The Plant Cell, Vol. 18, 3429–3442, December 2006, www.plantcell.org

Identification of Important Regions for Ethylene Binding and Signaling in the Transmembrane Domain of the ETR1 Ethylene Receptor of *Arabidopsis* [W] [OA]

Wuyi Wang,[a,1] Jeff J. Esch,[a,2] Shin-Han Shiu,[b] Hasi Agula,[a,3] Brad M. Binder,[a,c] Caren Chang,[d] Sara E. Patterson,[c,4] and Anthony B. Bleecker[a,2]

[a] Department of Botany, University of Wisconsin, Madison, Wisconsin 53706
[b] Department of Plant Biology, Michigan State University, East Lansing, Michigan 48824
[c] Department of Horticulture, University of Wisconsin, Madison, Wisconsin 53706
[d] Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland 20742

The ethylene binding domain (EBD) of the *Arabidopsis thaliana* ETR1 receptor is modeled as three membrane-spanning helices. We surveyed ethylene binding activity in different kingdoms and performed a bioinformatic analysis of the EBD. Ethylene binding is confined to land plants, *Chara*, and a group of cyanobacteria but is largely absent in other organisms, consistent with our finding that EBD-like sequences are overrepresented among plant and cyanobacterial species. We made amino acid substitutions in 37 partially or completely conserved residues of the EBD and assayed their effects on ethylene binding and signaling. Mutations primarily in residues in Helices I and II midregions eliminated ethylene binding and conferred constitutive signaling, consistent with the inverse-agonist model of ethylene receptor signaling and indicating that these residues define the ethylene binding pocket. The largest class of mutations, clustered near the cytoplasmic ends of Helices I and III, gave normal ethylene binding activity yet still conferred constitutive signaling. Therefore, these residues may play a role in turning off the signal transmitter domain of the receptor. By contrast, only two mutations were loss of function with respect to signaling. These findings yield insight into the structure and function of the EBD and suggest a conserved role of the EBD as a negative regulator of the signal transmitter domain.

INTRODUCTION

Ethylene, a gaseous phytohormone, regulates diverse developmental and physiological processes throughout the entire life cycle of plants, including seed germination, root initiation, flower and leaf senescence, abscission, fruit ripening, wounding response, and disease defense (Abeles et al., 1992). Over the last decade, a number of key components in the ethylene signaling pathway have been identified through the study of genetic mutants in the model plant *Arabidopsis thaliana*. The molecular and genetic analyses of these components have established a largely linear pathway at the early steps in transducing the ethylene signal in plants (Bleecker and Kende, 2000; Guo and Ecker, 2004).

In *Arabidopsis*, ethylene is perceived by a family of five membrane-bound receptors (ETR1, ERS1, ETR2, EIN4, and ERS2), which transmit the signal to downstream effectors. The receptors form homodimers and are believed to be located on the endoplasmic reticulum membrane (Chen et al., 2002). The five ethylene receptors share significant similarity in their amino acid sequences and structures. In all the receptors, the predicted transmembrane domain at the N terminus comprises the ethylene binding domain (EBD) (Rodriguez et al., 1999; O'Malley et al., 2005). The C-terminal half of the receptors consists of a His kinase domain and in some cases, a receiver domain (ETR1, ETR2, and EIN4), showing varying degrees of sequence similarity to the His kinase receptors and response regulators of the two-component system in prokaryotes (Chang et al., 1993). The EBD is connected to the His kinase domain by a linker region that shares sequence similarity to the GAF domain (Aravind and Ponting, 1997). Nevertheless, there are also features specific to different receptors. Residues that are required for His kinase activity are highly conserved in ETR1 and ERS1 but are not completely conserved in ETR2, EIN4, and ERS2 (Hua et al., 1998). The latter three members of the family are also distinguished by the presence of an additional hydrophobic region at the N terminus, which possibly constitutes a membrane-targeting signal peptide or a fourth membrane-spanning region. Based on these distinguishing features and the overall sequence similarity, the five receptors can be classified into two subfamilies: subfamily I (ETR1 and ERS1) and subfamily II (ETR2, EIN4, and ERS2).

[1] Current address: Ceres, 1535 Rancho Conejo Blvd., Thousand Oaks, CA 91320.
[2] Deceased.
[3] Current address: College of Life Sciences, Inner Mongolia University, Huhhot 010021, People's Republic of China.
[4] To whom correspondence should be addressed. E-mail spatters@wisc.edu; fax 608-262-4743.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Sara E. Patterson (spatters@wisc.edu).
[W] Online version contains Web-only data.
[OA] Open Access articles can be viewed online without a subscription.
www.plantcell.org/cgi/doi/10.1105/tpc.106.044537

The members of the receptor family coordinately and negatively regulate the ethylene response in plants (Hua and Meyerowitz, 1998); however, the mechanism by which this occurs is unknown. Recent studies have suggested that the two receptor subfamilies play different roles in mediating ethylene signal transduction (Hall and Bleecker, 2003; Wang et al., 2003). In vitro studies indicate that subfamily I receptors are capable of His kinase activity, while subfamily II members and ERS1 have Ser/Thr kinase activity (Gamble et al., 1998; Moussatche and Klee, 2004). However, no clear relationship has been established between kinase activity and ethylene response. Recent work directly or indirectly suggests that the canonical His kinase activity of ETR1 is not essential for primary receptor signaling (Gamble et al., 2002; Wang et al., 2003). However, we cannot rule out the possibility that kinase activity affects the signaling behavior in more subtle ways (Binder et al., 2004; Qu and Schaller, 2004). Despite these unresolved questions, it is likely that ethylene binding results in an alteration in the signaling state of the cytoplasmic transmitter domains of the receptors.

Genetic data support an inverse-agonist model for ethylene receptor signaling, in which the binding of ethylene by the EBD turns off the transmitter, whereas in the absence of ethylene binding, the transmitter actively represses ethylene response (Hua and Meyerowitz, 1998; Bleecker, 1999). This model is based on the fact that loss of the receptors results in constitutive ethylene response, and dominant mutations known to block ethylene binding result in ethylene insensitivity (Hua and Meyerowitz, 1998; Bleecker, 1999). The receptors act in concert with a homolog of eukaryotic Raf-like mitogen-activated Ser/Thr protein kinases, CTR1, which represses the ethylene response pathway in the absence of ethylene (Kieber et al., 1993; Clark et al., 1998; Gao et al., 2003). Although several key components downstream of the ethylene signaling pathway have been identified, it is not yet known how CTR1 regulates these elements to repress the ethylene response (Alonso and Stepanova, 2004; Chang and Bleecker, 2005).

Insights into the mechanism of ethylene binding have been provided by studies on wild-type and mutated forms of EBDs in yeast. When expressed in yeast, the ETR1 receptor binds ethylene with similar dose–response characteristics to ethylene responses in plants (Schaller and Bleecker, 1995). The EBD of ETR1 lies within the N-terminal 128 amino acids and is composed of three hydrophobic segments that have been modeled as membrane-spanning helices (Rodriguez et al., 1999). The five ethylene receptors in *Arabidopsis*, the five in tomato (*Solanum lycopersicum*), and the product of *Synechocystis* slr1212 have all been demonstrated to bind ethylene with similar high affinity (Schaller and Bleecker, 1995; Rodriguez et al., 1999; Hall et al., 2000; O'Malley et al., 2005). Analyses of mutations in the transmembrane domain of ETR1 have revealed that certain residues are crucial for ethylene binding (Schaller and Bleecker, 1995; Hall et al., 1999; Rodriguez et al., 1999) and that the binding requires copper as a cofactor (Rodriguez et al., 1999). Little is known, however, regarding the structure-function relationship of the EBD and receptor signaling.

The most divergent EBD-like domain that has been characterized is the slr1212 gene product of the cyanobacterium *Synechocystis* (Rodriguez et al., 1999). The presence of EBDs attached to His kinase domains in cyanobacteria has led to the hypothesis that the ethylene receptor in plants is derived from the chloroplast, a cyanobacterial symbiont (Bleecker, 1999; Mount and Chang, 2002). To further characterize the EBD, we surveyed for ethylene binding activity in representatives of all domains of life and examined all currently available sequences for the presence of EBDs. In addition, to investigate the structure-function relationship of the EBD and transmitter domain, we performed Ala scanning mutagenesis on highly conserved amino acid residues in the EBD, using ETR1 as the prototype. The effects of these mutations on ethylene binding were evaluated in yeast-expressed ETR1, while their effects on ethylene signaling were examined in transgenic *Arabidopsis* plants using the seedling growth response assay. By correlating ethylene binding activity with signaling output in planta, we identified regions of the EBD that are crucial for ethylene binding and/or its concomitant effects on ethylene signaling.

RESULTS

Survey of Ethylene Binding Activity in Organisms of All Kingdoms

The identification of the *Synechocystis* slr1212 protein, which binds ethylene, provided evidence that the EBD exists in nonplant species (Rodriguez et al., 1999) and raised questions concerning the origins of the EBD and how plants acquired it. To examine the phylogenetic distribution of the EBD, we analyzed displaceable ethylene binding activities in a variety of organisms representative of all kingdoms. The results are summarized in Table 1, and the phylogenetic relationships between organisms sampled are shown in Supplemental Figure 1 online.

