Requirement of the Histidine Kinase Domain for Signal Transduction by the Ethylene Receptor ETR1[1]

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In Arabidopsis, ethylene is perceived by a receptor family consisting of five members, one of these being ETR1. The N-terminal half of ETR1 functions as a signal input domain. The C-terminal region of ETR1, consisting of a His kinase domain and a putative receiver domain, is likely to function in signal output. The role of the proposed signal output region in ethylene signaling was examined in planta. For this purpose, the ability of mutant versions of ETR1 to rescue the constitutive ethylene-response phenotype of the *etr1-6;etr2-3;ein4-4* triple loss-of-function mutant line was examined. A truncated version of ETR1 that lacks both the His kinase domain and the receiver domain failed to rescue the triple mutant phenotype. A truncated ETR1 receptor that lacks only the receiver domain restored normal growth to the triple mutant in air, but the transgenic seedlings displayed hypersensitivity to low doses of ethylene. A mutation that eliminated His kinase activity had a modest effect upon the ability of the receptor to repress ethylene responses in air. These results demonstrate that the His kinase domain plays a role in the repression of ethylene responses. The potential roles of the receiver domain and His kinase activity in ethylene signaling are discussed.

The gaseous hormone ethylene plays important roles throughout the plant life cycle (Mattoo and Suttle, 1991; Abeles et al., 1992). Ethylene regulates seed germination, seedling growth, leaf and petal abscission, organ senescence, ripening, and responses to stress and pathogens. Plants have developed a sophisticated signal perception and transduction system to control ethylene responses. In Arabidopsis, ethylene is perceived by a receptor family consisting of five members: ETR1, ETR2, ERS1, ERS2, and EIN4 (Chang and Stadler, 2001; Schaller and Kieber, 2002). ETR1 was the first member of the receptor family identified and has been characterized in the most detail (Chang et al., 1993; Schaller and Bleecker, 1995).

The ethylene receptor ETR1 has a modular structure. The N-terminal portion of ETR1 contains three predicted transmembrane segments that function as a signal input domain based on their ability to bind ethylene (Schaller and Bleecker, 1995; Rodriguez et al., 1999). The functional unit of the receptor appears to be a disulfide-linked dimer, there being one ethylene-binding site per receptor dimer (Rodriguez et al., 1999), with dimerization mediated by two Cys residues located near the N terminus (Schaller et al., 1995). Recent studies indicate that the transmembrane seg-

ments also serve in localization of the receptor to the endoplasmic reticulum (ER), an unusual location for a hormone receptor but one compatible with the diffusion of ethylene in aqueous and lipid environments (Chen et al., 2002). Following the transmembrane segments, ETR1 contains a GAF domain; GAF domains have been shown to mediate cGMP binding and light regulation in some proteins, but its function in ETR1 is unknown (Aravind and Ponting, 1997).

The C-terminal region of ETR1 is likely to be involved in signal output. It consists of a His kinase domain and a receiver domain (Chang et al., 1993). These domains are evolutionarily related to signal transducing elements originally identified in the two-component systems of bacteria, which are now known to also exist in plants, fungi, and slime molds (Schaller, 2000; Stock et al., 2000). His kinase activity of ETR1 has been demonstrated (Gamble et al., 1998), but the role of His kinase activity in ethylene signal transduction is still not clear (Gamble et al., 2002; Wang et al., 2003). It has also been demonstrated that the His kinase and receiver domains of ETR1 can interact with the Raf-like kinase CTR1 (Clark et al., 1998; Gao et al., 2003; Huang et al., 2003). CTR1 is a negative regulator acting downstream of the ethylene receptors in the ethylene signaling pathway (Kieber et al., 1993). These results suggest that, as part of an ethylene receptor signaling complex, activity of CTR1 could be modulated enzymatically and/or allosterically by ETR1.

Despite an overall similarity in protein structure among the five members of the Arabidopsis ethylene receptor family, each ethylene receptor has distinctive

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features. The receptors form two subfamilies based on phylogenetic analysis and some shared structural features (Bleecker, 1999; Schaller and Kieber, 2002). ETR1 and ERS1 belong to subfamily 1 and contain canonical His kinase domains (Chang et al., 1993; Hua et al., 1995). ETR2, ERS2, and EIN4 belong to subfamily 2 and contain diverged His kinase domains that lack residues considered essential for His kinase activity (Hua et al., 1998; Sakai et al., 1998). Each member of subfamily 2 also has an additional hydrophobic segment at the N terminus that is predicted to function as a cleaved signal sequence for targeting to the secretory pathway (Schaller and Kieber, 2002). Two of the receptors (ERS1 and ERS2) lack a receiver domain at the C terminus. These structural differences suggest that individual receptors could function differently in ethylene signaling.

Loss-of-function and hypomorphic mutant alleles of the receptors have been isolated and characterized for their effects upon ethylene signal transduction (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002; Zhao et al., 2002; Wang et al., 2003). Single loss-of-function mutations have little or no effect upon ethylene signal transduction. In combination, however, the mutants display constitutive ethylene responses. These results indicate that there is functional overlap among the ethylene receptors and that the receptors act as negative regulators of ethylene signal transduction. The *etr1;etr2;ein4* triple loss-of-function mutant, for example, displays a constitutive ethylene-response growth phenotype (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002); this phenotype occurs because the two remaining receptor members in this mutant background (ERS1 and ERS2) are insufficient for suppression of ethylene responses.

