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Ethylene Receptors: Ethylene Perception and Signal Transduction

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Abstract

Ethylene is sensed by a family of receptors that can be divided into two subfamilies based on phylogenetic analysis and some shared structural features. In this review we focus on the mechanistic aspects of how the receptors function in plants to transduce the ethylene signal. Recent work has led to new insights into how ethylene binds to the receptors and how this binding may induce a conformational change to regulate signaling. Additional studies point to several possible mechanisms for signal output by the receptors, which may involve changes in enzymatic activity and/or conformational changes. Other studies indicate the importance of interactions, both physical and genetic, between the receptors and early components of the signaling pathway, in particular, the Raf-like kinase CTR1, which functions as an integral component of the ethylene receptor signaling complex. The current model for signaling in *Arabidopsis* supports differing contributions from the receptors, with subfamily-1 receptors playing a more significant role than the subfamily-2 receptors in transmitting the ethylene signal.

Key words: Ethylene; Receptors; Signal transduction

Introduction

Molecular genetic analysis of ethylene receptors was initiated about 20 years ago when a graduate student named Tony Bleecker, working in the laboratory of Hans Kende at Michigan State University, performed a mutant screen with *Arabidopsis* (Bleecker and others 1988). The immediate question in those early days, before *Arabidopsis* had achieved widespread acceptance as a model plant, was what would be the best screen to use for selection of a mutant affected in ethylene signaling. The role of ethylene in plant senescence was well established, so one possibility would be to look for mutants that exhibited delayed senescence in response to ethylene. Drawbacks to this screen were that it would require a lot of space and it might be difficult to assess the varying degrees of senescence among the plants. An alternative would be to look for changes in the response of dark-grown seedlings to ethylene, an effect of ethylene first noted almost a century before (Neljubov 1901). This screen took advantage of the triple response of seedlings to ethylene, which in *Arabidopsis* is characterized by a shortened and thickened hypocotyl, an inhibition of root elongation, and the formation of an exaggerated apical hook (Guzmán and Ecker 1990). The advantage here was that the screen could be performed on young seedlings, saving both time and space, and could be readily assessed visually.

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Indeed, when a Petri dish containing seedlings was pulled out of the darkened incubation chamber after four days of growth in ethylene, there was one spindly seedling that appeared to tower above a lawn of stunted wild-type seedlings, an image so clear and profound in its implications that it ended up on the cover of *Science* magazine (Bleecker and others 1988).

That mutant seedling was *etr1-1*. The *etr1-1* mutant provided a critical entry point into the ethylene signal transduction pathway. Not only did the mutation turn out to be in a gene encoding an ethylene receptor, but the nature of the mutant lesion turned out to offer crucial insights into the mechanism by which ethylene is perceived by the receptor.

We now know that ethylene perception is mediated by a family of receptors. In *Arabidopsis*, the receptor family is composed of ETR1, ERS1, ETR2, ERS2, and EIN4 (Bleecker 1999; O'Malley and others 2005; Schaller and Kieber 2002). Each ethylene receptor has a similar overall modular structure, with transmembrane domains containing the ethylene binding site near the N-terminus, followed by a GAF domain of unknown function, and then signal output domains in the C-terminal half (Figure 1). Although similar, the ethylene receptors can be divided into two subfamilies based on phylogenetic analysis and some shared structural features, subfamily 1 being composed of ETR1 and ERS1 and subfamily 2 being composed of ETR2, ERS2, and EIN4 (Bleecker 1999; Chang and Stadler 2001; Schaller and Kieber 2002). The same general features and division of the ethylene receptor family into two subfamilies is conserved in monocots and dicots (Bleecker 1999; Klee 2004; Yau and others 2004).

Two classes of mutations have been isolated in the ethylene receptors, both dominant gain-of-function mutations and recessive loss-of-function mutations. Ethylene receptors were originally identified based on mutations that resulted in a dominant ethylene-insensitive phenotype (Bleecker and others 1988; Chang and others 1993; Hua and others 1995, 1998; Sakai and others 1998; Wilkinson and others 1997). These mutations turn out to be missense mutations within the sensory portion of the receptors. The second class of mutations is loss-of-function, and these have now been identified for all five members of the ethylene receptor family in *Arabidopsis* (Hua and Meyerowitz 1998; Wang and others 2003; Xie and others 2006; Zhao and others 2002). Single loss-of-function mutations have little or no effect upon ethylene signal transduction. However, in combination with each other, the mutant plants exhibit constitutive ethylene response phenotypes. These results indicate that there is functional overlap among the receptor family members. These results also indicate that the receptors serve as negative regulators of the ethylene response pathway, because elimination of receptors activates ethylene responses. Both loss-of-function and gain-of-function mutations continue to be used as tools for the analysis of receptor signaling.

In this review we focus on the work that has gone into determining how the ethylene receptors function in signaling. Recent work has led to new insights into how ethylene binds to the receptors and how this binding may induce a conformational change to regulate signaling. We also consider several possible mechanisms for signal output by the receptors, a question that is still unresolved. Finally, we examine the importance of interactions, both physical and genetic, between the receptors and other early components of the signaling pathway, in particular, the Raf-like kinase CTR1, which functions as an integral component of the ethylene receptor signaling complex.

Subcellular Localization to Membranes of the Endoplasmic Reticulum (ER)

Most receptors are localized to the plasma membrane (PM), but ethylene is a gaseous hormone with the ability to diffuse in both aqueous and lipid environments (Abeles and others 1992), so its perception does not require a PM-localized receptor. In fact, several studies have now demonstrated that ethylene receptors localize to the endoplastic reticulum (ER) membrane (Chen and others 2002; Ma and others 2006). In the first study, localization of native AtETR1 was demonstrated by membrane fractionation and immunoelectron microscopy (Chen and others 2002). In a more recent study, membrane fractionation was also used to localize native CmERS1, an ethylene receptor from melon (Ma and others 2006). In addition, localization of CmERS1 was examined with a GFP-tagged version of the receptor expressed from the CaMV-35S promoter, with potential mislocalization due to overexpression being avoided by performing a time-course analysis with diluted plasmid. Fluorescence was observed in a reticular network characteristic of the ER in melon leaves, trichomes, leaf epidermal cells, and stomatal guard cells (Ma and others 2006).