Ethylene binding activity was detected in all land plants tested, including ferns, fern allies, and bryophytes. Ethylene binding activities among land plants are similar, ranging from three to five times over background (nonspecific) binding. Interestingly, *Chara*, a green alga that is the closest to the plant lineage, also binds ethylene (Karol et al., 2001). By contrast, no ethylene binding was detected in two other green algae belonging to other evolutionary lineages, *Chlamydomonas* and *Acetabularia* (Misler et al., 1994). Several cyanobacteria in addition to *Synechocystis* were assayed to determine their ethylene binding activities. They were chosen to ensure representation by species of various cyanobacterial subgroups and ecological niches (Wilmotte, 1994). Interestingly, the cyanobacteria that exhibited ethylene binding activity (*Anabaena*, *Fischerella*, *Lungbya*, *Nodularia*, *Nostoc*, *Oscillatoria*, and *Synechocystis*) all belong to a single clade, except *Spriulina*, and its relationship to the others is unclear (see Supplemental Figure 1 online). Among these cyanobacteria, their ethylene binding activities range from threefold above background in *Nodularia* to 80-fold in *Oscillatoria*. Only one member (*Pseudoanabaena*) tested in this clade did not bind ethylene. In addition, *Synechococcus* that is paraphyletic of this clade had no detectable ethylene binding activity. To determine if ethylene binding is a characteristic of just a subset of cyanobacteria or if other bacteria share this trait, several eubacteria, including both gram positive and negative species and one species of archeabacteria (*Halobacterium salinarium*), were

Table 1. Ethylene Binding Activity of Members within Various Kingdoms

Species	Ethylene Binding[a]	Species	Ethylene Binding[a]
Archeabacteria		Fungi	
Halobacterium salinarium	–	*Aspergillus flavus*	–
Eubacteria		*Neurospora crassa*	+
Agrobacterium tumefaciens	–	*Penicillum chrysogenum*	–
Bacillus luteus	–	*Rhizopus stolonifer*	+
Deinococcus radiodurans	–	*Saccharomyces cerevisiae*	–
Escherichia coli	–	*Schizophyllum commune*	–
Flavobacterium spp	–	Metazoan	
Streptomyces coelicolor	–	*Caenorhabditis elegans*	–
Protists		*Drosophila melanogaster*	–
Dictyostelium discoideum	–	Green algae	
Pythium torulosom	–	*Acetabularia acetabulum*	–
Rhodomonas sp	–	*Chara* spp	++
Tetrahymena thermophila	–	*Chlamydomonas reinhardtii*	–
Cyanobacteria		Plants	
Anabaena PCC 7122	+++	*Amblystegium* spp	++
Chamaesiphon PCC 7430	–	*Arabidopsis thaliana*	++
Fischerella PCC 7414	+++	*Elodea canadensis*	++
Lungbya PCC 7419	++	*Gingko biloba*	++
Nodularia PCC 73104	++	*Juniperus chinensis*	++
Nostoc PCC 7120	+++	*Lycopodium lucidulum*	++
Oscillatoria PCC 7105	+++	*Marchantia polymorpha*	++
Plectonema PCC 73110	–	*Marsilea drummondii*	++
Pseudanabaena PCC 6903	–	*Nephrolepis exaltata*	++
Spirulina PCC 6313	+++	*Nicotiana tobacum*	++
Synechococcus PCC 6301	–	*Physcomitrella patens*	++
Synechococcus PCC 6908	–	*Polytrichum* spp	++
Synechococcus PCC 7942	–	*Psilotum nudum*	++
Synechocystis PCC 6803	++	*Sphagnum* spp	++
		Vallisneria spp	++

[a] Binding level: +++, 10-fold or more binding over background; ++, 3- to 10-fold binding over background; +, 1.5- to 3-fold binding over background; –, no measurable binding.

examined. No ethylene binding activity above background levels was detected in any of these species (Table 1).

None of the tested metazoans or protists bound ethylene. Among the six fungi tested, only *Neurospora crassa* and *Rhizopus stolonifer* bound ethylene over background. However, these two species had the lowest binding activities among the species analyzed in this survey (1.8-fold and 1.9-fold above background, respectively), and the background ethylene binding levels for these fungi were 10 times higher than most other samples tested. Thus, it is uncertain whether the binding in the fungi is authentic.

Identification of EBD-Containing Genes from GenBank and Unfinished Genomes

To complement the targeted binding study above, we analyzed available sequence data for the presence of genes containing EBD-like sequences in untested organisms. The protein sequences of EBDs from *Arabidopsis* ethylene receptors and *Synechocystis* slr1212 were used to search against the annotated protein sequences in GenBank release 148. A total of 113 sequences had E values <1 and were regarded as candidate EBD-containing genes. The overall similarities of candidate EBD-containing proteins were determined using transformed E values as distance measures (see Supplemental Figure 2 online). The sequences reside in two clusters that are taxa specific. The first cluster consists of only sequences from various flowering plants and apparently forms two distinct subclusters: subfamilies I and II. The second cluster consists of only eubacterial sequences that have higher overall similarity to *Synechocystis* slr1212 than to plant EBD-containing proteins (see Supplemental Figure 2 online). Several candidate EBD-containing proteins fall outside of the two major clusters (referred to as outliers), including several metazoan and fungal sequences. To evaluate their likelihood to have EBDs, we constructed a hidden Markov model (HMM) using the alignments of representative EBDs (Figure 1A) and compared the model against all EBD candidates. All plant sequences have E values $<1 \times 10^{-17}$, as well as all bacterial sequences in the slr1212 clade except *Nostoc* all5173. On the other hand, all outlier sequences have E values >0.1. In addition, there is no obvious sequence conservation of these outliers with established EBDs.

To identify EBDs in genes that are not annotated or in unfinished genomes, we used the representative EBDs to conduct a translated BLAST search against the finished and unfinished genome sequences of metazoa, fungi, bacteria, and the green algae *Chlamydomonas*. Matches with BLAST E values <1 were regarded as candidate EBD-containing genes and were

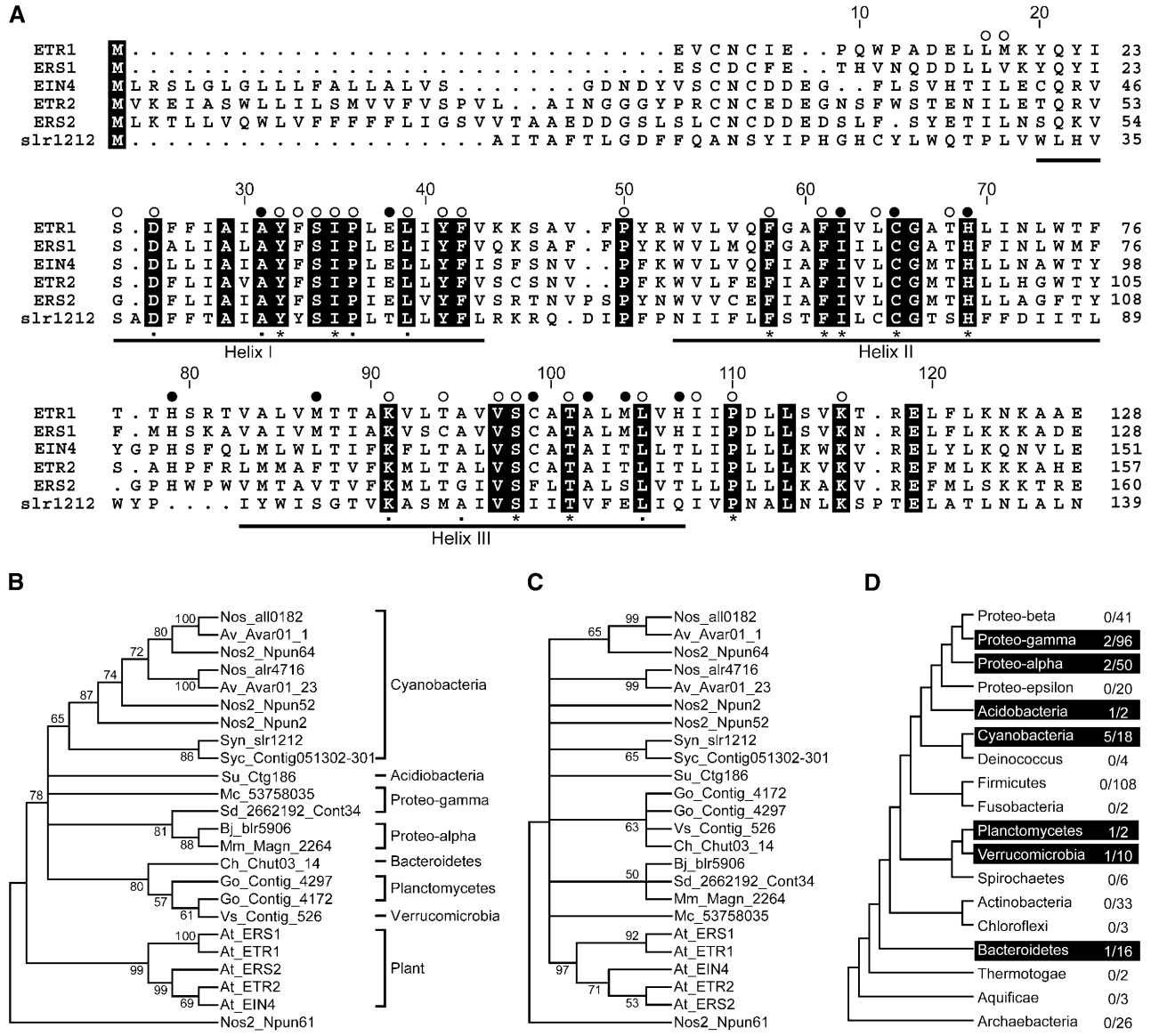

Figure 1. Alignments and Relationships between EBDs.

(A) Protein sequence comparison of the transmembrane region of the *Arabidopsis* ethylene receptors and *Synechocystis* slr1212. The three predicated hydrophobic segments are underlined. The amino acid residues of each protein are numbered at the right. The numbers on top of each alignment block indicate the residue numbers of ETR1. The closed circles above the alignments indicate that the residues were analyzed for their roles in the ethylene binding of ETR1 in the previous studies (Schaller and Bleecker, 1995; Hall et al., 1999; Rodriguez et al., 1999). The residues in which mutations were made in this study are marked by the open circles. The completely conserved residues among the six known EBDs are shaded. The asterisks below the alignments indicate amino acid residues completely conserved among the six known EBDs and the putative EBDs from bacteria (see Supplemental Figure 4 online); the periods indicate highly conserved residues (>90% identical among the six known EBDs and the putative EBDs from bacteria).