In this study, we used the *etr1;etr2;ein4* triple mutant as a genetic background to examine the ability of various *ETR1* mutants to rescue the constitutive ethylene-response phenotype found in the triple mutant. This analysis builds on a previous mutational analysis in which we focused on ethylene-insensitive mutations of *ETR1* (Gamble et al., 2002). Here we address the question as to what role the C-terminal domain of ETR1 plays in signal output. This question is of relevance to our understanding of ethylene signaling because prior genetic analysis has not uncovered a function for this proposed output domain. In one previous study, the ability of a dominant mutant *etr1-1* receptor to confer ethylene insensitivity was not affected by a mutation that eliminated kinase activity or by a truncation that eliminated the C-terminal half of the receptor (Gamble et al., 2002). In another previous study, a kinase-inactive version of ETR1 was able to restore normal growth and ethylene responsiveness to an *etr1;ers1* double mutant (Wang et al., 2003). Our results here demonstrate a role for the His kinase domain in ethylene signaling and lend insight into how the ethylene signal is transduced from ethylene receptors to downstream signaling components.

RESULTS

Addition of the Full-Length ETR1 Receptor Rescues the *etr1;etr2;ein4* Triple Mutant Phenotype

To study the in vivo effects of mutations in the ethylene receptor ETR1, we took advantage of the ethylene-induced "triple response" in seedlings (Knight et al., 1910). Ethylene has a profound impact upon the growth of dark-grown (etiolated) seedlings. In wild-type Arabidopsis, ethylene inhibits hypocotyl and root elongation, induces swelling of the hypocotyl, and leads to the formation of an exaggerated apical hook (Bleecker et al., 1988; Guzmán and Ecker, 1990). Although single loss-of-function mutations within members of the ethylene receptor family have little or no effect upon the triple response, mutant plants carrying combinations of receptor mutations display a triple response in the absence of ethylene (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002; Zhao et al., 2002; Wang et al., 2003). For instance, the *etr1-6;etr2-3;ein4-4* triple mutant exhibits a partial triple-response phenotype, including a stunted hypocotyl, in the absence of ethylene (Figs. 1A and 2A). We therefore used the *etr1-6;etr2-3;ein4-4* triple loss-of-function mutant line as a genetic background to characterize signal output by ETR1.

We first determined if wild-type *ETR1* could rescue the mutant phenotype of *etr1-6;etr2-3;ein4-4*. For this purpose, a genomic fragment that contained promoter and coding regions of ETR1 was cloned into a plant transformation vector and used to transform the triple mutant. Three independent lines homozygous for the transgene were isolated and characterized further. Initial analysis indicated that instead of displaying a constitutive ethylene-response phenotype, the transformed plants grew similarly to wild-type seedlings in air (Fig. 2A). Expression of the full-length ETR1 protein (ETR1-FL) in these transgenic lines was confirmed by immunoblot analysis, using membrane proteins isolated from 4-d-old etiolated seedlings. Protein expression levels of ETR1-FL in these transgenic lines varied from 2- to 3-fold higher than endogenous ETR1 in wild-type seedlings (Fig. 2B).

A quantitative analysis of ethylene responsiveness was performed on the transgenic lines. Seedlings were grown in the dark in ethylene at concentrations ranging from 0 to 1,000 μL L^{-1} and the hypocotyl lengths measured after 4 d growth. As shown in Figure 2C, all three transgenic lines exhibited similar responsiveness to ethylene as the wild-type seedlings, indicating that addition of the full-length ETR1 receptor fully restored ethylene responsiveness to the *etr1-6;etr2-3;ein4-4* mutant line.

These data indicate that the *etr1;etr2;ein4* line can be used as a testing platform to directly assay which part of the ethylene receptor ETR1 is crucial for signal output (Fig. 1A). In the subsequent experiments, we describe data obtained by adding mutant versions of the ETR1 receptor into this background and then
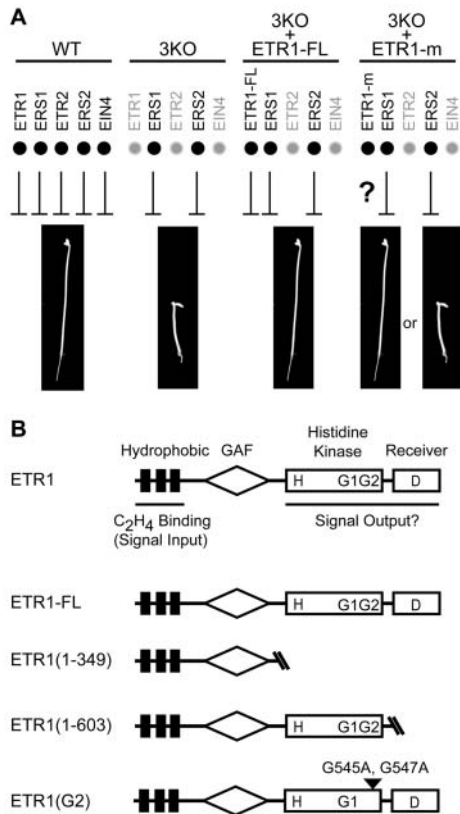
Figure 1. Experimental strategy and constructs used for analysis. A, Effect of receptor number on the repression of ethylene responses. In wild-type plants, all five ethylene receptors serve to repress ethylene responses. In the *etr1;etr2;ein4* triple mutant (3KO), the remaining two receptors (ERS1 and ERS2) are not sufficient to repress ethylene responses, and dark-grown seedlings show a constitutive ethylene-response phenotype. Transgenic expression of full-length ETR1 (ETR1-FL) in the *etr1;etr2;ein4* background should rescue the mutant phenotype. Other modified versions of ETR1 (ETR1-m) can then be tested to determine if they rescue the mutant phenotype. B, Structure of ETR1 and constructs used for analysis. The hydrophobic ethylene-sensing domain, the GAF domain, the His kinase domain, and the receiver domain are indicated. H indicates His-353 and D indicates Asp-659, the putative phosphorylation sites. G1 and G2 indicate positions of the G1 and G2 boxes within the kinase domain. The ETR1(1-349), ETR1(1-603), and ETR1(G2) constructs are all modified versions of ETR1 (ETR1-m).

analyzing their ability to rescue the triple mutant phenotype.