Localization to the ER membrane offers several possible advantages for the receptor. One of the most significant in terms of ethylene signaling is likely to be the speed with which the receptors can be delivered to their site of action, because the receptors do not have to pass out of the ER and travel to the plasma membrane. This may be of particular importance for the receptors that are induced by ethylene, such as ERS1, ERS2, and ETR2 in *Arabidopsis*, which as we discuss later are likely to play a key role in resensitizing plants to ethylene (Hua and others 1998). In addition, the ER is a multifunctional organelle and localization to the ER may allow for cross-talk with other pathways (Hara-Nishimura and Matsushima 2003; Staehelin 1997; Vitale and Denecke 1999).

Although the receptors appear to predominantly localize to the ER membrane, this does not exclude the possibility of low levels or transient receptor localization to other membrane systems such as the Golgi, PM, or vacuole. For instance, an ethylene receptor from tobacco (NTHK1) has been reported to localize to the PM (Xie and others 2003). Although this study relied upon overexpression of the receptor and may thus represent mislocalization, it does point to the possibility that receptors may be found at other points in the secretory pathway. Further studies are needed to determine if receptor localization to the PM or other subcellular locations occurs under native expression levels. Such studies are also needed to determine if subcellular localization differs between subfamily-1 and subfamily-2 receptors; subfamily-2 receptors carry an extra hydrophobic domain at their N-terminus that is predicted to be a signal sequence (Figure 1), but which may affect localization.

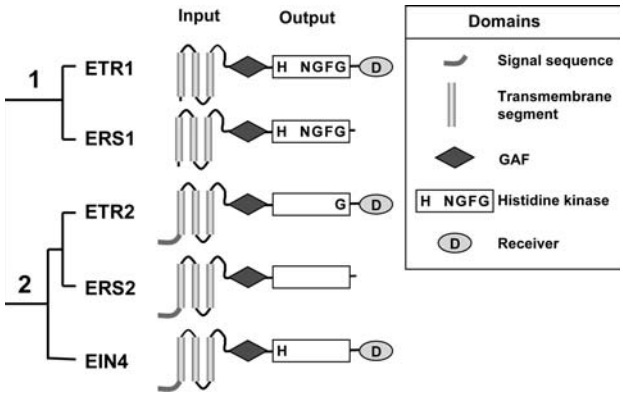

Figure 1. The ethylene receptor family of *Arabidopsis*. Primary structures of the five-member family are indicated along with their transmembrane, GAF, histidine-kinase, and receiver domains and putative signal sequences. The conserved phosphorylation sites upon histidine (H) and aspartate (D) are indicated if present. Conserved motifs (NGFG) within the histidine-kinase domain are indicated if present. There are two subfamilies of ethylene receptors (subfamily 1 and 2) based on sequence and phylogenetic analysis.

Membrane Topology

The transmembrane domains are required for both ethylene binding and membrane localization and, not surprisingly given this dual role, are among the most highly conserved regions of the ethylene receptors. Information on the number and topology of the transmembrane domains is thus an important factor in modeling the ethylene binding site. Initial computational methods suggested the presence of three transmembrane domains in the subfamily-1 receptors, with the N-terminus predicted to be localized to the extra-cytosolic space (that is, the lumen of the ER) and the C-terminus to the cytosol (Chang and others 1993; Schaller and others 1995). Only recently, however, has this topology been verified experimentally (Ma and others 2006) (Figure 2). Ma and others (2006) examined topology of both full-length and truncated versions of the melon receptor CmERS1 by two methods: (1) proteolytic sensitivity of the receptor following expression in microsomes and (2) N-glycosylation mutagenesis in which potential glyscosylation sites are introduced at various positions, with glycosylation indicating localization of that site to the ER lumen. Their finding that the N-terminus localizes to the ER lumen is consistent with the presence of disulfide bonds in this region (Schaller and others 1995), because these are introduced by protein disulfide isomerase, an ER-resident protein. This topology also places the GAF, histidine kinase, and receiver domains in the cytosol where they can participate in signal output from the receptor.

Ethylene Binding

The ethylene binding site, based on genetic and biochemical evidence, lies within the three conserved transmembrane domains. Much of our biochemical understanding of ethylene binding comes from the transgenic expression of the receptors in yeast. This approach has demonstrated that both subfamily-1 and -2 receptors bind ethylene and do so with similar affinities (Hall and others 2000; O'Malley and others 2005; Rodriguez and others 1999; Schaller and Bleecker 1995). Reconstitution of binding activity and affinity purification of