(B) and **(C)** Phylogenetic relationships between selected EBD-containing proteins determined with the neighbor-joining method and maximum parsimony, respectively. In **(B)**, the groups that the EBD-containing bacteria belong to are labeled at the right. The bootstrap values are shown at the bases of the nodes. Nodes with <50% bootstrap support are collapsed. The naming convention is provided in Supplemental Tables 1 to 3 online. The sequence alignment for generating the trees is provided in Supplemental Table 4 online.

(D) Phylogenetic relationships between major bacterial groups are listed in Table 2. The relationships are determined according to Brown et al. (2001), Brochier et al. (2002), Quaiser et al. (2003), and Wagnera and Horna (2006). The number of species containing EBD-like sequences versus the total number of species analyzed in the group is indicated at the right.

compared against the HMM of EBDs using E <1 × 10^{-10} as cutoff. With these criteria, no EBD was found in metazoan, fungal, and *Chlamydomonas* genome sequences currently available. On the other hand, we found 18 bacterial sequences containing EBD-like sequences from 13 species (Table 2; see Supplemental Figure 3 online), including EBDs in *Nostoc* and *Methylococcus* noted previously (Mount and Chang, 2002). Among the 18 candidate sequences, five were from finished genomes (see Supplemental Figure 2 online) and the remaining from nine unfinished genomes (see Supplemental Table 2 online). Among the nine species with EBD-like sequences in their unfinished genomes, three were from the cyanobacterial group (Table 2). Surprisingly, *Synechococcus* sp PCC 7002 contains an EBD-like sequence, although the non-PCC 7002 strains tested in our binding studies did not show ethylene binding activity. The other six species, together with the two that were identified from finished genomes, belong to six evolutionarily distant bacterial lineages (Table 2). It is noted that EBDs are significantly enriched in the cyanobacterial group (χ^2 tests of finished, unfinished, and all bacterial genomes against finished, unfinished, and all cyanobacterial genomes, $P < 0.001$, 0.01, and 0.001, respectively). It is unclear whether EBDs from plants are more similar to EBDs in cyanobacteria; phylogenetic trees of EBDs from *Arabidopsis* and eubacteria were generated using two methods rooted with a *Nostoc* sequence that is weakly similar to EBDs (Nos2_ Npun61). In the neighbor-joining method, bacterial and *Arabidopsis* EBDs belong to distinct clades with good bootstrap support (Figure 1B). The phylogeny is reconstructed using the maximum parsimony method (Figure 1C), and none of the bacterial EBDs have a closer relationship to plant EBDs. The phylogenetic relationship between the major bacterial groups as listed in Table 2 is shown in Figure 1D.

Identification of Amino Acid Residues Important for Ethylene Binding in ETR1

To functionally define the EBD, we performed site-directed mutagenesis on the EBD-coding region in a full-length *ETR1* cDNA clone and then assayed the altered forms for ethylene binding activity in yeast (Figure 2). It is interesting to note that the residues that are completely or highly conserved in known EBDs and putative EBDs from bacteria are almost exclusively located in the predicted transmembrane helices (Figure 1A; see Supplemental Figure 4 online). Twenty-five amino acid residues were

Table 2. Presence of EBD-Like Sequences in Different Bacterial Taxonomic Groups

	Sequencing Finished[b]		Sequencing in Progress[b]	
Group[a]	Total	w/EBD[c]	Total	w/EBD[c]
Archaebacteria				
Crenarchaeota	5	–	0	NA[d]
Euryarchaeota	16	–	4	–
Nanoarchaeota	1	–	0	NA
Eubacteria				
Acidobacteria	0	NA	2	*Solibacter usitatus* Ellin 6076
Actinobacteria	19	–	14	–
Aquificae	1	–	2	–
Bacteroidetes/Chlorobi	5	–	11	*Cytophaga hutchinsonii*
Chlamydiae/Verrucomicrobia	9	–	1	*Verrucomicrobium spinosum* DSM 4136
Chloroflexi	1	–	2	–
Cyanobacteria	9	*Nostoc* sp PCC 7120 *Synechocystis* sp PCC 6803	9	*Anabaena variabilis* ATCC 29413 *Nostoc punctiforme* PCC 73102 *Synechococcus* sp PCC 7002
Deinococcus-Thermus	3	–	1	–
Firmicutes	58	–	50	–
Fusobacteria	1	–	1	–
Planctomycetes	1	–	1	*Gemmata obscuriglobus* UQM 2246
Proteobacteria, α	26	*Bradyrhizobium japonicum* USDA 110	24	*Magnetospirillum magnetotacticum* MS-1
Proteobacteria, β	12	–	29	–
Proteobacteria, ε	10	–	10	–
Proteobacteria, γ	55	*Methylococcus capsulatus* strain Bath	41	*Saccharophagus degradans* 2-40
Spirochaetes	6	–	0	NA
Thermotogae	1	–	1	–
Other bacteria	0	NA	5	–
Total	212	4	203	9

[a] Group assignments are based on the classification in the National Center for Biotechnology Information (NCBI) taxonomy database (http://www.ncbi.nlm.nih.gov/taxonomy/taxonomyhome.html/).

[b] The statuses of sequencing among the bacterial genomes analyzed were obtained from the NCBI Genome Biology section (http://www.ncbi.nlm.nih.gov/genomes/).

[c] The presence of EBD is determined by an E value of 1 × 10^{-10} or lower when compared against the HMM models of the known EBDs.

[d] NA, not applicable.

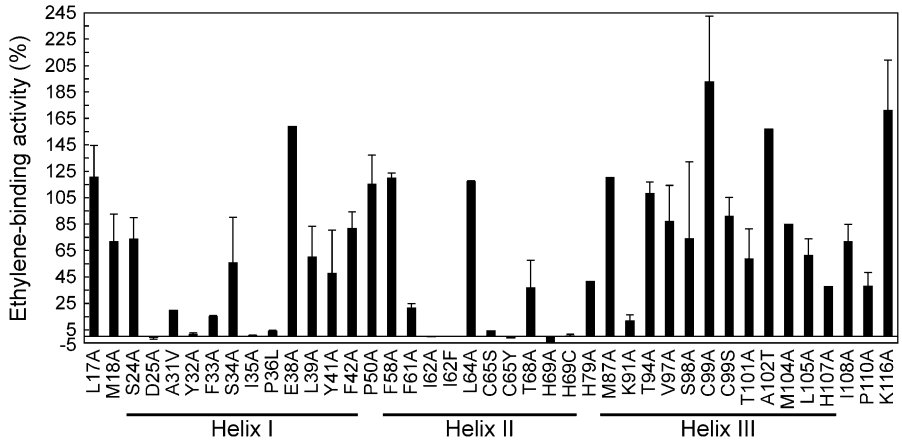

Figure 2. Ethylene Binding Activity of the ETR1 Mutants in Yeast.

The ethylene binding activities were measured using whole yeast cells expressing ETR1 wild type and mutants. Binding activity is presented as the percentage of wild-type ETR1 binding activity. The ETR1 mutants are designated by amino acid substitution. Point mutants are denoted by the native amino acid and its position, followed by the mutant amino acid. The binding data for 11 amino acid substitutions (A31V, E38A, I62A, I62F, C65S, H69A, H79A, M87A, A102T, M104A, and H107A) were obtained from previous studies (Hall et al., 1999; Rodriguez et al., 1999). Residues within the three hydrophobic segments (Helices I to III) are shown. Data represent the average with SD from at least three samples.

replaced with Ala, and one residue (P36) was changed to Leu to mimic the mutation in both *Arabidopsis etr2-1* and tomato *Nr*. Most of these residues are highly conserved in known and/or putative EBDs (Figure 1A; see Supplemental Figure 4 and Supplemental Table 3 online). Like the wild-type ETR1 protein (Schaller et al., 1995), all of the ETR1 mutants formed homodimers in yeast and could be converted into monomers by treatment with the reducing agent DTT (data not shown), indicating that the mutations did not dramatically disturb the native structure of the receptor and that the ETR1 mutants were still membrane-bound proteins. Figure 2 includes the ethylene binding activities of 11 previously reported mutants (Rodriguez et al., 1999; Hall et al., 2000). A second mutation was made on each of the four residues (I62, C65, H69, and C99), giving a total of 41 mutants for which ethylene binding activity was measured (Figure 2).

The majority of ETR1 mutants (27 out of the 41 mutants) retained more than one-third the ethylene binding capacity of the wild-type ETR1, while 10 mutations in seven amino acid residues had dramatic effects on ethylene binding and abolished ethylene binding capacity (<5% of the ethylene binding activity of the wild-type ETR1). Among the seven residues with dramatic effects in the mutational analysis, three in Helix II (I62, C65, and H69) had been identified in previous studies (Schaller et al., 1995; Hall et al., 1999; Rodriguez et al., 1999). The four identified in this study, D25, Y32, I35, and P36, are all located in Helix I. These residues are either completely (Y32, I35, I62, C65, and H69) or highly (D25 and P36) conserved among all known and putative EBDs (Figure 1A; see Supplemental Figure 4 and Supplemental Table 3 online). Interestingly, these two groups of residues distribute periodically on the α-helix and occur along a single helical face of each predicted helix (Figure 5B), indicating that these two conserved surfaces are essential for ethylene binding. Their importance in binding ethylene was also manifested by the fact that mutations in amino acid residues (such as A31, F33, and F61) situated on adjacent helical faces (Figure 5B) severely reduced ethylene binding (10 to 30% of the ethylene binding activity of the wild-type ETR1). The only other residue that significantly influenced ethylene binding when mutated was K91 in Helix III (Figure 2).