The C-Terminal Half of the Ethylene Receptor ETR1 Is Required for Ethylene Signaling

The proposed signal output region, including His kinase domain and receiver domain, is located in the C-terminal half of the ETR1 receptor (Fig. 1B). To address whether the proposed signal output region of ETR1 is indeed involved in ethylene signaling, a truncated version of the receptor, named ETR1(1-349), was generated (Gamble et al., 2002). *ETR1(1-349)* is a genomic fragment of *ETR1*, driven by the native promoter,

that encodes the N-terminal half of the receptor and lacks sequences encoding the His kinase and receiver domains (Fig. 1B). ETR1(1-349) localizes to the ER, like full-length wild-type ETR1, indicating that the truncation does not result in mislocalization of the receptor (Chen et al., 2002).

As shown in Figure 3A, homozygous transgenic lines carrying *ETR1(1-349)* in the *etr1;etr2;ein4* triple mutant background still exhibited the partial triple-response phenotype in the absence of ethylene. These results are in contrast to those obtained with *ETR1-FL*. To determine whether the failure of *ETR1(1-349)* to rescue the triple mutant phenotype arises from an absence of the ETR1(1-349) protein or from a truncated receptor incapable of signaling, membrane proteins were isolated and protein expression of ETR1(1-349) was determined by immunoblot analysis (Fig. 3B). ETR1(1-349) was detected by the anti-ETR1(165-400) antibody at the expected molecular mass of 36 kD for the monomer and at 68 kD for the disulfide-linked
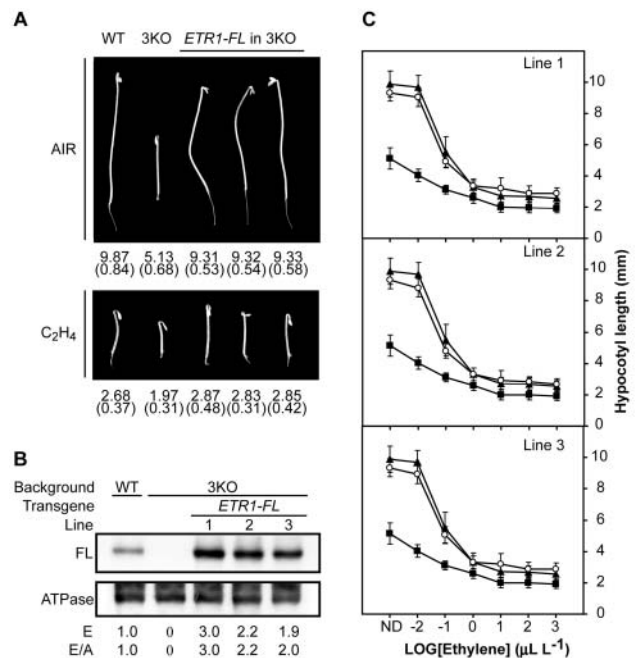

Figure 2. Effect of the ETR1-FL receptor upon the triple response of *etr1-6;etr2-3;ein4-4* dark-grown seedlings. *ETR1-FL* was transformed into the triple mutant line and three independent transgenic lines compared to wild-type and the triple mutant (3KO). A, Phenotypes of 4-d-old seedlings grown in the absence of ethylene (air) or in the presence of 100 μL L^{-1} ethylene. Mean hypocotyl length is given in mm based on measurement of at least 25 seedlings with SD in parentheses. B, Protein expression of ETR1-FL based on immunoblot analysis using the anti-ETR1(401-738) antibody. The relative expression level of full-length immunodetectable receptor (FL) for each plant line was quantified densitometrically (E) and also normalized against the ATPase control (E/A). C, Ethylene dose-response curves of hypocotyl growth for the three lines of ETR1-FL (white circles). For comparison, ethylene dose-response curves are shown for wild type (black triangles) and 3KO (black squares). Values represent the means $\pm$ SD of at least 25 measurements. ND, No detectable ethylene.

Figure 3. Effect of the ETR1(1-349) mutant receptor upon the triple response. *ETR1(1-349)* was transformed into the triple mutant line (3KO) and three independent transgenic lines analyzed. A, Phenotypes of 4-d-old seedlings grown in the absence of ethylene (air) or in the presence of 100 μL L^{-1} ethylene. Mean hypocotyl length is given in mm based on measurement of at least 25 seedlings with SD in parentheses. B, Protein expression of ETR1(1-349) based on immunoblot analysis using the anti-ETR1(165-400) antibody. In the absence of reducing agent, the truncated receptor migrates as the 68-kD homodimer. In the presence of the reducing agent DTT, the truncated receptor migrates as a 36-kD monomer. C, Ethylene dose-response curves of hypocotyl growth for the three lines of ETR1(1-349); white circles) compared to wild type (black triangles) and 3KO (black squares). Values represent the means $\pm$ SD of at least 25 measurements. ND, No detectable ethylene.
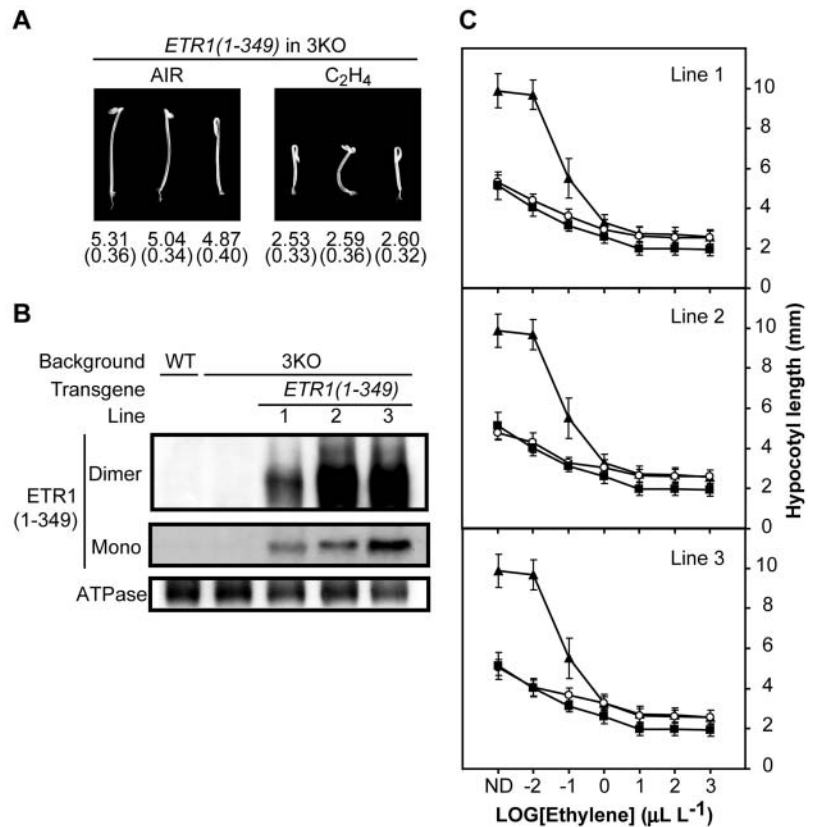
dimer (Gamble et al., 2002). Under the immunoblot exposures where the ETR1(1-349) protein was readily detected, we did not detect full-length ETR1 in the wild-type control, indicating that ETR1(1-349) is expressed at substantially higher levels than wild-type ETR1. Thus, the inability of the transgene to rescue the triple mutant is due to an inability of the truncated receptor to signal properly.