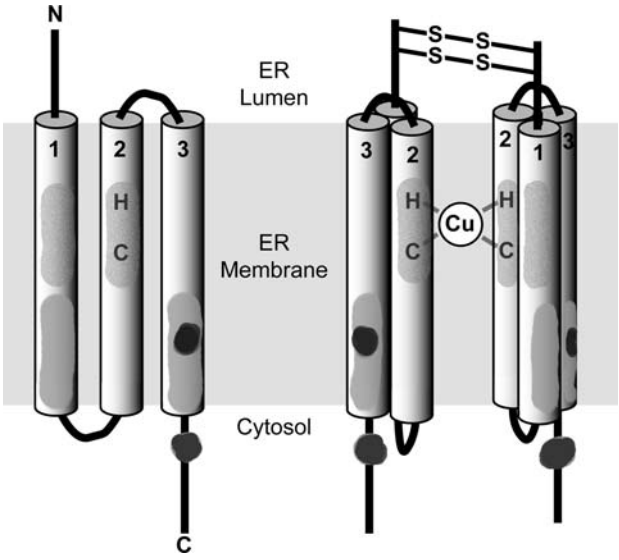

Figure 2. Model of ethylene binding site. Models are shown for the membrane topology of the ETR1 monomer (left) and for the ethylene binding site contained within an ETR1 homodimer (right). There is one copper binding site and consequently one ethylene binding site per receptor homodimer. Each monomer of the receptor homodimer contains three transmembrane segments. Cys65 and His69 are thought to coordinate the copper ion within the second transmembrane domains of each monomer. Sandy gray highlights indicate the region involved in ethylene binding based on the finding that mutations in this region substantially reduce or eliminate ethylene binding, along with conferring ethylene insensitivity. Light gray highlights indicate a region involved in transmitting the conformational change brought about by ethylene binding based on the finding that mutations in this region still allow for ethylene binding but nevertheless confer dominant ethylene insensitivity. Dark gray highlights indicate a region involved in maintaining the receptor in the conformation normally exhibited in the absence of ethylene (in air) based on the finding that mutations result in a loss-of-function phenotype.

the receptors from transgenic yeast has also demonstrated the requirement of a copper cofactor for ethylene binding (Rodriguez and others 1999), a finding in agreement with theoretical predictions from decades ago (Beyer 1976; Burg and Burg 1967) and the discovery that ethylene signaling in plants requires the function of a copper-transporting ATPase (RAN1) (Hirayama and others 1999; Woeste and Kieber 2000). The basic functional unit for ethylene perception is apparently a dimer, based on the finding that there is one copper ion, and thus the ability to bind one molecule of ethylene per receptor dimer (Rodriguez and others 1999) (Figure 2).

The utility of this biochemical analysis is greatly increased when coupled with molecular genetic analysis in plants, and this two-pronged approach has been essential to developing a model for ethylene binding and signal transduction by the receptors. The *etr1-1* mutation, which confers dominant ethylene insensitivity in plants, arises from a single amino acid change (Cys65 to Ala) within the second transmembrane domain of the receptor (Bleecker and others 1988; Chang and others 1993). The site of this mutation pointed to the importance of the transmembrane domains in ethylene perception, and biochemical analysis of the etr1-1 mutant protein in yeast demonstrated that this lesion not only abolished ethylene binding by the receptor, but did so because the receptor could no longer chelate the copper cofactor required for ethylene binding (Rodriguez and others 1999; Schaller and Bleecker 1995). Employing a similar approach, the missense mutations *etr1-3* and *etr1-4*, which also affect amino acids in the transmembrane domains, have been found to reduce or eliminate ethylene binding (Hall and others 1999).

The above analysis might seem to suggest that all mutations in the receptors that confer ethylene insensitivity arise from an inability to bind ethylene. This, however, turns out not to be the case as demonstrated by analysis of the *etr1-2* mutation. Receptors carrying the *etr1-2* mutation, which results in a missense mutation in the third transmembrane domain, still bind ethylene but nevertheless confer dominant ethylene insensitivity on plants. This finding suggests that the transmembrane domain may contain regions responsible for both ethylene binding and intramolecular transduction of the ethylene signal (Hall and others 1999).

Recently, this type of structure/function analysis was extended (Wang and others 2006), resulting in a significant leap forward in our understanding of how the transmembrane domains contribute to ethylene binding and signaling. Alanine-scanning mutagenesis was performed on conserved residues of the ethylene receptor ETR1. These ETR1 mutants were then tested for their ability to bind ethylene following transgenic expression in yeast. In addition, the mutants were introduced into an *Arabidopsis* line that contains loss-of-function mutations in *ETR1*, *ETR2*, and *EIN4*. The *etr1;etr2;ein4* mutant line exhibits a partial ethylene-response phenotype (Hua and Meyerowitz 1998), which allowed the investigators to assess (1) whether the mutant versions of ETR1 were competent for signaling based on their ability to rescue the mutant phenotype, and (2) whether the mutant versions of ETR1 were

able to confer dominant ethylene insensitivity. Three different mutant phenotypes were observed among the mutations that perturbed function of the receptor. First, seven amino acid residues essential for ethylene binding were identified in the first and second transmembrane helices; these *etr1* mutations all conferred ethylene insensitivity when expressed in plants. The amino acids affected by these mutations are likely to form the binding pocket for ethylene and include Cys65 and His69, which are thought to be involved in chelating the copper cofactor (Rodriguez and others 1999) (Figure 2). Second, 13 residues were identified that when mutated did not affect ethylene binding but still resulted in ethylene insensitivity when examined *in planta*. These residues are primarily in the first and third transmembrane helices and are localized toward the cytosolic face of the membrane. These residues are thus likely to be required for transmitting the conformational change induced by ethylene binding to the signal output domain. Third, two residues were identified that when mutated did not affect ethylene binding but which caused a partial loss of ETR1 function when examined *in planta*. These residues may be required for maintaining the conformation that ETR1 normally exhibits in the absence of ethylene (in air) such that, upon mutation, the receptor adapts the conformation induced upon ethylene binding.

The use of ethylene agonists and antagonists represents an alternative approach by which to characterize ethylene binding and signaling. The strained alkene 1-methylcyclopropene (1-MCP) has been previously demonstrated to be one of the most effective antagonists of ethylene action in plants (Sisler and others 1996). Analysis of its action upon ETR1 expressed in yeast indicates that 1-MCP functions as a competitive inhibitor for the ethylene binding site, but with higher affinity than ethylene (a K_i of 10.7 nL/L compared with K_d of 36 nL/L for ethylene) (Hall and others 2000). Recently, a series of 1-alkyl cyclopropenes were prepared in which the side chain was extended by up to ten carbons in length and their effectiveness against ethylene action in plants examined (Sisler and others 2003). The perhaps counterintuitive result from this study was that increasing the hydrocarbon chain by five or more carbon residues actually increased the effectiveness of the cyclopropene. It is likely that the extended hydrocarbon tail results in increased incorporation and retention of the inhibitor in membranes, thereby placing the inhibitor in proximity to the membrane-localized ethylene binding site of the receptor. This study also suggests that the ethylene binding site may be fairly accessible because creating a more bulky inhibitor did not reduce its apparent efficacy.