Mutations in a number of amino acid residues outside of the three hydrophobic helices were examined, including L17 and M18 just before Helix I, P50 in the loop between Helices I and II, H79 in the loop between Helices II and III, and I108, P110, and K116 presumably in the cytoplasmic region adjacent to Helix III. Most are highly conserved between the ethylene receptors (Figure 1A; see Supplemental Figure 4 and Supplemental Table 3 online). Although the mutations on H79 and P110 reduced the ethylene binding activity to ~40% compared with the wild type, none of these substitutions eliminated ethylene binding, indicating that the residues outside the hydrophobic helices are not directly involved in ethylene binding.

Effect of ETR1 Transmembrane Domain Mutations on Signaling in Planta

Previous studies on the transmembrane domain of ETR1 have focused primarily on the identification of residues involved in ethylene binding, yet little is known about the effect of such mutations on receptor signaling in plants (Hall et al., 1999). To evaluate their effects on signaling in planta, we transformed the 37 mutant *ETR1* constructs (from the above ethylene binding analysis) and wild-type *ETR1* (as a control) into the triple receptor null mutant line *etr1-6 etr2-3 ein4-4*. The transgenes were full-length genomic DNA clones containing the native *ETR1* promoter. Because the triple null mutant *etr1 etr2 ein4* has a partial constitutive ethylene-response phenotype in both light-grown plants and dark-grown seedlings (Hua and Meyerowitz, 1998), transformation with in vitro–mutagenized forms of ETR1 allowed

us to determine both the ability of the mutant receptor to suppress the constitutive ethylene response phenotype in the absence of ethylene treatment and the capacity to respond when treated with ethylene. Another advantage of this system is that recessive loss-of-function mutations, which would likely be undetected in the wild-type background, could be detected in the triple mutant.

The results of the etiolated seedling growth response of a representative homozygous line for each transgene are shown in Figure 3. As expected, the hypocotyl length of the transgenic lines carrying the wild-type genomic *ETR1* gene was comparable to that of the double receptor null mutant line *etr2-3 ein4-4*, indicating that the wild-type *ETR1* transgene was fully functional. By evaluating the two aspects of receptor signaling, the ETR1 mutants could be divided into four classes. The first class contained mutants that restored the growth defects of the triple mutant and ethylene-sensitive growth of the triple mutant to a level similar to the *etr2 ein4* double mutant, thus indicating normal receptor function. This group included M18A, S24A, F33A, S34A, H79A, M87A, H107A, and K116A. The second class was comprised of mutants that are similar to those in the first class except that they conferred reduced sensitivity or partial insensitivity to ethylene. The mutations in this group include L17A, F61A, L64A, T68A, and C99A, and we propose that they may partially disturb the signal-response coupling of the receptor. The third class consisted of mutants that suppressed the constitutive ethylene response in the triple mutant, but the transgenic plants were completely insensitive to ethylene. This group included D25A, A31V, Y32A, I35A, P36L, E38A, L39A, Y41A, F42A, P50A, F58A, I62A, C65S, H69A, K91A, T94A, V97A,

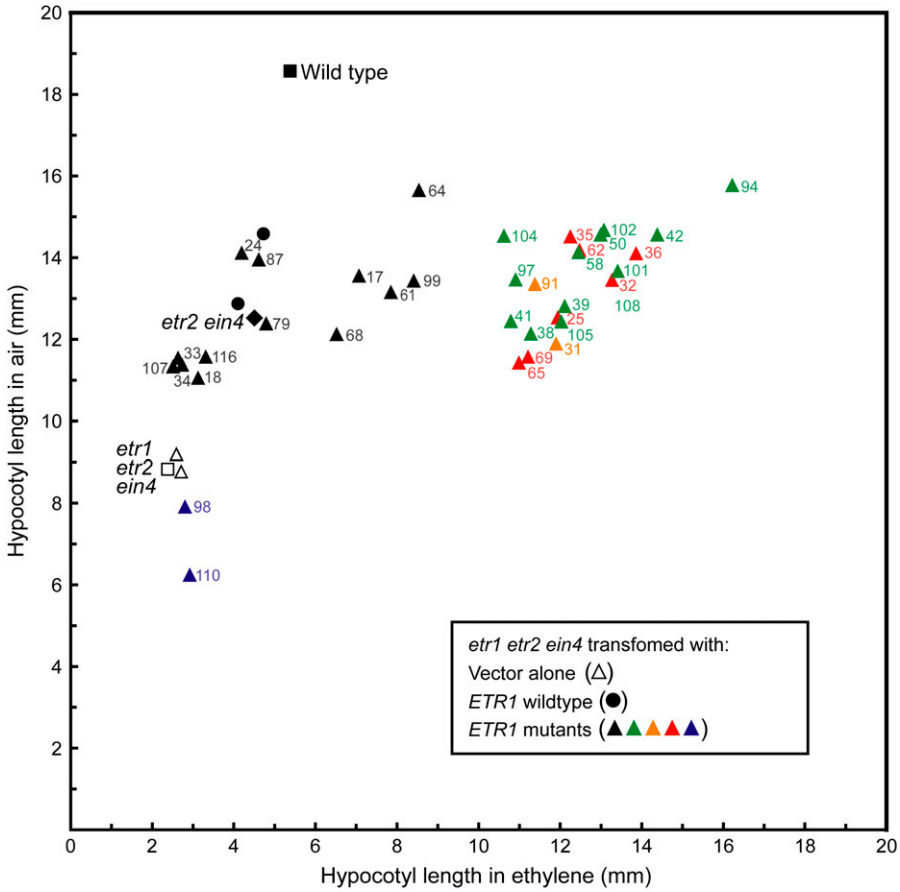

Figure 3. Etiolated Seedling Growth Response of the Transgenic Lines in the *etr1 etr2 ein4* Triple Receptor Null Mutant Background.

The growth response was measured in transgenic lines harboring various ETR1 mutants in the presence and absence of ethylene. The *Arabidopsis* Columbia wild type (closed square), the *etr2 ein4* double receptor null mutant line (diamond), the triple mutant *etr1 etr2 ein4* (open square), the triple mutants transformed with the empty vectors (pPZP211 and pPZP221) (open triangles), and the wild-type *ETR1* (closed circles) are included in the analysis for comparison. A representative homozygous transgenic line is shown for each of the mutant *ETR1* transgenes. Black: mutants restoring ethylene-sensitive growth to the triple mutant but conferring no or only partial ethylene insensitivity. Green, orange, and red: mutants with different ethylene binding activities conferring strong dominant insensitivity to ethylene. Blue: mutants failing to restore ethylene-sensitive growth to the triple mutant. For the four residues that were mutated to different residues, the data from I62A, C65S, H69A, and C99A are presented here. Seedlings were grown on agar plates in the dark for 4 d in either air or 10 ppm ethylene. Hypocotyl length of at least 20 etiolated seedlings of each transgenic line for each treatment was quantified. The SD of the mean was calculated; however, for clarity of the figure, data are not shown.

T101A, A102T, L105A, M104A, and I108A. Similar to the dominant ethylene-insensitive mutant alleles (Bleecker and Kende, 2000), these ETR1 mutants were converted into a constitutively active state, which could no longer be turned off by the ligand. The fourth class contained the S98A and P110A mutants, which failed to rescue the growth defects of the triple mutant as effectively as wild-type *ETR1*. Unlike the previous three classes, the phenotype of the transgenic plants carrying either of these two mutant transgenes was very similar to the triple mutant in both light-grown plants and etiolated seedlings, indicating that the mutations resulted in the loss of ETR1 function in the triple mutant background (Figure 3; data not shown).

Mutants S98A and P110A Partially Rescue the Double Mutant *ers1 etr1*

To further investigate the signaling capabilities of the S98A and P110A mutants, these two *ETR1* mutants were transformed into the subfamily I receptor null double mutant line *ers1-2 etr1-7*. Unlike the *etr1 etr2 ein4* triple mutant, the *ers1 etr1* double mutant exhibits distinct developmental and growth defects in light-grown seedlings and is stunted and infertile as an adult, in addition to the partial constitutive ethylene response phenotype in etiolated seedlings (Hall and Bleecker, 2003; Wang et al., 2003). The severe constitutive ethylene response phenotype of the double mutant lacking both subfamily I receptors cannot be compensated by the subfamily II receptors, indicating that ETR1 and ERS1 play a unique role in receptor signaling (Wang et al., 2003). Transforming the S98A and P110 mutants into the subfamily I receptor null mutant background provided an additional and alternative approach to assess any functional defects of the two ETR1 mutant forms.

Homozygous transgenic lines carrying wild-type *ETR1* and the two mutants were generated in the *ers1 etr1* double mutant background. The two transgenes complemented the growth defects of the double mutant to a certain degree, though not completely (Figure 4). Under light-grown conditions, the 4-d-old seedlings of the transgenic plants of the mutants grown on the agar plate showed no significant difference compared with those carrying the wild-type *ETR1*, except for slightly shorter roots and smaller cotyledons (Figure 4A). However, when grown in soil for 15 d, an obvious difference was observed in their rosette size (Figure 4A). The transgenic plants carrying the mutants have much smaller stature than those transformed with the wild-type *ETR1* at the later developmental stages. One of the most striking differences in these transgenic plants is that fertility was only partially restored, whereas the transgenic plants carrying the wild-type *ETR1* were completely fertile (data not shown). When grown in the dark on agar plates containing 5 μM aminoethoxyvinylglycine (AVG), an ethylene biosynthesis inhibitor, the hypocotyl of the transgenic plants of both S98A and P110A mutants are significantly shorter than that of the transgenic plant carrying wild-type *ETR1* but longer than that of the *ers1-2 etr1-7* double mutant in both air and ethylene (Figure 4B), indicating a partial rescue by the two *ETR1* mutant forms. Since the etiolated seedlings were grown on the agar plates in the presence of AVG, which prevents production of endogenous ethylene, this ruled out that the partial complementation of growth defect of the

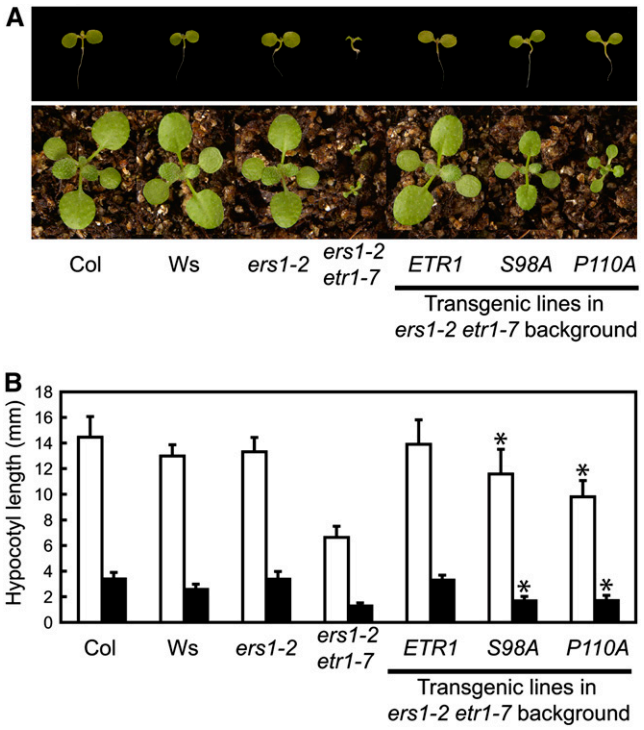

Figure 4. Complementation of Growth Defects of the Double Mutant *ers1 etr1* with the Wild-Type and Mutant *ETR1* Transgenes.