A dose-response curve using dark-grown seedlings confirmed the similarity of the transgenic lines to the triple mutant control when grown in air or in low levels of ethylene (Fig. 3C). Interestingly, in the presence of increased levels of ethylene (1–1,000 μL L^{-1}), the hypocotyl lengths of the transgenic lines were significantly longer than those of the triple mutant ($P < 0.0003$, $P < 6.78 \times 10^{-6}$, $P < 5.10 \times 10^{-6}$, and $P < 8.83 \times 10^{-6}$, for 1–1,000 μL L^{-1} ethylene, respectively; as judged by Student's t test; Fig. 3C). Over the same ethylene concentration range, hypocotyls of the transgenic lines were not significantly different in length from those of wild-type plants ($P \geq 0.351$, $P \geq 0.401$, $P \geq 0.202$, and $P \geq 0.760$, respectively). Furthermore, when grown in light, rosettes of the triple mutant containing the *ETR1(1-349)* transgene were slightly larger than those of the triple mutant itself, although still substantially smaller than the triple mutant lines containing *ETR1-FL*, which were wild type in appearance (results not shown).

Based on these results, we concluded that the truncated ETR1(1-349) receptor was incapable of rescuing the constitutive ethylene-response phenotype of the *etr1;etr2;ein4* mutant observed with dark-grown seedlings in air. The truncated receptor, however, still rescued the subtle growth effect observed at higher ethylene concentrations with the dark-grown seedlings and partially rescued the growth of the triple mutant when grown in the light. These data indicate that the C-terminal half of the receptor, containing His kinase and receiver domains, is required for ethylene signaling in Arabidopsis. These data also point to a role for the N-terminal half of the receptor in mediating some growth responses in the plant.

Effect of the Receiver Domain of ETR1 on Rescue of the *etr1;etr2;ein4* Triple Mutant Phenotype

ETR1, ETR2, and EIN4 are the only members in the Arabidopsis ethylene receptor family that possess a receiver domain, and thus there are no ethylene receptors with receiver domains in the *etr1;etr2;ein4* triple mutant. To further assess the role of the C-terminal half of ETR1 in ethylene responses, another truncated version of the receptor, named *ETR1(1-603)*, was generated and transformed into the *etr1;etr2;ein4* background. *ETR1(1-603)* is a genomic fragment of *ETR1*, driven by its native promoter, that encodes

a truncated ETR1 receptor that lacks the receiver domain (Fig. 1B).

Homozygous lines carrying the *ETR1(1-603)* transgene in the *etr1-6;etr2-3;ein4-4* mutant background were isolated and characterized. As shown in Figure 4A, dark-grown seedlings for all three transgenic lines display a wild-type-like growth phenotype in air. When grown in light, the transgenic *ETR1(1-603)* lines produced rosettes similar in size to those of transgenic *ETR1-FL* lines (results not shown). The presence of the truncated ETR1(1-603) protein in the triple mutant transgenic lines was confirmed by immunoblot analysis with the anti-ETR1(165-400) antibody (Fig. 4B), which recognized a 63-kD polypeptide consistent with the predicted molecular mass of 65 kD. This polypeptide was also recognized by the anti-ETR1(401-738) antibody (results not shown). Based on immunoblot analysis, the expression of ETR1(1-603) in the transgenic lines varied between 1.8- and 3.5-fold that found for full-length ETR1 in the wild-type control. Thus, the receiver domain is not required for ETR1 to repress ethylene responses in the air.

Despite their normal seedling growth response in air, all of the *ETR1(1-603)* transgenic lines exhibited hypersensitivity to low doses of ethylene but not to higher doses (Fig. 4C). For example, as the ethylene concentration was increased from 0 to 0.01 μL L^{-1}

ethylene, hypocotyl length of the *ETR1(1-603)* transgenic seedlings decreased from 9.8 to 6.5 mm, whereas wild-type seedlings showed little change in hypocotyl length. It is this hypersensitivity to low ethylene concentrations that accounts for the difference between the dose-response curves for the *ETR1(1-603)* transgenic seedlings and the wild-type control.