Dimerization of the Receptors

At an early stage in the characterization of ethylene receptors it was discovered that the basic functional unit for ETR1 is a disulfide-linked homodimer (Schaller and Bleecker 1995). The ETR1 homodimer is maintained through cysteine residues (Cys4 and Cys6) located at the N-terminal end of the protein and which form a covalent disulfide bond. Cysteine residues in the analogous position are conserved in other plant ethylene receptors (Schaller and Bleecker 1995) and formation of a covalent dimer has also been demonstrated for ERS1 from *Arabidopsis* (Hall and others 2000) and for an ethylene receptor in *Cucumis* (Takahashi and others 2002).

Recent experiments, however, indicate that the disulfide bonds are not required for ethylene signaling (Xie and others 2006). Mutant versions of ETR1 in which the cysteines were mutated to alanine rescued the constitutive ethylene-response phenotype found in the *etr1-7;ers1-2* double mutant. Similarly, the cysteine residues were also not required for the mutant etr1-1 protein to confer dominant ethylene insensitivity. However, given that the functional unit of ETR1 is a dimer, with one ethylene binding site per dimer, other noncovalent mechanisms may serve to form and maintain ETR1 dimers. The possibility for such noncovalent interactions among receptors is supported by two experiments. First, it was shown through yeast two-hybrid analysis that the GAF domain of ETR1 can interact with the soluble portion of ETR1 (Xie and others 2006). Second, X-ray crystallography of the ETR1 receiver domain indicates that the receiver domain can dimerize (Muller-Dieckmann and others 1999).

What then is the role of the disulfide linkage, given that the residues that participate in formation of the disulfide bond are so highly conserved among the ethylene receptors? In general, disulfide bonds serve roles in folding and stability of proteins (Gilbert 1997) and it is likely, even if not essential, that they serve a similar purpose in the ethylene receptors. They may facilitate assembly of the homodimer during translation, particularly given their presence at the N-terminus, in which case they could increase the rate of formation and/or the percentage of functional receptors. They may also stabilize the protein under conditions of stress and, as such, their role may not be obvious under optimal growth conditions.

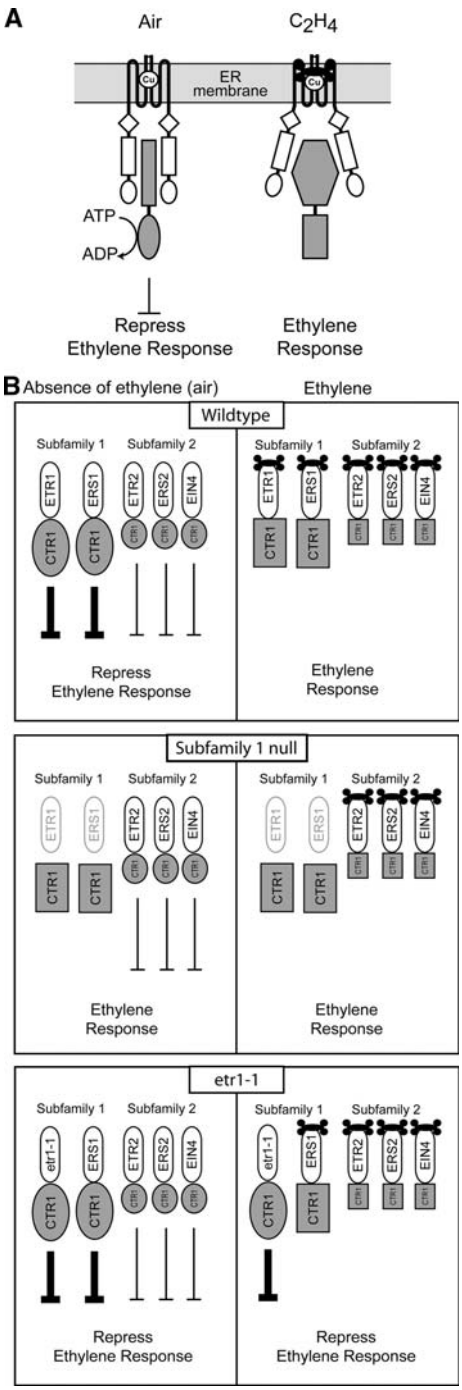

Figure 3. Model for signaling by ethylene receptors. **A** CTR1 (shown in gray) interacts with the histidine-kinase domain and receiver domains of the receptor. In air, the kinase domain of CTR1 actively represses ethylene responses. Binding of ethylene by the receptor induces a conformational change in CTR1 that reduces its kinase activity, thereby relieving repression of the ethylene response pathway. **B** In the absence of ethylene, all five receptors interact with CTR1, but subfamily-1 receptors activate CTR1 to a greater extent than subfamily-2 receptors. Ethylene binding to the receptors results in the inactivation of CTR1. In the figure active CTR1 is indicated by a circle and inactive CTR1 by a square; the size of the CTR1 symbol indicates its relative contribution to signaling. Loss of subfamily-1 receptors (the *etr1;ers1* mutant) leads to the stimulation of ethylene responses because there is not enough active CTR1 to suppress the pathway. An ethylene-insensitive receptor mutant (etr1-1) may no longer bind ethylene, and as such continues to activate CTR1, even in the presence of ethylene, so that CTR1 represses ethylene responses.

Signal Output of the Receptors Through Physical Interactions with the Raf-like Kinase CTR1

A key question is how the receptors transmit information to downstream signaling components in the pathway. The initial discovery that the receptors contain histidine kinase and receiver domains, motifs known to participate in His-Asp phosphorelays in prokaryotes, suggested that a similar phosphorelay might operate in ethylene signaling (Chang and others 1993), but genetic analysis did not point in this direction. Instead, the next immediate element in the signaling pathway appeared to be the Raf-like kinase CTR1 (Kieber and others 1993). Subsequent analysis has not only confirmed the importance of CTR1 to signaling but also indicates that the receptors are able to directly bind to this downstream signaling component (Cancel and Larsen 2002; Clark and others 1998; Gao and others 2003). In this section we consider the importance of this physical interaction with CTR1 to signaling by the receptors, taking up the question of receptor kinase activity and its potential signaling role in the next section.

