 gene maps. To identify its function, we used a reverse genetics approach. Its sequence homology to the ethylene receptor gene ETR1 suggested that ERS2 might also play a role in ethylene perception. The functions of other members of the gene family were suggested by their dominant mutant phenotypes resulting from missense mutations in their N-terminal domains. Therefore, we engineered similar missense mutations into the ERS2 gene and introduced the mutated genes into wild-type Arabidopsis plants.

We isolated a 6-kb EcoRI genomic fragment of the ERS2 gene containing the coding region, 4 kb of 5' upstream sequence, and 230 bp of 3' sequence after the translation stop codon. Because the 3' fragment of the cloned ERS2 gene might not have been sufficient for RNA processing, a 1-kb 3' end of the nopaline synthase (NOS) gene was added. Two mutant ERS2 genes were generated by in vitro mutagenesis. The mutant ERS2 P67L gene has proline substituted by leucine at position 67, which is a mutation similar to that of etr2-1 (Sakai et al., 1998). The mutant ERS2 I94F gene has isoleucine changed to phenylalanine at position 94, which is similar to the mutations in etr1-4, ein4-1, and ers1-1 (Chang et al., 1993; Hua et al., 1995). These two mutated ERS2 genes and the wild-type form were separately transformed into wild-type Arabidopsis, and the transgenic lines were designated ers2-1 (containing ERS2 P67L), ers2-2 (containing ERS2 I94F), and ers2-T (containing the wild-type ERS2 gene). All analyzed T1 lines (>50) of ers2-1 and ers2-2 showed ethylene insensitivity in the triple response assay (Figure 1B). No such phenotype was observed in any of the ers2-T plants. In the T2 generation, the ethylene-insensitive phenotype cosegregated with the transgene, indicating that the phenotype is caused by the mutated ERS2 genes.

The ethylene-insensitive phenotype of ers2-1 and ers2-2 extends to responses other than the triple response. Leaf expansion of the mutant plants was not inhibited by the exogenous application of ethylene (data not shown). Moreover, mutant plants had larger leaves than did the wild type when grown in air, which might be due to their insensitivity to endogenous ethylene.

Double mutant analysis was performed to place ERS2 in the ethylene signal transduction pathway. ctr1 mutants have the constitutive ethylene response phenotype. Etiolated ctr1 seedlings show the triple response when grown in air, and the adult ctr1 plants have stunted growth as if they were constantly being treated with ethylene. Double mutants between ers2 and ctr1 mimicked the ctr1 phenotype. Etiolated seedlings of the double mutants had constitutive triple response when grown in air (Figure 1C). The adult plants basically resembled the ctr1 single mutants, although they had slightly more expanded leaves than did ctr1. From these results, we determined that ERS2 acts upstream of CTR1 and thus occupies a position in the ethylene signal transduction pathway similar to other putative ethylene receptors. Thus, ERS2 defines a new member of the putative ethylene receptor gene family.

Expression Patterns of the Ethylene Receptor-Related Gene Family

ein4 and ers2 mutations affect ethylene responses in many tissues and at several developmental stages, suggesting that EIN4 and ERS2 would be broadly expressed both spatially and temporally. Reverse transcription followed by polymerase chain reaction (PCR) and/or RNA gel blot assays showed that both genes are indeed expressed in many organs. RNA signals of EIN4 and ERS2 genes were detected in etiolated seedlings, leaves, stems, roots, and inflorescences (data not shown).

To further analyze the expression of each member of this gene family at the cellular level, we performed RNA in situ hybridization with a number of tissues. The RNA levels of the five genes were generally low and ubiquitous, and they had basically similar expression patterns in most of the tissues (Figures 3 and 4).

ERS1 was ubiquitously expressed. In general, the signals appeared higher in younger and smaller cells than they did in older and more expanded cells. It remains to be determined
whether the number of RNA molecules expressed per cell is
the same in both types of cells. ERS1 was expressed in em-
byos (Figures 3A and 3B), leaves, and etiolated seedlings,
including cotyledons (Figures 3C and 3D), hypocotyls (Fig-
ures 3E and 3F), and roots (Figures 3G and 3H). ERS1 ex-
pressions was observed in stems, especially in procambium
cells (Figures 3I and 3J). Young floral primordia and floral or-
gan primordia expressed ERS strongly (Figures 3K and 3L).
Very strong expression was observed in the locules of the
anthers (Figures 3M to 3P). ERS1 also was expressed in the

Figure 3. RNA Localization of the ERS1 Gene.