Effect of His Kinase Activity of ETR1 on Rescue of the *etr1;etr2;ein4* Triple Mutant Phenotype

The previous experiments demonstrated that the His kinase domain of ETR1 is required for rescue of the triple mutant phenotype. To determine if His kinase activity of ETR1 is required for rescue, we used a site-directed mutant of ETR1 containing a lesion in the G2 box (Fig. 1C). Mutations in the G2 box (G545A and G547A) affect the ability of ATP to bind to the kinase and thus abolish His kinase activity of ETR1 (Bilwes et al., 1998; Gamble et al., 2002). *ETR1(G2)* was transformed into the *etr1-6;etr2-3;ein4-4* mutant to determine whether, without its kinase activity, the full-length receptor could still rescue the constitutive ethylene-response phenotype of the mutant line.

As shown in Figure 5A, dark-grown seedlings from all three homozygous transgenic lines containing the *ETR1(G2)* transgene displayed normal growth in air
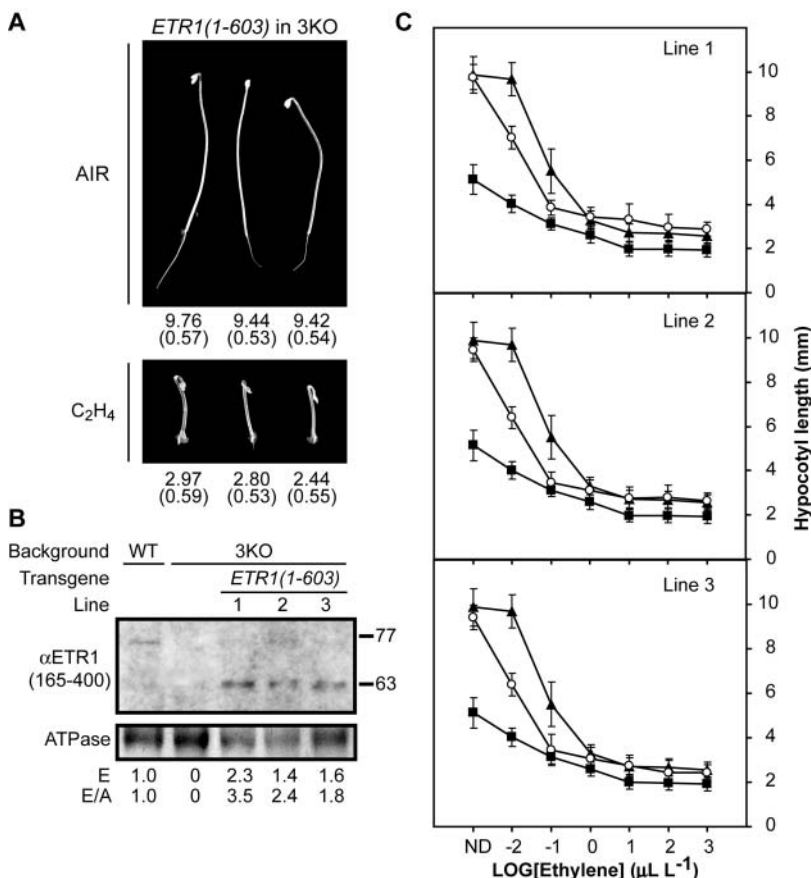

Figure 4. Effect of the ETR1(1-603) mutant receptor upon the triple response. *ETR1(1-603)* was transformed into the triple mutant line (3KO) and three independent transgenic lines analyzed. A, Phenotypes of 4-d-old seedlings grown in the absence of ethylene (air) or in the presence of 100 μL L^{-1} ethylene. Mean hypocotyl length is given in mm based on measurement of at least 25 seedlings with SD in parentheses. B, Protein expression of ETR1(1-603) based on immunoblot analysis using the anti-ETR1(165-400) antibody. The wild-type receptor migrated at 77 kD and the truncated receptor at 63 kD. The relative expression level of immunodetectable receptor for each plant line was quantified densitometrically (E) and also normalized against the ATPase control (E/A). C, Ethylene dose-response curves of hypocotyl growth for the three lines of ETR1(1-603; white circles) compared to wild type (black triangles) and 3KO (black squares). Values represent the means $\pm$ SD of at least 25 measurements. ND, No detectable ethylene.