CTR1 is a negative regulator of ethylene signaling and shows similarity to the Raf family of serine/threonine protein kinases in its C-terminal half (Huang and others 2003; Kieber and others 1993). Loss-of-function mutations in CTR1 result in a constitutive ethylene-response phenotype and can result from nonsense mutations and from missense mutations that eliminate key residues required for kinase activity (Huang and others 200; Kieber and others 1993). The ability of CTR1 to interact with the ethylene receptors was initially identified based on yeast two-hybrid analysis. From this analysis it was found that the N-terminal half of CTR1 could interact with the histidine kinase and receiver domains of ETR1 and with the histidine kinase domain of ERS1 (Clark and others 1998). Subsequent analysis using a similar strategy revealed a weak

interaction with the subfamily-2 receptor ETR2 (Cancel and Larsen 2002).

More recently, the association of CTR1 with the receptors has been confirmed *in planta*. From this analysis it was found that CTR1, although predicted to be a soluble protein, is associated with the ER, consistent with a physical association between CTR1 and the ER-localized ethylene receptors (Gao and others 2003). Direct evidence for the *in planta* interaction of CTR1 with ETR1 came from the finding that affinity purification of TAP-tagged CTR1 resulted in copurification of ETR1 (Gao and others 2003). Ethylene treatment does not affect the association of CTR1 with the membranes, suggesting that CTR1 normally forms stable associations with the receptors.

The importance of the interaction between CTR1 and the receptors for signal output is supported by the analysis of mutations in both CTR1 and the receptors. A mutation that disrupts the interaction of CTR1 with the receptors (*ctr1-8* mutation) resulted in a loss of CTR1 protein from the ER membrane and its appearance in the soluble protein fraction (Gao and others 2003; Huang and others 2003). Similarly, mutations that eliminated ethylene receptors also resulted in a loss of CTR1 from the ER membrane (Gao and others 2003). All these mutations result in a constitutive ethylene-response phenotype, indicating that the interaction of CTR1 with the receptors is required for maintaining the activity of CTR1.

The means by which the receptors regulate CTR1 activity is thus a key mechanism in ethylene signal transduction. Such regulation could be purely through conformational changes brought about by the receptors following ethylene binding and passed on to the associated CTR1, or regulation may involve kinase activity of the receptors. What is becoming clear is that regulation of CTR1 by the receptors is likely to be nonstoichiometric in several ways. First, the amount of CTR1 bound by the receptors may not occur in a consistent ratio (that is, there may not be one CTR1 per receptor homodimer). This proposal is based on the finding that elimination of the receptor ETR1 actually resulted in a significant increase in the level of CTR1 found at the membrane in *Arabidopsis* (Gao and others 2003). Second, signal output from CTR1 may be partially dependent on the receptor with which CTR1 interacts (that is, an Animal Farm hypothesis in which all receptors are equal, but some are more equal than others), as the level of membrane-associated CTR1 did not always correlate with the level of signaling through the ethylene pathway. For example, loss of all three subfamily-2 receptors results in only a mild constitutive ethylene-response phenotype (Cancel and Larsen 2002; Hua and Meyerowitz 1998) even though there is substantial loss of CTR1 from the membrane (Gao and others 2003). In contrast, a stronger constitutive ethylene-response phenotype is found in mutant combinations containing a loss-of-function *etr1* mutant (Cancel and Larsen 2002; Hua and Meyerowitz 1998), even though these do not show the same reduction in CTR1 levels at the membrane (Gao and others 2003). These results suggest that subfamily-1 receptors, such as ETR1, may be better able to activate CTR1 than the subfamily-2 receptors.

The Role of Enzymatic Activity in Signal Output

In the C-terminal halves of the ethylene receptors are domains with homology to histidine kinases and receiver domains (Figure 1), motifs originally identified in the two-component signaling pathways of prokaryotes (Stock and others 2000). Two-component systems allow organisms to rapidly respond to various stimuli by the use of a phosphorelay mechanism to transduce the signal. Two-component signaling systems usually consist of a membrane-localized histidine protein kinase that senses input signals, and a response regulator that mediates signal output (Schaller and others 2002; Stock and others 2000). Stimuli induce autophosphorylation of a conserved histidine residue in the histidine kinase domain. The phosphate group is then transferred to an aspartate residue located within the receiver domain of the response regulator, which in many cases is a transcription factor. The transfer of the phosphate group can be either direct or through a histidine-containing phosphotransfer protein. Plants mediate cytokinin signal transduction through such a two-component system (Hwang and others 2002), and thus it is possible that plants also make use of a phosphorelay mechanism for ethylene signaling (Mason and Schaller 2005).

Truncation studies using ETR1 indicate the importance of the histidine kinase domain for signaling and have served to establish this as a signal output domain as predicted from the sequence (Qu and Schaller 2004; Xie and others 2006). This raises the question as to what feature(s) of the histidine kinase domain are important for signal output. As described in the previous section, the histidine kinase domain serves as a site for physical interaction with CTR1, but the sequence and biochemical analysis also indicate that this domain has

enzymatic activity. Clarifying the role of this enzymatic activity in signaling has posed a challenge, and while evidence has begun to accumulate that phosphorylation affects ethylene signal transduction, it is still unclear if it plays a major role. In this section we consider the possible roles of enzymatic activity found in the receptors, first considering the subfamily-1 receptors, which contain conserved histidine kinase domains, then considering the subfamily-2 receptors, which contain diverged kinase domains that may function as ser/thr kinases (Moussatche and Klee 2004).