(A), (C), (E), (G), (I), (K), (M), (O), and (Q) represent bright-field microscopy. (B), (D), (F), (H), (J), (L), (N), (P), and (R) are double exposures of bright- and dark-field microscopy. Radioactive signals are indicated by bright grains in the double-exposure photographs.

(A) and (B) Embryo in an imbibed seed.
(C) and (D) Longitudinal section of the cotyledons and the shoot apical meristem of an ethylene-treated etiolated seedling.
(E) and (F) Longitudinal section of the hypocotyl of an ethylene-treated etiolated seedling.
(G) and (H) Longitudinal section of the root of an ethylene-treated etiolated seedling.
(I) and (J) Longitudinal section of the stem of an inflorescence.
(K) and (L) Longitudinal section of an inflorescence with flowers younger than stage 7 (Smyth et al., 1990).
(M) and (N) Longitudinal section of stage 6 and 9 flowers.
(O) and (P) Transverse section of a stage 10 flower with developing microspores.
(Q) and (R) Longitudinal section of a stage 10 flower with developing ovules.
developing septum of the carpels (Figures 3Q and 3R) and was later expressed in carpels, including the ovules.

ETR1 had an expression pattern similar to ERS1 in etiolated seedlings and leaves but appeared to be expressed at a lower level. Expression of ETR1 in stems and flowers was of similar pattern and comparable level to that of ERS1. Like ERS1, ETR1 was strongly expressed in the locules of the anthers (Figures 4A and 4B) and the developing carpels (Figures 4C and 4D). It is also expressed in the ovules and funiculi in late carpel development (Figures 4E and 4F).

Figure 4. RNA Localization of ETR1, ETR2, EIN4, and ERS2 in Floral Tissues.

(A), (C), (E), (G), (I), (K), (M), (O), and (Q) represent bright-field microscopy. (B), (D), (F), (H), (J), (L), (N), (P), and (R) are double exposures of bright- and dark-field microscopy. Radioactive signals are indicated by bright grains in the double-exposure photographs.

(A) and (B) ETR1 expression in a stage 10 flower with developing microspores (a medial transverse section).

(C) and (D) ETR1 expression in a stage 10 flower with developing carpels (a distal transverse section).

(E) and (F) ETR1 expression in carpels of stage 12 to 13 flowers (a transverse section).

(G) and (H) ETR2 expression in stage 8 and 10 flowers (a longitudinal section).

(I) and (J) ETR2 expression in carpels of stage 12 to 13 flowers (a transverse section).

(K) and (L) EIN4 expression in a stage 10 to 11 flower (a transverse section).

(M) and (N) ERS2 expression in a stage 10 to 11 flower (a transverse section).

(O) and (P) ERS2 expression in carpels of stage 12 to 13 flowers (a transverse section).

(Q) and (R) ERS2 expression in a stage 10 to 11 flower (a longitudinal section).
The ETR2 expression pattern was similar to that of ETR1 and ERS1 in the embryos, etiolated seedlings, leaves, stems, and young floral primordia, but the flower expression pattern was slightly different. Strong expression was not observed in the stamens (Figures 4G and 4H). In contrast to ETR1 (Figures 4A and 4B) and ERS1 (Figures 3M and 3N), ETR2 was strongly expressed in the developing carpels, especially in the funiculi and ovules (Figures 4I and 4J; Sakai et al., 1998).

EIN4 was expressed in embryos, etiolated seedlings, leaves, roots, and inflorescences. Strong signals were detected in the locules of stamens, including the developing pollen cells and tapetum cells (Figures 4K and 4L). Developing carpels expressed EIN4 at moderate levels (data not shown).

ERS2 expression was detected in the etiolated seedlings, leaves, roots, and stems. Higher expression was observed in flowers. Stamens, especially the tapetum cells and developing pollen cells, as well as developing carpels, ovules, and funiculi expressed ERS2 strongly (Figures 4M to 4P). Later in flower development, ERS2 expression was concentrated in the epidermal layers of the septum (Figures 4Q and 4R), where strong expression was not observed with the other four genes.