(A) Phenotype of light-grown seedlings. Top panel: seeds were plated on agar plates and then grown under constant fluorescent light for 4 d. Bottom panel: seeds were directly sown in soil and grown under a 16-h-light regime for 2 weeks. The wild-type Columbia (Col-0) and *etr1 ers1* double mutant seedlings are shown for comparison. *ETR1* indicates the double mutant transformed with the wild-type genomic *ETR1*. The mutant *ETR1* transgenes are represented by the mutations.

(B) Etiolated seedling hypocotyl growth response of the transgenic lines carrying the wild-type and mutant *ETR1* transgenes. For each transgenic line, hypocotyls of at least 20 etiolated seedlings grown on agar plates for 4 d in air (open bars) or 1.4 ppm ethylene (closed bars) were measured. Error bars represent the SD. The asterisks indicate that the hypocotyl length is significantly shorter than that of the transgenic plants carrying wild-type *ETR1* but longer than that of the *ers1 etr1* double mutant under the same growth conditions using a Student's *t* test ($P < 0.01$).

double mutant with the S98A and P110A transgenes was due to alteration in endogenous ethylene levels in the transgenic plants. Taken together, these results suggested that the S98A and P110A mutations caused partial loss of *ETR1* function (Figures 3 and 4).

The mutant forms S98A and P110A were still capable of binding ethylene (73.9 and 37.9%, respectively) (Figure 2), indicating that the overall structure was not dramatically disturbed in either mutant. Multiple independent transgenic plants carrying the two mutants in the double mutant background were also generated and analyzed. They all displayed a similar phenotype as shown in Figure 4. It is worthwhile to note that the two residues are among a few amino acids that are completely conserved in all known and putative EBDs (Figure 1A; see Supplemental Figure 4 and Supplemental Table 3 online). This may implicate that they play a

unique role in transmitting signals in this class of receptors so that their conservation has been maintained during the course of evolution.

DISCUSSION

Analysis of the EBD in Nonplant Species

An analysis of in vivo ethylene binding in a wide range of species, together with sequence analysis, was performed to identify evolutionarily conserved amino acids in the EBD and provide insight into the origin and function of the EBD. Based on in vivo ethylene binding studies, we identified a single clade of the cyanobacteria whose members are capable of binding ethylene. This clade includes *Anabaena*, *Fischerella*, *Lungbya*, *Nodularia*, *Nostoc*, *Oscillatoria*, *Pseudoanabaena*, and *Synechocystis* (Wilmotte, 1994; Honda et al., 1999). Of these, only *Pseudoanabaena* showed no ethylene binding activity. No ethylene binding was detected in the cyanobacteria from other clades or in the eubacteria analyzed. In addition, among eukaryotes, only plants and *Chara* have significantly above-background binding. These studies suggest that EBDs have a cyanobacterial origin, consistent with the hypothesis that the ethylene receptors in plants may have derived from a plastid lineage (Bleecker, 1999; Mount and Chang, 2002). Whether ethylene serves as a signaling molecule in the cyanobacterial species is unknown. Since the EBD is capable of binding metals (Rodriguez et al., 1999), an alternate possibility is that the EBD could serve to buffer copper to maintain proper homeostasis of copper levels or act as a metal sensor or transporter in these species.

Sequence analyses have uncovered EBD-like sequences in eight other bacteria that are noncyanobacteria (Mount and Chang, 2002; this study). Most of these noncyanobacterial species belong to highly divergent eubacterial groups. The plastids in plants are likely derived from the *Nostoc* lineage of cyanobacteria after its divergence from *Synechocystis* (Martin et al., 2002), although it is unclear whether *Nostoc* EBDs are more similar to the plant EBDs than to the *Synechocystis* EBD. Additional bacterial genomes representing the entire phylogenetic breadth of eubacteria will eventually help to determine whether plant EBDs could have arisen from horizontal gene transfer rather than from a plastid lineage.

The EBD of *Arabidopsis* ETR1

The EBD of ETR1 in *Arabidopsis* has previously been shown to be within the first 128 amino acid residues of the N terminus (Rodriguez et al., 1999). Various transmembrane helix prediction programs consistently identified three hydrophobic segments in this transmembrane domain. However, the predicted boundaries of the segments were slightly different. Our mutational analysis targeting evolutionarily conserved amino acids in this domain has allowed us to further refine our understanding about the EBD in ETR1. Our results largely support the predicted topological model of the transmembrane domain in ETR1 (Rodriguez et al., 1999). We have identified residue D25 as being crucial for ethylene binding, suggesting that the first transmembrane helix might be extended to Y20 (Figures 1A, 2, and 5B).

Mutational analysis showed that the seven residues essential for ethylene binding are located exclusively within the first and second transmembrane helices (Figures 2 and 5B). This suggests that the ethylene binding pocket may be formed within these first two helices. The observation that the four residues in Helix I (D25, Y32, I35, and P36) are on the same face of the helix, while the three residues of Helix II (I62, C65, and H69) also align along one face of the helix, supports this idea. We speculate that these two conserved surfaces come together in the membrane to form the ethylene/copper binding pocket. This hypothesis is also supported by the fact that mutations in the amino acids surrounding the seven residues (such as A31, F33, and F61) caused severe reduction in the ethylene binding capacity. In Helix III, none of the Ala substitutions completely eliminated ethylene binding, indicating that the contribution of the third transmembrane helix to ethylene binding is minimal compared with the first two helices. Nevertheless, K91 in the third helix severely reduced the ethylene binding capacity, suggesting that the third transmembrane helix still plays a role in facilitating ethylene binding or perhaps in helping to stabilize the ethylene binding pocket.

It has been proposed that the EBD contains a copper cofactor coordinated in an electron-rich hydrophobic pocket formed by membrane-spanning helices of the ETR1 protein (Rodriguez et al., 1999). However, how the EBD and the copper binding domain are organized in the membrane is unknown. Since copper is essential for ethylene binding, alterations of any copper binding ligands should completely eliminate ethylene binding. A number of amino acids analyzed in this study possess the characteristics of metal binding and the preference of providing ligands for copper binding. So far, only the mutation in C65 has been demonstrated experimentally to eliminate copper binding (Rodriguez et al., 1999). Among other potential copper binding coordinates, mutations in D25 and Y32 in Helix I and H69 in Helix II also completely abolished ethylene binding. It will be interesting to determine whether they are also involved in binding the copper cofactor. It is very likely that the EBD and the copper binding domain are adjacent if not overlapping.

Transmembrane Signaling of ETR1

According to the inverse-agonist model for ethylene receptor signaling, the receptor signals as a negative regulator of ethylene response in the absence of ethylene binding, and upon binding of ethylene mediated by the copper cofactor, a conformational change in the membrane-spanning domain is presumably propagated to the cytoplasmic transmitter domain to affect a change in signaling status, resulting in loss of signaling and thus giving rise to ethylene response (Hua and Meyerowitz, 1998; Bleecker, 1999). Consistent with the inverse-agonist model for ethylene receptor signaling, the few EBD mutations that eliminated ethylene binding (10 mutations in seven residues) all conferred dominant ethylene insensitivity in plants (Figure 5A). In other words, these mutations converted the receptor into a constitutive signaling-on state. Interestingly, mutations in many EBD residues (22 of the 37 residues in this study) conferred complete insensitivity to ethylene in *Arabidopsis* (Figure 5A) regardless of ethylene binding ability, thus indicating that these 22 residues are crucial for transmitting conformational changes in ETR1. The