Histidine kinase activity has been shown for the subfamily-1 receptors ETR1 and ERS1 *in vitro* and so these are the receptors that have been focused on when examining the role of histidine kinase activity *in planta* (Gamble and others 1998, 2002; Moussatche and Klee 2004). The general approach used in several studies has been to test the ability of kinase-inactive versions of the receptors to rescue the constitutive ethylene-response phenotype found in mutant combinations of receptors. For example, the *etr1-7;ers1-2* double mutant, in which both subfamily-1 receptors contain loss-of-function mutations, shows a constitutive ethylene-response phenotype (Hall and Bleecker 2003; Wang and others 2003; Xie and others 2006; Zhao and others 2002). In an initial study it was found that both wild-type and kinase-inactive versions of ETR1 were able to rescue the constitutive ethylene-response phenotype of the double mutant (Wang and others 2003), indicating that the histidine kinase activity of ethylene receptors does not play a substantial role in the ethylene response. It is now known, however, that the *ers1-2* allele used in this study is a partial loss-of-function allele rather than a null (Qu and others 2007; Xie and others 2006; Zhao and others 2002). The residual level of ERS1 protein found in the *ers1-2* mutant background recently has been shown to have significant effects upon signaling (Xie and others 2006). In addition to autophosphorylation, ERS1 could potentially transphosphorylate the kinase-inactive version of ETR1 used to evaluate the role of histidine kinase activity in ethylene signaling, which still contained a phosphorylatable Asp residue in the receiver domain (Wang and others 2003). Thus, the degree to which histidine kinase activity contributes to ethylene signaling is currently unknown and awaits testing in a background completely lacking in histidine kinase activity. It should be noted, however, that a residual level of ethylene responsiveness was still detected in a recently generated line that is null for both *ETR1* and *ERS1* (Qu and others 2007), indicating that at least a portion of ethylene signal output does not require histidine kinase activity and that subfamily-2 receptors can independently contribute to signaling.

Two studies have found subtle effects attributable to kinase activity upon ethylene responses in *Arabidopsis* seedlings. First, when a kinase-inactive mutant of ETR1 was examined in the triple mutant *etr1;etr2;ein4*, it was found to rescue the mutant phenotype but showed increased sensitivity to ethylene (Qu and Schaller 2004). Second, kinase activity may play a role in the ability of seedlings to recover normal growth following ethylene treatment. The growth rate of wild-type etiolated seedlings is inhibited when exposed to ethylene, but once ethylene is removed, the seedlings can return to their normal growth rate. The *etr1-7;ers1-2* double mutant is delayed in its ability to recover normal growth rate, and this can be rescued by introducing a wild-type version of ETR1 but not by a kinase-inactive version of ETR1 (Binder and others 2004b).

If receptors signal through a phosphorelay, then one would predict that mutations in the receiver domains would affect signaling. This is because after autophosphorylation on histidine, the phosphate is predicted to be transferred to the conserved aspartate residue in the receiver domain (Mason and Schaller 2005). Two studies point to roles for the receiver domains in signaling. First, although the constitutive response phenotype of the triple mutant *etr1;etr2;ein4* (which eliminates the three receptors with receiver domains) can be rescued by a truncated ETR1 lacking a receiver domain, the seedlings show enhanced ethylene sensitivity (Qu and Schaller 2004). Second, the receiver domain also plays a role in the ability of seedlings to recover normal growth following ethylene treatment. The *etr1;etr2;ein4* triple mutant, in which no ethylene receptors containing receiver domains are present, requires 4 h to recover normal growth following treatment and removal of ethylene, in contrast to wild-type plants that take 1.5 h (Binder and others 2004b). Introducing a wild-type copy of ETR1 rescues the growth phenotype in these mutants, but a mutant copy of ETR1 lacking a receiver domain does not. Again, these are subtle roles, but they are similar to what is found when examining kinase-inactive versions of the receptors and are thus suggestive that phosphorylation may play a role in signaling.

If a two-component system operates in ethylene signaling, then one would predict that phosphotransfer would next occur from the receptors to a histidine-containing phosphotransfer protein and from there to a response regulator. Thus, studies of these genes in *Arabidopsis* might reveal roles in

ethylene signaling. One study has pointed to a role for the response regulator ARR2 in signaling by the ethylene receptor ETR1 (Hass and others 2004). ARR2 incubated with cellular extracts from *Arabidopsis* can be phosphorylated *in vitro*, but this does not occur when extracts are obtained from an *etr1-7* loss-of-function background. In addition, initial analysis of an *arr2* mutant suggested a decreased response to ethylene (Hass and others 2004), but this result could not be confirmed in subsequent analysis (Mason and others 2005). Thus, the role of downstream two-component elements in ethylene signaling is still an open question.

An alternative to histidine kinase activity as a means of enzymatic regulation is the ser/thr kinase activity that has been detected *in vitro* for some members of the ethylene receptor family (Moussatche and Klee 2004; Xie and others 2003; Zhang and others 2004). This activity primarily is found associated with the subfamily-2 receptors that lack functional histidine-kinase domains, suggesting that these domains have evolved into kinases with new enzymatic function (Figure 1). In addition, the subfamily-1 receptor ERS1, which contains a conserved histidine kinase domain, was found to be bifunctional, with both histidine and ser/thr kinase activity detected (Moussatche and Klee 2004). It is tempting to speculate about how ser/thr kinase activity may mediate downstream signal transduction, but there are still some unresolved questions in this relatively new area of research. In particular, mutations predicted to eliminate kinase activity did not do so, suggesting either that the conserved residues targeted for mutagenesis are not required for catalysis or that a contaminating kinase activity was not removed during purification from the transgenic yeast expression system (Moussatche and Klee 2004; Zhang and others 2004). Another unusual but potential source of contaminating activity is the heat-shock protein Hsp70, which generally serves as a chaperone bound to unfolded regions of proteins. Hsp70 copurified with most of the receptors (Moussatche and Klee 2004) but is itself an ATPase thought to autophosphorylate on histidine as part of its reaction cycle (Hiromura and others 1998; Lu and others 2006); whether Hsp70 can transphosphorylate the receptors is not known. Thus, additional studies are needed to resolve the importance of the ser/thr kinase activity in signaling by the receptors.