Figure 5. Effect of the ETR1(G2) mutant receptor upon the triple response. *ETR1(G2)* was transformed into the triple mutant line (3KO) and three independent transgenic lines analyzed. A, Phenotypes of 4-d-old seedlings grown in the absence of ethylene (air) or in the presence of 100 μL L^{-1} ethylene. Mean hypocotyl length is given in mm based on measurement of at least 25 seedlings with SD in parentheses. B, Protein expression of ETR1(G2) based on immunoblot analysis using the anti-ETR1(401-738) antibody. The relative expression level of full-length immunodetectable receptor (FL) for each plant line was quantified densitometrically (E) and also normalized against the ATPase control (E/A). C, Ethylene dose-response curves of hypocotyl growth for the three lines of ETR1(1-603; white circles) compared to wild type (black triangles) and 3KO (black squares). Values represent the means ± SD of at least 25 measurements. ND, No detectable ethylene.
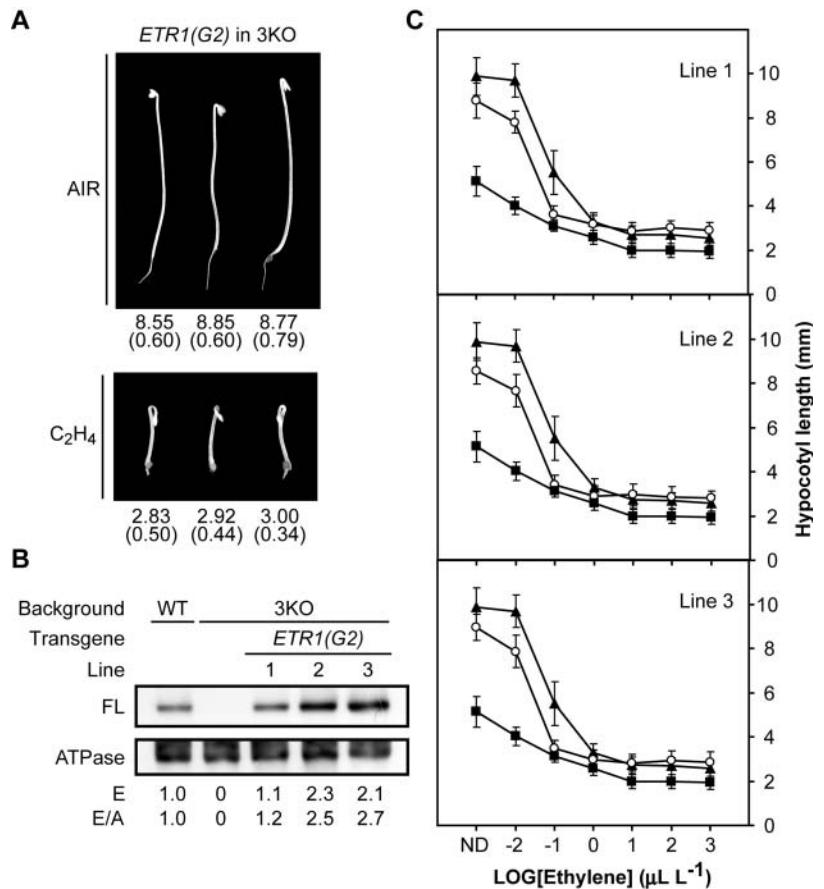
with straightened apical hooks and elongated hypocotyls and roots. When grown in light, the *ETR1(G2)* transgenic lines produced rosettes similar in size to those of *ETR1-FL* transgenic lines (results not shown). Immunoblot analysis confirmed that the transgene was expressed in the transgenic lines. Based on this analysis, the expression of ETR1(G2) in the transgenic lines varied between 1.2- and 2.7-fold that found for ETR1 in the wild-type control (Fig. 5B).

To further assess the effect of the *ETR1(G2)* transgene in conferring ethylene responses, a quantitative analysis of the ethylene dose response was performed (Fig. 5C). This analysis revealed that the hypocotyl length of the transgenic seedlings was slightly shorter than that of the wild-type controls in air (ND for no detectable ethylene in Fig. 5C). The 1- to 1.3-mm differences in hypocotyl length between the *ETR1(G2)* transgenic lines and the wild-type control in air were significant ($P \leq 0.0004$, as judged by Student's *t* test). The protein levels of ETR1(G2) were equal to or greater than those observed with ETR1 in the wild-type control (Fig. 5B), and thus the inefficiency in completely restoring hypocotyl elongation by the mutant ETR1(G2) receptor was not due to low receptor levels. This result with the *ETR1(G2)* transgenic lines contrasts with that observed with the *ETR1(1-603)* transgenic lines in which there was no difference between

the transgenic lines and the wild-type control when analyzed in air (ND for no detectable ethylene in Fig. 4C). Dose-response analysis also revealed that, in comparison with the wild-type control, the *ETR1(G2)* transgenic seedlings displayed slightly increased ethylene sensitivity (Fig. 5C) but not the pronounced hypersensitivity found with the *ETR1(1-603)* transgenic lines (compare the change in response that occurs between 0 and 0.01 μL/L ethylene in Figs. 4C and 5C). The difference in the dose-response curves between the *ETR1(G2)* transgenic lines and the wild-type control is primarily accounted for by the inability of the ETR1(G2) transgene to completely rescue the triple mutant phenotype in the absence of ethylene.

DISCUSSION

The function of ethylene receptors in ethylene signal transduction can be considered in terms of their roles in (1) the repression of ethylene responses in the absence of ethylene (in air), and (2) the establishment of ethylene responses in the presence of ethylene. The ethylene receptors do not act alone to regulate ethylene signal transduction, and both genetic and biochemical evidence supports a physical association between ethylene receptors and CTR1 (Clark et al.,

1998; Cancel and Larsen, 2002; Gao et al., 2003). CTR1 is a Raf-like kinase that acts as a negative regulator of ethylene signaling, with loss-of-function mutations in CTR1 resulting in a constitutive ethylene response (Kieber et al., 1993; Huang et al., 2003). According to the current model, ethylene receptor/CTR1 signaling complexes are localized to the ER membrane. In air, the kinase domain of CTR1 actively represses ethylene responses. Binding of ethylene by the receptor leads to a conformational change in CTR1 that reduces its kinase activity, thereby relieving repression of the ethylene-response pathway. Higher order ethylene receptor loss-of-function mutations result in a constitutive ethylene-response phenotype (Hua and Meyerowitz, 1998), apparently due to the loss of CTR1 from the ER membrane (Gao et al., 2003). Thus, the role of ethylene receptors in air may be achieved by maintaining both the activity and the correct location for action of CTR1, this occurring via their association with CTR1 within the same protein complex. The role of the receptors in establishing ethylene responses upon binding ethylene may be achieved by down-regulating the kinase activity of CTR1, a process that could involve enzymatic and/or allosteric mechanisms.

In this study, we used a triple mutant lacking three members of the ethylene receptor family and analyzed the ability of various mutant forms of ETR1 to rescue the constitutive ethylene-response phenotype found in the triple mutant, focusing on the triple response shown by dark-grown seedlings in response to ethylene. By performing ethylene dose-response analysis on the transgenic lines, we were able to detect subtle deviations from the wild-type ethylene response. Importantly, we observed virtually identical ethylene dose-response curves for multiple independent transgenic lines. The reproducibility of these dose-response curves, despite varying expression levels of the transgenic receptors, indicates that the observed perturbations in ethylene signal transduction are most likely due to the mutations introduced into ETR1.