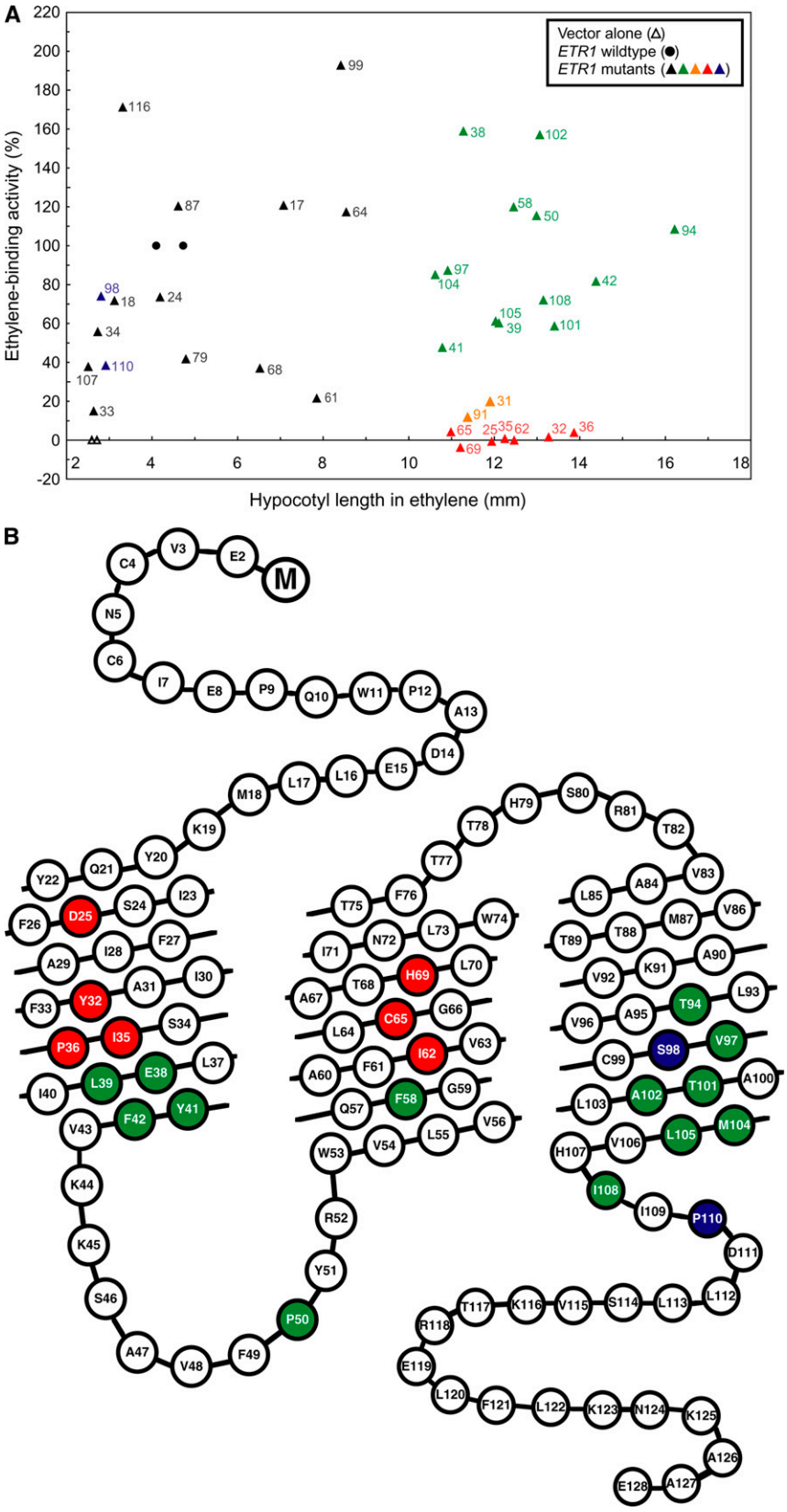

Figure 5. Relationship between Ethylene Binding and Signaling of the ETR1 Receptor.

large number of mutations (13 of the 22 residues) that cause constitutive signaling without significantly impairing ethylene binding suggests that the transduction of transmembrane signaling in the ethylene receptors requires only subtle changes in steric structure, since large changes in steric structure would be expected to affect ethylene binding ability as well. These findings also suggest that the conserved general function of the EBD may be to control the conformation of an attached signaling domain, since so many highly conserved residues of the EBD do not appear to play a role in ethylene binding, yet affect signaling.

The residues crucial for maintaining the proper structural conformation of the receptor to ensure effective signal transduction are clustered both at the bottom of Helix I (toward the cytoplasmic face) and in Helix III. These residues might form a domain that is essential for switching off the receptor. For example, P50A, which is located in the loop between Helices I and II, converted the receptor into the signaling-on state but did not affect ethylene binding. In addition, the two novel partial loss-of-function mutations uncovered in this study (S98A and P110A) are located in Helix III and in the cytoplasmic region proximal to Helix III, respectively. This raises the possibility of there being a domain that is also required for maintaining the signaling-on state of the receptor.

The results of this mutational analysis are represented in a model of ethylene receptor signaling as shown in Figure 6. Previous analyses of ethylene receptor gain-of-function and null mutations led to the inverse-agonist model for ethylene receptor signaling, in which the transmitter domain is constitutively signaling when ethylene is unbound and is shut off when ethylene is bound. Here, our analysis of numerous additional mutations has revealed that the ethylene receptor can be held in an intermediate state (State II in Figure 6) in which the transmitter remains on even when ethylene is bound. While the existence of a transitional state between transmitter on and off states is to be expected, it was not known that a substantial number of mutations could be found to affect the transmitter without affecting ethylene binding. Based on these results, we propose that the ethylene receptor resides in one of the three conformational states during ethylene response and that it functions through conversion between the three states in the presence or absence of ethylene. Moreover, we can assign residues that are important to each of the states. Conformational State I: in the absence of ethylene, the receptor is in a transmitter-on state that actively signals to suppress the ethylene response pathway. We consider this the low-energy or default state for the receptor because mutations in 22 out of 37 residues favor this functional state of the receptor, as evidenced by complete dominant ethylene insensitivity conferred to transgenic *Arabidopsis* (Figure 5A). Residues marked in blue in Figures 5B and 6 are required to achieve this state. Conformational State II: ethylene is bound to the receptor but the transmitter is maintained in the on state. We consider this a quasistable transitional state that can be stabilized by mutations in 13 out of the 37 residues marked in green in Figures 5B and 6, leading to receptor isoforms that confer ethylene insensitivity even when saturated with ethylene. Residues marked in red required for binding ethylene are essential for achieving this state (Figures 5B and 6). Conformational State III: the ethylene-bound receptor undergoes a conformational change in the EBD that is propagated to the transmitter domain, resulting in a transmitter-off state. We consider this a higher-energy state for the receptor because most mutations (in 22 out of the 37 residues) prevent this state from occurring in transgenic *Arabidopsis*. Residues marked in green are required to achieve this state by the ethylene-bound receptor (Figures 5B and 6). These residues cluster near the cytoplasmic side of membrane-spanning Helices I and III in the receptor and may define interaction sites required to achieve the transmitter-off conformational state.

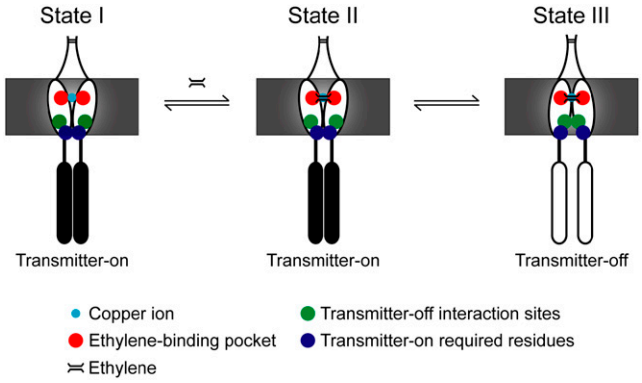

Figure 6. Model of Ethylene Receptor Signaling.

The identification of a possible intermediate state (State II) in which ethylene-bound receptors are maintained in the transmitter-on state may help explain why receptor deficiency caused by null mutations in receptors causes a more severe constitutive response phenotype than wild-type plants saturated by ethylene (Hua and Meyerowitz, 1998; Hall and Bleecker, 2003; Wang et al., 2003). According to our model (Figure 6), wild-type receptors are expected to be in equilibrium between State II and State III in the presence of ethylene; therefore, some suppression of the response pathway would occur even under conditions of saturating

Figure 5. (continued).

(A) Effect of mutations on ethylene binding activity and ethylene response of ETR1. For each *ETR1* mutant, the hypocotyl length of the etiolated seedlings of the transgenic line carrying the transgene grown in the presence of 10 ppm ethylene as shown in Figure 3 is plotted against the ethylene binding activity in yeast transformed with the same mutant as shown in Figure 2. The numbers indicate the positions of the mutated residues in ETR1. Black: mutants with relatively normal binding activity and showing no or only partial ethylene insensitivity. Green: ethylene-insensitive mutants showing substantial binding activity. Orange: ethylene-insensitive mutants with substantially reduced ethylene binding activity. Red: ethylene-insensitive mutants showing negligible ethylene binding activity. Blue: mutants with partial loss of function showing substantial binding activity.
(B) Helical net representation of the transmembrane domain of ETR1. The three hydrophobic segments are modeled as α-helices. The three classes of residues are mapped and colored as shown in **(A)**.

ethylene. By contrast, null mutations would remove all suppression of the response pathway by those receptors removed.

The intermediate State II may also provide a mechanism by which unbound receptors in a large cluster could alter the signaling states of surrounding ethylene-bound receptors. Current models for the evolutionarily related bacterial chemotaxis systems propose that two-component receptors form higher-order clusters composed of receptor dimers. Through direct contact, receptor dimers can influence the signaling state of neighboring dimers so that the signaling state of these neighbors is altered by a single ligand binding event for signal amplification (Bray et al., 1998; Thomason et al., 2002). While there is no direct evidence for such cooperative receptor-clustering associated with the ethylene receptor system, clustering models have been suggested as a possible explanation for the ability of the system to sense small changes in receptor occupancy (Binder et al., 2004) and for dominant truncated mutant alleles of the receptor genes to confer high levels of ethylene insensitivity (Gamble et al., 2002).

METHODS

Ethylene Binding Assay in Organisms of Various Kingdoms

Ethylene binding assays were performed using techniques previously described (Schaller and Bleecker, 1995; Rodriguez et al., 1999) with minor modifications in tissue collection. All of the organisms were tested using [^{14}C]-labeled ethylene except *Acetabularia acetabulum*, *Chara* spp, and *Rhodomonas* sp cells, which were tested using [^{3}H]-labeled ethylene (American Radiolabeled Chemicals). The ethylene binding experiments were performed on 10 to 20 g fresh weight of plant leaf tissue, 1 to 5 g of dry fungi biomass, or 1 to 2 g dry weight of bacterial and algal cultures. The *Caenorhabditis elegans* samples consisted of ~10 million adults and 30 to 40 million larvae, while the *Drosophila melanogaster* samples consisted of ~50,000 adult flies. The source and growth conditions for the various species used for the survey of ethylene binding will be provided as requested.