Although the specific roles of enzymatic activity in ethylene signaling are still unclear, some basic conclusions can be made about signal output from the receptors. First, the histidine-kinase domain is essential to regulating signal output. Second, the receiver domain is not essential but does play a modulating role. Third, enzymatic activity, although not yet demonstrated to play a major role, appears to play at least a modulating role. It should be noted that the three mechanisms for signal output discussed in this section and the last (interaction with CTR1, histidine kinase activity, and ser/thr kinase activity) are not mutually exclusive and it is possible that multiple mechanisms are involved in signal output from the receptors. In addition, autophosphorylation of the receptors need not function only to regulate a downstream two-component signaling system but could, due to the physical association of CTR1 with the receptors, also potentially regulate CTR1 activity.

Genetic Interactions with Other Pathway Components

Studies of the ethylene receptors have revealed genetic interactions with additional signaling elements, including other ethylene receptors and the recently identified membrane protein RTE1, pointing to the possibility of a physical interaction. Several studies using dominant ethylene-insensitive mutant versions of the receptors suggest that receptors may have the ability to interact with each other. In an initial study, the surprising observation was made that a truncated version of the etr1-1 protein, lacking the signal output domain, was still able to confer dominant ethylene insensitivity (Gamble and others 2002). One potential explanation for this capability is that the truncated etr1-1 protein can convert full-length wild-type receptors to an ethylene-insensitive signaling state. This hypothesis is supported by recent experiments that made use of different receptor loss-of-function backgrounds, in which the ability of truncated etr1-1 to exhibit an effect was found to be primarily dependent on the presence of other subfamily-1 receptors (Xie and others 2006). In a separate set of experiments, it was found that the dominant ethylene-insensitive phenotype of *etr2-1* is partially dependent on the presence of *ETR1* (Cancel and Larsen 2002). Interactions of this type are not limited just to ethylene-insensitive mutant receptors, for it has also been found that a truncated version of the wild-type receptor ETR1 can also enhance signal output from the subfamily-1 receptors (Xie and others 2006).

The mechanism by which one receptor affects signaling from other family members has not yet been determined, but it could be mediated by

physical interaction. The potential for physical interaction among the receptors, in higher-order clusters than their dimeric functional unit, would be consistent with what is known about signaling by the histidine-kinase linked chemoreceptors of prokaryotes (Baker and others 2006). The signaling state of one bacterial chemoreceptor in a cluster can affect the signaling state of other chemoreceptors in the cluster, such that signal output from a single ligand binding event is amplified. Clustering has been proposed as one possible explanation for the ability of receptors to mediate sensing of ethylene across a wide concentration range (Binder and others 2004a).

Receptor function also appears to be modulated by action of a small membrane protein recently identified in two independent studies that converged upon a gene conserved in plants and other eukaryotes, identifying *REVERSION-TO-ETHYLENE-SENSITIVITY* (*RTE1*) in *Arabidopsis* and *GREEN-RIPE* (*Gr*) in tomato (Barry and Giovannoni 2006; Barry and others 2005; Resnick and others 2006). In *Arabidopsis*, *RTE1* was identified based on mutations that suppressed the dominant ethylene insensitivity conferred by the *etr1-2* mutation (Resnick and others 2006), a mutation that affects signal transduction but not ethylene binding in the receptor (Hall and others 1999). Interestingly, *rte1* appears fairly specific in its effect upon *etr1-2* because it does not revert the *etr1-1* or *ers1-10* ethylene-insensitive mutations (Resnick and others 2006). This specificity suggests that *RTE1* may regulate receptor signaling at the protein level rather than modulate transcription. Overexpression of *RTE1* in *Arabidopsis* and of *GR* in tomato results in reduced ethylene sensitivity, consistent with a role for *RTE1* as a negative regulator of ethylene signaling (Barry and Giovannoni 2006; Resnick and others 2006). The finding that RTE1 is a membrane protein is particularly intriguing given that the ethylene receptors are also membrane proteins.

A Model for Receptor Signaling

Genetic, molecular, and biochemical studies indicate that the initial steps in ethylene signal transduction involve both positive and negative regulation of the pathway. A general model for signaling by a single ethylene receptor is shown in Figure 3A. The ethylene receptor functions as part of a protein complex with CTR1 (Clark and others 1998; Gao and others 2003; Huang and others 2003). In the absence of ethylene (in air), the receptor maintains CTR1 in an active conformation so that the kinase domain of CTR1 actively represses ethylene responses (Gao and others 2003; Huang and others 2003; Kieber and others 1993). Binding of ethylene by the receptor induces a conformational change in the receptor, possibly involving a change in the receptor's kinase activity, and this is transmitted to CTR1. The conformational change in CTR1 reduces its own kinase activity, thereby relieving repression of the ethylene response pathway.

Any model for ethylene signaling must also take into account that there is not one receptor but several belonging to two subfamilies (Figure 3B). Genetic analysis of loss-of-function mutations involving the five ethylene receptors of *Arabidopsis* has shown that ethylene receptors are negative regulators and function redundantly in ethylene signaling (Hua and Meyerowitz 1998). Analysis of single mutants and higher-order mutant combinations supports a greater role for the subfamily-1 receptors (ETR1 and ERS1) in signaling than the subfamily-2 receptors (ETR2, ERS2, and EIN4). First, of the single loss-of-function mutants examined, only those of ETR1 and ERS1 demonstrate any difference from wild type in terms of their ethylene response, the mutants exhibiting slight increases in ethylene sensitivity (Cancel and Larsen 2002; Hua and Meyerowitz 1998; Qu and others 2007). Second, higher-order mutant combinations of subfamily 1 demonstrate stronger phenotypes than higher-order mutant combinations of subfamily 2. Specifically, the subfamily-1 double mutant (*etr1;ers1*) displays a strong constitutive ethylene-response phenotype (Hall and Bleecker 2003; Wang and others 2003; Xie and others 2006; Zhao and others 2002). In contrast, a triple mutant of the subfamily-2 receptors (*etr2;ers2;ein4*) is primarily distinguished by an increase in its ethylene sensitivity such that it exhibits a partial triple-response phenotype due to its responsiveness to endogenous ethylene levels in the seedling (Cancel and Larsen 2002). Third, the severe phenotype found in *etr1;ers1* double mutants can be rescued by expressing subfamily-1 but not subfamily-2 ethylene receptors, pointing to a unique role for the subfamily-1 receptors in mediating signal transduction (Wang and others 2003).