Analysis of the truncated ETR1(1-349) and ETR1(1-603) receptors in the *etr1;etr2;ein4* triple loss-of-function mutant background indicates that the His kinase domain is needed for the role of the receptor in repressing ethylene responses in air. The truncated ETR1(1-349) receptor lacking both His kinase and receiver domains failed to rescue the triple mutant phenotype of dark-grown seedlings in air. In contrast, a truncated ETR1(1-603) receptor lacking only the receiver domain restored normal growth to the triple mutant in air. This result is consistent with a role for the His kinase domain in activation of CTR1. Previous studies indicate that CTR1 can physically associate with the His kinase domain of ETR1 (Clark et al., 1998; Gao et al., 2003) and that the interaction between CTR1 and ethylene receptors is required for the ability of CTR1 to repress ethylene responses. The *ctr1-8* mutant contains a missense mutation that disrupts the interaction of CTR1 with ethylene receptors, resulting in

mislocalization of CTR1 to the cytosol and a constitutive ethylene-response phenotype (Gao et al., 2003; Huang et al., 2003). Our data suggest that the His kinase domain of ETR1 may help recruit CTR1 to its site of action. Although His kinase activity of ETR1 is not required for the interaction between ETR1 and CTR1 (Gao et al., 2003), this does not preclude the possibility that the enzymatic activity of ETR1 may play a role in regulating the kinase activity of CTR1.

Although the truncated ETR1(1-603) receptor is able to repress ethylene responses in air, the transgenic seedlings display hypersensitivity to ethylene, suggesting an involvement of the receiver domain in the establishment of ethylene responses. One possible mechanism, based on the ability of the receiver domain of ETR1 to interact with CTR1 (Clark et al., 1998), is that the truncated ETR1(1-603) receptor may not be as effective as the wild-type receptor at maintaining CTR1 in an active state. This could increase the sensitivity of the seedlings to ethylene because CTR1 would be less effective at repressing the ethylene responses. Alternatively, the receiver domain could modulate activity of other potential downstream targets such as CTR1-like proteins (Tang and Innes, 2002; Huang et al., 2003) and/or Arabidopsis His-containing phosphotransfer proteins (Urao et al., 2000). Regulation of the receiver domain activity could potentially be elicited by conformational changes, resulting from binding of ethylene to the receptor, or by phosphorylation occurring at the Asp residue within the receiver domain.

Even though the ethylene responses of the triple mutant carrying ETR1(1-603) were perturbed, these transgenic lines still repressed the ethylene response in air and established the response in ethylene. The active receptors in these transgenic lines would include ERS1, ERS2, and ETR1(1-603), none of which contain a receiver domain. Thus, analysis of these transgenic lines suggests that a multistep phosphorelay is not required for ethylene signaling, although it could potentially modulate the signaling kinetics. In a multistep phosphorelay, signal transduction involves phosphorylation upon the conserved His in the His kinase domain of the receptor, subsequent transfer of the phosphate to the receiver domain of the receptor, then phosphotransfer to a separate His-containing phosphotransfer protein, then finally phosphotransfer to the receiver domain of a response regulator (Schaller et al., 2002). It might still be possible, however, for phosphotransfer to occur directly from the His kinase domain of the receptor to the receiver domain of a separate response regulator.

Previous genetic analyses have not revealed a role for the His-kinase activity of ETR1 in ethylene signal transduction (Gamble et al., 2002; Wang et al., 2003). Our results are suggestive that the kinase activity of ETR1 may play a role in modulating ethylene signal transduction, because the full-length kinase-inactive ETR1(G2) receptor only partially rescued the *etr1;etr2;ein4* triple loss-of-function mutant phenotype.

Dose-response curves indicate that the primary perturbation of ETR1(G2) transgenic seedlings is that they are shorter than wild-type seedlings in air, although a slightly increased sensitivity to ethylene is also observed.

The inability of ETR1(G2) to fully rescue growth of the triple mutant in air can be accounted for by two general models that are not mutually exclusive, one based on repression and the other upon hypersensitivity. In the first model, ETR1(G2) is not as effective as the wild-type receptor in the repression of ethylene responses. Repression could occur by several mechanisms. For example, because ETR1 interacts directly with CTR1 (Clark et al., 1998; Gao et al., 2003), it is possible that the His kinase activity of ETR1 may modulate CTR1 activity, thereby affecting its ability to repress ethylene responses. It is, however, also possible that the G2 mutation itself could physically affect the interaction between CTR1 and the receptor such that ETR1(G2) is less effective at activating CTR1. Alternatively, the mutant phenotype of the transgenic seedlings could arise from a partial dependence of ETR1 on phosphorylation as part of a two-component signaling system involving a His to Asp phosphorelay mechanism. In the second model, the ETR1(G2) plants are hypersensitive to very low concentrations of ethylene. We included the ethylene biosynthesis inhibitor aminoethylvinyl-Gly in all assays, but this does not preclude the production of ethylene below readily detectable levels. This model would imply the existence of a mechanism for sensing very low ethylene concentrations, below those normally associated with the triple response, because the ETR1(G2) transgenic seedlings did not show pronounced hypersensitivity to ethylene between 0.01 and 1,000 μL L^{-1} ethylene.