The bacteria, archaea, and protists were pipetted or spread onto glass microfiber filters, whereas fungi were collected by filtration. Since ethylene production increases in wounded plants, cut leaf tissues were aired under moist conditions for 2 h prior to ethylene binding experiments. The mobility of the *Caenorhabditis* and *Drosophila* resulted in necessary modifications. *Caenorhabditis* were cultured in liquid until its food source (*Escherichia coli*) was scarce so that the majority of the *Caenorhabditis* stayed on the glass microfiber filter containing *E. coli. Drosophila* were anesthetized by adding carbon dioxide to the binding chamber just prior to removing the flies. The flies were kept anesthetized by constant carbon dioxide flow throughout the duration of the airing step. Carbon dioxide does not measurably alter ethylene binding to the ETR1 ethylene receptor expressed in yeast (data not shown). Saturable ethylene binding was determined from each organism. Each assay was conducted at least two times with a maximum deviation of 20% between experiments.

Identification of Genes Containing EBD-Like Sequences

The transmembrane regions of *Arabidopsis thaliana* ETR1, ERS1, EIN4, ETR2, ERS2, and *Synechocystis* slr1212 were used as query sequences in BLAST (Altschul et al., 1997) searches against GenBank release 148 (protein sequences) and in translated BLAST searches against genome sequences of metazoa, fungi, bacteria, and the green algae *Chlamydomonas* deposited in GenBank (nucleotide sequences). Matching sequences with E values <1 were included in the initial analysis. Redundant and highly similar entries (identity >97% in the same species) were eliminated from this analysis. To generate a similarity cluster, the full-length protein sequences of the candidates were searched against a database constructed with the same sequence set. The E values of pairwise comparisons were transformed to the logarithmic scale. The absolute values of transformed E values were regarded as distance measures between sequence pairs and were used to generate similarity clusters with the UPGMA algorithm implemented in MEGA2 (Kumar et al., 2001). For evaluation of the significance of similarity between known EBDs and candidate sequences, the EBD protein sequences of *Arabidopsis* ethylene receptors and *Synechocystis* slr1212 were aligned and used for generating an HMM using HMMER (Eddy, 1998). Candidate sequences with a HMM E value of 1×10^{-10} or smaller were regarded as EBDs. The EBD protein sequences were aligned with the profile alignment mode of ClustalX (Higgins et al., 1996). The profile used is shown in Figure 1A. The alignments generated (see Supplemental Figure 4 online) were used for phylogenetic reconstruction without any adjustment. Phylogenies were generated using the neighbor-joining method and maximum parsimony each with 1000 bootstrap replicates with MEGA2 (Kumar et al., 2001). The sequence naming convention is provided in Supplemental Tables 1 to 3 online.

Site-Directed Mutagenesis and Construction of the *ETR1* Mutants

The majority of site-directed mutations were generated using the Altered Sites Mutagenesis System (Promega). The *Eco*RI-*Sac*I fragment, which encodes the first 128 amino acids of ETR1, was subcloned into the pAlter vector. Oligonucleotides (30 to 35 mers) containing desired mutations were annealed to single-strand templates to generate changes according to the manufacturer's protocol. To construct the full-length cDNA containing the mutations, the altered fragments were ligated in frame with the rest of the *ETR1* coding region in pBluescript II SK− (Stratagene) using a *Sac*I site from ETR1 and a *Sca*I site within the β-lactamase gene in both pAlter and pBluescript II SK−. The full-length *ETR1* mutants were then removed and inserted into an *Eco*RI cloning site of the yeast expression vector pYcDE-2 (amp^{r}, 2 μ, *TRP1*, *ADC1* promoter and terminator) (Hadfield et al., 1986). For a few mutations, including P50A, H69C, and T94A, site-directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene). In this case, a pair of complementary oligonucleotides was used to generate each mutation on the template of pYcDE-2 expressing the full-length ETR1 (Schaller and Bleecker, 1995).

To generate constructs for plant transformation, an 800-bp *Msc*I-*Bst*XI fragment containing each mutation was isolated from the *ETR1* mutants in pYcDE-2 and was used to replace the corresponding region in a 7.3-kb genomic clone of the wild-type *ETR1* in pBluescript II SK− (Chang et al., 1993). The resulting genomic *ETR1* was removed by digestion with *Kpn*I and *Bam*HI or *Pst*I and inserted into the binary vector pPZP211 or pPZP221 (Hajdukiewicz et al., 1994). All of the constructs transformed into the triple receptor null mutant line *etr1-6 etr2-3 ein4-4* (Hua and Meyerowitz, 1998) were made in the binary vector pPZP211 except those containing the mutations S24A, P50A, and T94A, which were made in pPZP221. The transgenes that were transformed into the double receptor null mutant line *ers1-2 etr1-7* (Hall and Bleecker, 2003; Wang et al., 2003) were constructed in pPZP221. All of the mutations and constructs were confirmed by DNA sequencing, and no additional undesired mutations were found.

Ethylene Binding Assay in Yeast

Plasmids expressing wild-type *ETR1* and the *ETR1* mutants were transformed into the yeast strain 2908 (*MATα, his3-200 leu2 trp1-101 ura3-52 ade5*) (Yuan et al., 1995). The yeast transformation was conducted by electroporation using a Gene Pulser (Bio-Rad). The ETR1 mutants containing the mutations C65Y and C99S tested previously (Schaller and Bleecker, 1995) were included in this analysis. Yeast growth and ethylene

binding assays were performed as described before (Schaller and Bleecker, 1995). At least three independent yeast colonies harboring each mutant *ETR1* were tested. Three replicates were performed on each colony.

The protein expression level of the wild-type and mutant ETR1 in yeast was determined by immunoblotting using the ETR1-specific antibody (Ab-HRR) as described (Schaller and Bleecker, 1995). The expression of ETR1 was quantified densitometrically using the NIH Image program (version 1.62). In most cases, the protein expression levels of the wild-type ETR1 and mutants were approximately equivalent with variance <10%. Any yeast colony with a significant higher or lower ETR1 expression level by 50% in comparison with the wild type was excluded from further analysis. The ethylene binding activities of the wild-type and mutant ETR1 were normalized to the protein expression levels after the background binding activity was subtracted. The ethylene binding activity of the ETR1 mutants was expressed as the percentage of that of the wild-type ETR1.

Generation of Transgenic *Arabidopsis* Lines

The wild-type and mutant *ETR1* constructs in the pPZP211 or pPZP221 vector were transformed into the *Agrobacterium tumefaciens* strain ABI. The resulting strains were used to transform the triple receptor null mutant line *etr1-6 etr2-3 ein4-4* or the double receptor null mutant *ers1-2 etr1-7* by the floral dipping method (Clough and Bent, 1998). T1 transgenic plants were selected on agar plates containing either kanamycin (50 μg/mL) for constructs in pPZP211 or gentamycin (100 μg/mL) for constructs in pPZP221. Multiple T1 plants were obtained for each *ETR1* transgene. Six to 12 independent lines transformed with each transgene were further analyzed on agar plates to determine the copy number of transgenes based on the segregation ratio of plants resistant and susceptible to the antibiotics in the T2 progeny. An etiolated-seedling assay was also performed on these transgenic lines in the T2 generation to analyze their response to ethylene and to assess whether the partial constitutive ethylene response phenotype of the triple and double mutants was rescued. Based on these analyses, at least two representative lines with a single copy insertion of each transgene were selected to generate homozygous lines for further analysis.

To confirm that homozygous transgenic lines carried the desired transgenes, a fragment of *ETR1* coding for the transmembrane region was amplified using PCR with the primers 5′-CTGCAATTGTATTGAACCGCAATGGCC-3′ and 5′-GCAACATTCTGCTCCATGAGAAGGTCCC-3′. PCR products were purified and used for DNA sequencing. For genotyping the double mutant *ers1-2 etr1-7*, a PCR-based approach was conducted as described (Wang et al., 2003).

Ethylene Response Assay in Etiolated Seedlings

Seedlings of each homozygous transgenic line were grown in the dark on agar plates containing 5 μM AVG to analyze their response to ethylene. Hypocotyls of etiolated seedlings grown in the presence or absence of ethylene were measured as described previously (Wang et al., 2003).

Accession Numbers

The Arabidopsis Genome Initiative locus identifier for *ETR1* is At1g66340. Other Arabidopsis Genome Initiative accession numbers are within Supplemental Tables 1 to 3 online.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Phylogenetic Relationships between Organisms Analyzed for Ethylene Binding.

Supplemental Figure 2. Similarity Clustering, Taxonomic Distribution, and Domain Organization of Candidate Genes with Ethylene Binding Domains.

Supplemental Figure 3. EBD Phylogeny.

Supplemental Figure 4. Sequence Alignment of EBDs from *Arabidopsis* and Bacteria.

Supplemental Table 1. Sequences Used for Similarity Clustering in Supplemental Figure 2.

Supplemental Table 2. EBD-Like Sequences from Unfinished Bacterial Genomes.

Supplemental Table 3. ClustalX (1.83) Multiple Sequence Alignment for Generating Supplemental Figure 3.

Supplemental Table 4. Species Abbreviations for All EBDs and EBD-Like Sequences.

ACKNOWLEDGMENTS

This article is in memory of our mentor, Anthony B. Bleecker, and our colleague, Jeff J. Esch. The *Arabidopsis etr1-6 etr2-3 ein 4-4* and *etr2-3 ein4-4* mutants were kindly provided by Elliot Meyerowitz. We thank Dennis Milutinovic, Beth Raboin, and Ted Anderson for help with plant care, Colleen Hetzel and Barrett Foat for assisting with ethylene binding assays for several bacterial species, and Claudia Lipke for assistance with photography. We also thank Ronan O'Malley and other members of the Bleecker lab for thoughtful discussions. S.-H.S. was supported by a National Institutes of Health fellowship and the Michigan State University Foundation. H.A. was supported by a fellowship from the China Scholarship Council. This work was funded by a National Science Foundation grant (MCB-0131564 to A.B.B.) and a grant from the American Floral Endowment.