Because these five genes are involved in ethylene signaling, we analyzed the regulation of their RNA expression by ethylene. It has been noted that ETR1 is not regulated by ethylene treatment (Chang et al., 1993), whereas putative ethylene receptors in tomato and Rumex palustris are upregulated by ethylene (Wilkinson et al., 1995; Vriezen et al., 1997). Total RNAs were isolated from leaves of 1-month-old plants before and after ethylene treatment at a concentration of 5 μL/L for 12 hr. Gene-specific probes for each member of this gene family were used to probe RNA gel blots made from the above samples. A gene probe for the β subunit of coupling factor 1 (atpB; Isono et al., 1997) was used for the normalization of RNA loading. The expression of ETR1 and EIN4 was not appreciably affected by ethylene treatment (Figure 5). In contrast, the RNA levels of the ERS1, ETR2, and ERS2 genes were elevated in leaves by the ethylene treatment (Figure 5).

**DISCUSSION**

Three members of the putative ethylene receptor gene family have been found in Arabidopsis; they are ETR1, ERS1, and ETR2 (Chang et al., 1993; Hua et al., 1995; Sakai et al., 1998). We have now isolated two additional members by their cross-hybridization with ETR2. Their encoded proteins, EIN4 and ERS2, exhibit sequence homology to the three putative ethylene receptors in both the N-terminal domain and the C-terminal region. EIN4 was identified previously as a dominant ethylene-insensitive mutant. Although mutations in the ERS2 gene were not isolated in the genetic screens, ers2 mutant transgenic plants had an ethylene-insensitive phenotype similar to those of etr1, etr2, ein4, and ers1. Sequence homology and their dominant mutant phenotypes indicate that EIN4 and ERS2 are involved in ethylene perception as well and that they are two new members of the putative ethylene receptor gene family in Arabidopsis.

The five genes appear to belong to two subfamilies (Figure 6). One subfamily consists of ETR1 and ERS1, whereas the other contains ETR2, EIN4, and ERS2. Members in the same subfamily have higher amino acid sequence similarity to each other than to members in the other subfamily. The five proteins share 79% to 86% similarity within each subfamily, respectively, and 57 to 65% similarity between subfamilies. Moreover, genes in the same subfamily have conserved intron positions that are not shared between the two subfamilies (Figure 6). ETR1 has five introns that correspond in length and position to introns in ERS1, with the exception of the sixth intron in the receiver domain, which ERS1 does not contain. ETR2, EIN4, and ERS2, on the other hand, exhibit one intron at a location where no introns are found in either ETR1 or ERS1. This division of subfamilies is not related to the presence or absence of the receiver domain, because the two members without the receiver domain (ERS1 and ERS2) belong to different subfamilies.

The most conserved domain of these proteins is the N-terminal domain, with similarities ranging from 64 to 82% among members. The three hydrophobic segments at the N terminus exhibit even higher sequence conservation (Figure 2A). These putative transmembrane segments may poten-
tially form the ethylene binding pocket, because the first 165 amino acids of ETR1, which is made up mostly of these segments, are capable of ethylene binding in yeast (Schaller and Bleecker, 1995). This possible ethylene binding activity of each member also is suggested by the similarity of their dominant mutations. For all five proteins, the amino acids, which when mutated confer ethylene insensitivity, reside in these segments. Members in the second subfamily have an extra 20 to 30 amino acids at the N terminus when compared with members of the first subfamily (Figure 2A). This stretch of amino acids is hydrophobic and could potentially form a fourth transmembrane segment. The amino acid sequences in this segment are not well conserved, and the functional significance of this segment is not clear.

The putative histidine kinase domains of this gene family differ greatly in their sequence similarities to the bacterial histidine kinases (Figure 2B). ETR1 and ERS1 have substantial homology to the bacterial proteins in this region. All of the five signature motifs, H, N, G1, F, and G2 (Parkinson and Kofoid, 1992), found in the bacterial histidine kinases are well conserved in ETR1 and ERS1, and the putative autophosphorylation sites (H-353) are present at the predicted positions. The putative histidine kinase domains in the second subfamily are very diverged from the canonical bacterial histidine kinase sequences. ETR2 and ERS2 do not have histidine residues at the predicted sites, although the H motifs are recognizable. A few examples exist in bacteria, where the autophosphorylated histidine is present not in the H motif but adjacent to it (Parkinson and Kofoid, 1992). Aligning the five protein sequences revealed a histidine residue (H-360 in ETR1) that was seven amino acids downstream of the histidine position found in the majority of the bacterial proteins. However, this histidine is not conserved in all five proteins, because an arginine occupies this position in ERS2. Motifs N, G1, and G2 are essential for autokinase activity, as suggested by mutational analysis of some bacterial histidine kinases (Parkinson and Kofoid, 1992). Limited sequence similarity to the N motif is present in the second subfamily, but the two N amino acids are absent. The G1 and G2 motifs may comprise nucleotide binding sites. No sequences match the G1 motif well in the second subfamily. The G2 motif is present in ETR2 but barely recognizable in EIN4 and ERS2. The F motif is not recognizable in any member of the second subfamily.