The greater role of the *Arabidopsis* subfamily-1 receptors in signaling does not seem to be due to differences in their ethylene binding ability or expression level compared with the subfamily 2 receptors (O'Malley and others 2005; Wang and others 2003). The difference is thus likely to reside within sequence-specific information found in their signal output domains and relates to their ability to

activate CTR1, the key component acting immediately downstream of the receptors. As discussed earlier, interactions with CTR1 do not appear to be purely stoichiometric and the level of output through CTR1 does not directly correspond to the expression level of CTR1. Thus, it may be that the subfamily-1 members are able to activate CTR1 better than are subfamily-2 members; this gives rise to their greater relative contribution to ethylene signal transduction (Figure 3B).

The studies in *Arabidopsis* suggest that on a per molecule basis, the subfamily-1 receptors are more effective in signaling than are subfamily-2 receptors. It remains to be seen if this is a general property of subfamily-1 and -2 receptors that applies to other plants outside of *Arabidopsis*. In tomato, for example, a reduction in the expression level of *LeETR4* (a gene for a subfamily-2 receptor) results in symptoms of increased ethylene sensitivity, including epinasty of petioles and leaves, premature senescence of flowers, accelerated fruit ripening, and enhanced ethylene sensitivity in a seedling growth assay (Tieman and others 2000). Thus, loss of a single subfamily-2 receptor in tomato, unlike the case in *Arabidopsis*, results in a phenotypic change. This clearly demonstrates a more pronounced role for the subfamily-2 receptors in tomato than that found in *Arabidopsis*, but this observation does not necessarily mean that the intrinsic properties of subfamily-1 and subfamily-2 receptors differ between tomato and *Arabidopsis*. For example, the subfamily-2 receptors may be expressed at a higher level in tomato than is found in *Arabidopsis*, which will result in an increase in their contribution to signaling (that is, reduced signal output on a per molecule basis may be made up for by increasing the expression level). In the tomato study the authors found that the loss of *LeETR4* (subfamily 2) could be compensated for by increasing the expression of *LeNR* (subfamily 1) (Tieman and others 2000), indicating that a subfamily-1 receptor can functionally compensate for the loss of a subfamily-2 receptor and pointing to the key role of expression levels in signal output from the receptors.

The model for signaling by the ethylene receptors indicates how loss-of-function and gain-of-function mutations in the receptors affect signal output, resulting in either a constitutive ethylene response or ethylene insensitivity (Figure 3B). Elimination of ethylene receptors through loss-of-function mutations results in a redistribution of CTR1 from the membrane to the cytosol (Gao and others 2003). Because there is a family of ethylene receptors, elimination of a single family member has little effect on redistribution of CTR1, but in double- and triple-receptor mutants, significant amounts of CTR1 are found in the cytosol instead of at the membrane. Such higher-order receptor mutants show a constitutive ethylene-response phenotype (Hua and Meyerowitz 1998) apparently because of this loss of CTR1 from the membrane. In the cytosol, CTR1 may adapt a kinase-inactive conformation or may not be proximate to the appropriate phosphorylation substrate. By way of contrast, a gain-of-function mutation such as that found in *etr1-1* either eliminates the ability of the receptor to bind ethylene or the ability of the receptor to transduce the information that it has bound ethylene (Wang and others 2006). In such a case, the CTR1 associated with the mutant receptor never becomes inactivated and thus continues to repress the ethylene response, even in the presence of ethylene, thereby resulting in a plant displaying ethylene insensitivity. The dominance of the mutation arises due to the negative regulation in the pathway, such that CTR1 needs to be inactivated to induce an ethylene response.

This model is obviously not the whole story and there are still unanswered questions about the mechanism of signal output, how receptors may modulate each other's ability to transduce the signal, and how the sensitivity to the ethylene signal may change in response to other cellular factors such as RTE1. In addition, the ethylene signal transduction system also requires a means for recovery and resensitization once ethylene levels decrease. Kinetic analysis indicates that *Arabidopsis* seedlings respond within minutes and return to normal growth rates within 90 min following removal of ethylene (Abeles and others 1992; Binder and others 2004b). This time of recovery is much faster than the rate of dissociation of ethylene from the receptors, the apparent half-life for dissociation of ethylene from ETR1 being at least 12 h (Schaller and Bleecker 1995). Thus, other mechanisms are needed for recovery besides simple diffusion of ligand away from receptor. A key to recovery is likely to be the receptors that are induced at the transcriptional level by ethylene (ERS1, ETR2, and ERS2 in *Arabidopsis*) (Hua and others 1998). If ethylene levels have decreased, the newly synthesized ''empty'' receptors will not bind ethylene. As a result they will activate CTR1, which will in turn suppress ethylene responses in the plant, even under conditions where other receptors still retain bound ligand. Thus, subsets of the receptors are likely to have functions not shared equally with other members of the family, and research in the future will undoubtedly clarify how differences

among the receptors are integrated into the dynamic process of ethylene signal transduction. Of particular interest will be the development and refinement of computational models for the ethylene signaling process. In a recent computational model (Diaz and Alvarez-Buylla 2006), the receptors are treated as functionally uniform, but the model still effectively demonstrates that the phenotypic effects of ethylene upon seedling growth can be modeled based on the activation through the pathway of ethylene-induced transcription factors such as ERF1.

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