Received June 6, 2006; revised September 9, 2006; accepted November 10, 2006; published December 22, 2006.

REFERENCES

Abeles, F.B., Morgan, P.W., and Saltveit, M.E., Jr. (1992). Ethylene in Plant Biology, 2nd ed. (New York: Academic Press).

Alonso, J.M., and Stepanova, A.N. (2004). The ethylene signaling pathway. Science **306,** 1513–1515.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. **25,** 3389–3402.

Aravind, L., and Ponting, C.P. (1997). The GAF domain: An evolutionary link between diverse phototransducing proteins. Trends Biochem. Sci. **22,** 458–459.

Binder, B.M., O'Malley, R.C., Wang, W., Moore, J.M., Parks, B.M., Spalding, E.P., and Bleecker, A.B. (2004). Arabidopsis seedling growth response and recovery to ethylene. A kinetic analysis. Plant Physiol. **136,** 2913–2920.

Bleecker, A.B. (1999). Ethylene perception and signaling: An evolutionary perspective. Trends Plant Sci. **4,** 269–274.

Bleecker, A.B., and Kende, H. (2000). Ethylene: A gaseous signal molecule in plants. Annu. Rev. Cell Dev. Biol. **16,** 1–18.

Bray, D., Levin, M.D., and Morton-Firth, C.J. (1998). Receptor clustering as a cellular mechanism to control sensitivity. Nature **393,** 85–88.

Brochier, C., Bapteste, E., Moreira, D., and Philippe, H. (2002). Eubacterial phylogeny based on translational apparatus proteins. Trends Genet. **18,** 1–5.

Brown, J.R., Douady, C.J., Italia, M.J., Marshall, W.E., and Stanhope, M.J. (2001). Universal trees based on large combined protein sequence data sets. Nat. Genet. **28,** 281–285.

Chang, C., and Bleecker, A.B. (2005). Ethylene biology. More than a gas. Plant Physiol. **136,** 2895–2899.

Chang, C., Kwok, S.F., Bleecker, A.B., and Meyerowitz, E.M. (1993). *Arabidopsis* ethylene response gene *ETR1*: Similarity of product to two-component regulators. Science **262,** 539–544.

Chen, Y.F., Randlett, M.D., Findell, J.L., and Schaller, G.E. (2002). Localization of the ethylene receptor ETR1 to the endoplasmic reticulum of *Arabidopsis.* J. Biol. Chem. **277,** 19861–19866.

Clark, K.L., Larsen, P.B., Wang, X.X., and Chang, C. (1998). Association of the *Arabidopsis* CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. Proc. Natl. Acad. Sci. USA **95,** 5401–5406.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana.* Plant J. **16,** 735–743.

Eddy, S.R. (1998). Profile hidden Markov models. Bioinformatics **14,** 755–763.

Gamble, R.L., Coonfield, M.L., and Schaller, G.E. (1998). Histidine kinase activity of the ETR1 ethylene receptor from *Arabidopsis.* Proc. Natl. Acad. Sci. USA **95,** 7825–7829.

Gamble, R.L., Qu, X., and Schaller, G.E. (2002). Mutational analysis of the ethylene receptor ETR1, role of the histidine kinase domain in dominant ethylene insensitivity. Plant Physiol. **128,** 1428–1438.

Gao, Z., Chen, Y.F., Randlett, M.D., Zhao, X.C., Findell, J.L., Kieber, J.J., and Schaller, G.E. (2003). Localization of the Raf-like kinase CTR1 to the endoplasmic reticulum of *Arabidopsis* through participation in ethylene receptor signaling complexes. J. Biol. Chem. **278,** 34725–34732.

Guo, H., and Ecker, J.R. (2004). The ethylene signaling pathway: New insight. Curr. Opin. Plant Biol. **7,** 40–49.

Hadfield, C., Cashmore, A.M., and Meacock, P.A. (1986). An efficient chloramphenicol-resistance marker for *Saccharomyces cerevisiae* and *Escherichia coli.* Gene **45,** 149–158.

Hajdukiewicz, P., Svab, Z., and Maliga, P. (1994). The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol. Biol. **25,** 989–994.

Hall, A.E., and Bleecker, A.B. (2003). Analysis of combinatorial loss-of-function mutants in the Arabidopsis ethylene receptors reveals that the *ers1 etr1* double mutant has severe developmental defects that are EIN2 dependent. Plant Cell **15,** 2032–2041.

Hall, A.E., Chen, Q.H.G., Findell, J.L., Schaller, G.E., and Bleecker, A.B. (1999). The relationship between ethylene binding and dominant insensitivity conferred by mutant forms of the ETR1 ethylene receptor. Plant Physiol. **121,** 291–299.

Hall, A.E., Findell, J.L., Schaller, G.E., Sisler, E.C., and Bleecker, A.B. (2000). Ethylene perception by the ERS1 protein in *Arabidopsis.* Plant Physiol. **123,** 1449–1457.

Higgins, D.G., Thompson, J.D., and Gibson, T.J. (1996). Using CLUSTAL for multiple sequence alignments. Methods Enzymol. **266,** 383–402.

Honda, D., Yokota, A., and Sugiyama, J. (1999). Detection of seven major evolutionary lineages in cyanobacteria based on the 16S rRNA gene sequence analysis with new sequences of five marine *Synechococcus* strains. J. Mol. Evol. **48,** 723–739.

Hua, J., and Meyerowitz, E.M. (1998). Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana.* Cell **94,** 261–271.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q.H.G., Bleecker, A.B., Ecker, J.R., and Meyerowitz, E.M. (1998). *EIN4* and *ERS2* are members of the putative ethylene receptor gene family in Arabidopsis. Plant Cell **10,** 1321–1332.

Karol, K.G., McCourt, R.M., Cimion, M.T., and Delwiche, C.F. (2001). The closest living relatives of land plants. Science **294,** 2351–2352.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). CTR1, A negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. Cell **72,** 427–441.

Kumar, S., Tamura, K., Jakobsen, I.B., and Nei, M. (2001). MEGA2: Molecular evolutionary genetics analysis software. Bioinformatics **17,** 1244–1245.

Martin, W., Rujan, T., Richly, E., Hansen, A., Cornelsen, S., Lins, T., Leister, D., Stoebe, B., Hasegawa, M., and Penny, D. (2002). Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. Proc. Natl. Acad. Sci. USA **99,** 12246–12251.

Misler, B.D., Lewis, L.A., Buchheim, M.A., Renzaglia, K.A., Garbary, D.J., Delwiche, C.F., Zechman, F.W., Kantz, T.S., and Chapman, R.L. (1994). Phylogenetic relationships of the ''green algae,'' and the ''bryophytes.''. Ann. Mo. Bot. Gard. **81,** 451–483.

Mount, S.M., and Chang, C. (2002). Evidence for a plastid origin of plant ethylene receptor gene. Plant Physiol. **130,** 10–14.

Moussatche, P., and Klee, H. (2004). Autophosphorylation activity of the Arabidopsis ethylene receptor multigene family. J. Biol. Chem. **279,** 48734–48741.

O'Malley, R.C., Rodriguez, F.I., Esch, J.J., Binder, B.M., O'Donnell, P., Klee, H.J., and Bleecker, A.B. (2005). Ethylene-binding activity, gene expression levels, and receptor system output for ethylene receptor family members from Arabidopsis and tomato. Plant J. **41,** 651–659.

Qu, X., and Schaller, G.E. (2004). Requirement of the histidine kinase domain for signal transduction by the ethylene receptor ETR1. Plant Physiol. **136,** 1–10.

Quaiser, A., Ochsenreiter, T., Lanz, C., Schuster, S.C., Treusch, A.H., Eck, J., and Schleper, C. (2003). Acidobacteria form a coherent but highly diverse group within the bacterial domain: Evidence from environmental genomics. Mol. Microbiol. **50,** 563–575.

Rodriguez, F.I., Esch, J.J., Hall, A.E., Binder, B.M., Schaller, G.E., and Bleecker, A.B. (1999). A copper cofactor for the ethylene receptor *ETR1* from *Arabidopsis.* Science **283,** 996–998.

Schaller, G.E., and Bleecker, A.B. (1995). Ethylene-binding sites generated in yeast expressing the *Arabidopsis ETR1* gene. Science **270,** 1809–1811.

Schaller, G.E., Ladd, A.N., Lanahan, M.G., Spanbauer, J.M., and Bleecker, A.B. (1995). The ethylene response mediator ETR1 from *Arabidopsis* forms a disulfide-linked dimer. J. Biol. Chem. **270,** 12526–12530.

Thomason, P.A., Wolanin, P.M., and Stock, J.B. (2002). Signal transduction: Receptor clusters as information processing arrays. Curr. Biol. **12,** 339–401.

Wagnera, M., and Horna, M. (2006). The Planctomycetes, Verrucomicrobia, Chlamydiae and sister phyla comprise a superphylum with biotechnological and medical relevance. Curr. Opin. Biotechnol. **17,** 241–249.

Wang, W., Hall, A.E., O'Malley, R., and Bleecker, A.B. (2003). Canonical histidine kinase activity of the transmitter domain of the ETR1 ethylene receptor from *Arabidopsis* is not required for signal transmission. Proc. Natl. Acad. Sci. USA **100,** 352–357.

Wilmotte, A. (1994). Molecular evolution and taxonomy of the cyanobacteria. In The Molecular Biology of Cyanobacteria, D.A. Bryant, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 1–25.

Yuan, D.S., Stearman, R., Dancis, A., Dunn, T., Beeler, T., and Klausner, R.D. (1995). The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. Proc. Natl. Acad. Sci. USA **92,** 2632–2636.