Despite the similarity and divergence in sequences, all of these five genes when mutated confered similar dominant mutant phenotypes. Mutant plants are insensitive to ethylene in a variety of responses, including triple response, leaf growth, and leaf senescence. There does not seem to be any tissue specificity associated with mutants of different genes, except for the variation in strengths. Our in situ hybridization data are consistent with the pleiotropic effects of these mutations: the genes are expressed ubiquitously, and they have largely overlapping expression domains.

It is desirable to have loss-of-function mutations to analyze the functions of EIN4 and ERS2. Thus far, only dominant mutations of the two genes are available, like the other three members of the gene family. Depending on the mechanisms of the dominance of these mutations, several scenarios regarding their functions can be envisioned. It remains possible that EIN4 and ERS2 may not encode ethylene receptors. The dominant ein4 and ers2 mutations may be neomorphic, that is, the mutant EIN4 and ERS2 proteins may exhibit a function normally not associated with their wild-type activities. The mutant proteins could interfere with the function of the ethylene receptor, such as ETR1, because they exhibit sequence homology to the real receptor.

Alternately, EIN4 and ERS2 may encode ethylene receptors, and the five genes in the family may perform similar functions. This functional redundancy could explain the failure to isolate recessive alleles of any of the genes in the genetic screens. In this scenario, the mutations induce constitutive activity of the proteins or cause dominant interference of the wild-type activities of other receptors. Total redundancy raises the question of how all of these genes could be kept from becoming pseudogenes during evolution. It appears more likely that these genes are somewhat different, while sharing common functions in ethylene sensing. Differences in expression are indeed observed among family members. These genes are differentially regulated by ethylene: the RNA levels of the ERS1, ETR2, and ERS2 genes are upregulated by ethylene, whereas the expression of ETR1 and EIN4 is not affected by ethylene treatment. In addition, the spatial expression patterns of these genes are overlapping but not identical. For instance, ERS2 has a unique expression domain in the epidermal layer of the septum, and ETR2 expression is below detection in the stamen locules, where the other four members show strong expression.

Ethylene response has been shown to be regulated at the level of ethylene synthesis. But this regulation does not
account for all of the regulation in ethylene responses. It has been observed in several plant species that ethylene sensitivity varies widely between tissues, between classes of response within a tissue, and even within a single class of response under different physiological conditions (Goeschl and Kays, 1975). We have shown here the existence of a family of putative ethylene receptors. They are homologous in sequence but may possibly possess different ethylene binding affinities and signaling activities. They are differentially regulated by ethylene and perhaps also by other environmental or developmental factors. This differential regulation of expression of the receptor family may provide a mechanism to achieve differential sensitivities even in the same response under different conditions.

It is conceivable that these genes may not act independently if they all encode ethylene receptors. Heterodimer or heteromultimer forms of these receptors could be used for ethylene sensing. They could even act in several linear pathways, with some members acting on the others. Multi-step phosphorelay systems consisting of H-to-D-to-H-to-D have been described in two-component systems, such as Kin A-Spo0 and BvgS-BvgA in bacteria and Slr1p-Ypd1p-Ssk1p in yeast (Appleby et al., 1996). The presence of a receiver domain in some members of the ethylene receptor family provides a structural potential for such an extended relay.

More in-depth studies on ethylene signal transduction are now made possible by the identification of this putative ethylene receptor gene family and by the availability of mutants with altered response due to changes in each member. Isolation and characterization of loss-of-function alleles of these genes should reveal the functions of the wild-type proteins and further our understanding of the process of ethylene perception.

METHODS

Plant Growth and Genetic Studies

All Arabidopsis thaliana strains used are of ecotype Columbia unless otherwise mentioned. Etiolated seedlings were grown as follows: seeds were sterilized and sown on Petri plates with medium containing 1% agar (Difco, Detroit, MI) and Murashige and Skoog salts (at pH 5.7; Sigma). For selection of transgenic plants, kanamycin was added to the medium to 50\( \mu \)g/mL. Seeds were cold-treated for 3 days, exposed to light for 6 hr, and incubated in the dark at 22 to 24°C for 3 days. For ethylene treatment, plants were placed in a flowthrough chamber with an ethylene concentration of ~1 to 10 \( \mu L/L \). An 8:10:10 mixture of perlite (Therm-o-Rock Industries, Chandler, AZ)vermiculite (Therm-o-Rock Industries)soil (Rod McClellan Co. Supersoil, South San Francisco, CA) was used for plants grown in pots. Plants were grown at 22 to 24°C under 600-ft candles of constant cool-white fluorescent light.

ein4-3 was crossed to ecotype Landsberg erecta for mapping. \( F_3 \) families homozygous for the ethylene-insensitive phenotype were used for simple sequence length polymorphism mapping (Bell and Ecker, 1994). To construct ers2 and ctrl double mutants, we crossed ers2-1 and ers2-2 separately onto ctrl-1. Ethylene-insensitive \( F_1 \) progeny, which were also kanamycin resistant, were selfed. Etiolated \( F_2 \) seedlings were scored in air on medium containing Murashige and Skoog salts (Sigma) and 50 \( \mu g/mL \) kanamycin. Approximately 15% of the \( F_2 \) plants exhibited the ctrl phenotype, and ~75% of these ctrl-like plants were kanamycin resistant, indicating that they contained the ers2 mutant gene.

Library Screening and DNA Analysis

A genomic DNA library (Sakai et al., 1998) representing approximately five genomes was screened with ETR2, ETR1, and ERS1 probes. Reduced stringency wash was performed at 60°C and 2 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4). A high-stringency wash was performed at 65°C in 1 × SSPE. Approximately 10\(^5\) plaques of an Arabidopsis flower cDNA library (Weigel et al., 1992) were screened with the coding regions of EIN4 and ERS2. Six positive cDNA clones were isolated for EIN4, and seven positive cDNA clones were isolated for ERS2. Nucleotide sequences were determined using Sequenase version 2.0 (United States Biochemical Co.) or the ABI PRISM dye terminator cycle sequencing Ready Reaction (Perkin-Elmer, Foster City, CA) and synthetic oligonucleotides.

To identify mutations in the ein4 alleles, we amplified the EIN4 coding regions from wild-type and mutant plant tissues by using polymerase chain reaction (PCR). PCR products were excised from low-melting-point gels and used directly for sequencing. The alterations in sequence were verified by independent PCR amplifications.

Restriction fragment length polymorphism (RFLP) mapping was done with the MAPMAKER program (Chang et al., 1988). CITd39 and pCITY31 are two random \( \lambda \) clones isolated in the Meyerowitz laboratory.

In Vitro Mutagenesis and Plant Transformation

A 2.2-kb BamHI genomic fragment of ERS2 containing 1.5 kb of 5' sequence and 0.7 kb of coding sequence was cloned into the pBlue-script vector SK+ (Stratagene, La Jolla, CA). Mutagenic primers were used individually to introduce CCA-\( \rightarrow \)CTA and ATT→TTT mutations by using the MORPH kit (5 prime Inc., Boulder, CO). A 6-kb EcoRI fragment of the ERS2 genomic DNA was cloned in pCR2.1 (Invitrogen, Carlsbad, CA). The wild-type 2.2-kb BamHI fragment in this 6-kb fragment was replaced by the two mutated 2.2-kb BamHI fragments individually to generate two 6-kb mutant ERS2 genes. The wild-type 6-kb fragment and its two mutant forms were excised by KpnI and XbaI digestions and ligated into vector pCGN1547 (McBride and Summerfelt, 1990) with the 3' nopaline synthase (NOS) end cloned between XbaI and PstI sites. The constructs in pCGN1547-NOS were transformed into AEs Agrobacterium tumefaciens strains (Fraley et al., 1985). The resulting strains were used to transform wild-type Arabidopsis by in-the-plant vacuum infiltration (Bechtold et al., 1993).

RNA in Situ Hybridization and RNA Isolation

Samples were fixed, embedded, sectioned, and hybridized as described previously (Drews et al., 1991; Sakai et al., 1995). DNA fragments coding for the C-terminal domains (putative histidine kinase
and receiver domains of ETR1, ETR2, and EIN4, and histidine kinase domains of ERS1 and ERS2) of the five genes were amplified from their respective cDNA clones and cloned into either pBluescript SK+ (Stratagene) or pCR2.1 (Invitrogen) vectors. Sense and antisense probes were synthesized with T7 or T3 RNA polymerases. Total RNAs were isolated using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer’s instruction.

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