Our ability to detect an effect of the kinase-deficient ETR1(G2) on signaling likely arises because of the genetic background employed in these studies. Other studies in which the kinase activity of ETR1 was shown to be dispensable for signaling used as a genetic background either wild type, a single loss-of-function etr1 mutant (Gamble et al., 2002), or a double etr1;ers1 mutant (Wang et al., 2003). None of these backgrounds has as pronounced an effect upon the dark-grown growth of seedlings as does the triple mutant etr1;etr2;ein4. It should be noted that the triple mutant background we employed still contains the ethylene receptor ERS1, which, like ETR1, contains all the conserved residues required for His kinase activity (Gamble et al., 1998; Schaller and Kieber, 2002). It is also possible that the ers1-2 mutation, previously used in an etr1;ers1 background to study the effect of a kinase-deficient version of ETR1 on signaling (Wang et al., 2003), is a hypomorphic allele rather than a complete loss of function. The ers1-2 mutant still produces transcript and, although this transcript contains several additional ATGs in the 5'-untranslated region, the wild-type gene itself contains two ATGs in the 5'-untranslated region, indicating that the upstream ATGs in ers1-2 may still allow for translation

and production of the ERS1 receptor (Gamble et al., 2002; Wang et al., 2003). Thus, all studies on the role of His kinase activity in signaling, including this one, may have been hampered by residual activity from ERS1. Further studies will be required to determine if the His kinase activity of ETR1 plays a larger role in signaling than that found here.

It has been previously reported that, besides a shift in ethylene sensitivity, the etr1 loss-of-function mutations also lead to enhanced responsiveness to ethylene; mutant seedlings display an exaggerated reduction in hypocotyl length in comparison with wild type (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002). This exaggerated reduction in hypocotyl length is also seen in higher order mutants such as the etr1;etr2;ein4 triple mutant that we used in our studies. Interestingly, we found that the exaggerated ethylene response was fully reversed by transformation of the triple mutant line with all the mutant versions of ETR1 that we tested, including the truncated receptor ETR1(1-349; Fig. 3C). Thus, all the constructs can rescue a phenotype found in the triple mutant at higher concentrations of ethylene. In addition, when grown in light, rosettes of the triple mutant containing the ETR1 (1-349) transgene were slightly larger than those of the triple mutant itself, although still substantially smaller than the triple mutant lines containing ETR1-FL, ETR1(1-603), and ETR1(G2), which were wild type in appearance (results not shown). These data suggest that the N-terminal half of the receptor may play some role in modulating plant growth that does not require signal output through the C-terminal domain. Such an effect could be mediated by the GAF domain, which was present in all constructs tested, and for which no function has been yet determined. Alternatively, it could be mediated by the transmembrane ethylene-binding domain that was also present in all constructs, potentially through interactions with other membrane proteins.

etr1-1 is a dominant ethylene-insensitive mutant allele of ETR1 (Chang et al., 1993). Previously, we found that a truncated version of etr1-1 lacking the His kinase domain, etr1-1(1-349), still conferred dominant ethylene insensitivity in both the wild-type and the etr1-7 loss-of-function mutant background (Gamble et al., 2002). Thus, etr1-1(1-349) is able to repress ethylene responses even though it lacks the C-terminal half. We proposed two models that could account for this ability: (1) the etr1-1(1-349) receptor might be directly capable of signal output; or (2) the truncated etr1-1(1-349) receptor might be incapable of signal output, but be able to "convert" other wild-type receptors to an ethylene-insensitive signaling state. Our data here demonstrate that the N-terminal half of the receptor ETR1 is not sufficient for the repression of ethylene responses in air, a result that lends support to the second of the proposed models. On the other hand, we also found a possible role for the N-terminal half of ETR1 in modulating plant responsiveness to higher concentrations of ethylene, which thus leaves open the

possibility that the N-terminal half of the receptor may be capable of some signal output independent of the C-terminal half.

In summary, the results described here establish the importance of the proposed signal output region of ETR1 in ethylene signaling. The His kinase domain of the receptor is required for repression of ethylene responses in dark-grown seedlings. The receiver domain is not required for repression of ethylene responses in air but may play a regulatory role in the establishment of ethylene responses. The role of these domains in modulating ethylene signaling is likely to involve their interaction with the Raf-like kinase CTR1 with which the ethylene receptor ETR1 forms a signaling complex (Clark et al., 1998; Gao et al., 2003). Although these data confirm a role for the His kinase domain of ETR1 in ethylene signaling, they cannot fully resolve the question as to what role enzymatic activity of this domain may play. The mutant background used for these experiments still contains *ERS1*, another member of ethylene receptor subfamily 1, which is predicted to contain His kinase activity. Further analysis of mutant versions of ETR1 in different mutant backgrounds should resolve the role(s) of kinase activity and phosphorylation in ethylene signal transduction.

MATERIALS AND METHODS

Plasmid Constructions and Plant Transformation

All *ETR1* constructs were driven by their native genomic promoter. *ETR1-FL*, *ETR1(1-349)*, and *ETR1(1-603)* were amplified by using PCR from a 7.3-kb genomic *ETR1* fragment (Chang et al., 1993) with the forward primer 5′-ATGCTCATGATCTGTCTACGCTACG-3′ and the reverse primers 5′-GTCGACCCTTTACATGCCCTCGTA-3′, 5′-GTCGACTTAAACCGCTAGGAAATCATTG-3′, and 5′-GTCGACTTATCCAGTGAAATTTGAATGTC-3′, respectively. The PCR products were cloned into the *Bam*HI and *Sal*I sites of the binary vector pCAMBIA1380 (GenBank accession no. AF234301). Construction of *ETR1(G2)* has been previously described (Gamble et al., 2002). Constructs were introduced into *Agrobacterium tumefacians* strain GV3101 and used to transform the *etr1-6;etr2-3;ein4-4* triple mutant (Hua and Meyerowitz, 1998) by the floral-dip method (Clough and Bent, 1998). Independent homozygous lines for each transformation were obtained based on segregation of the acquired antibiotic resistance: kanamycin for the *ETR1(G2)* construct and hygromycin for the other constructs.

Seedling Growth-Response Assays

To examine the triple response of seedlings to ethylene (Chen and Bleecker, 1995; Gamble et al., 2002), seeds were grown on petri dishes containing one-half-strength Murashige and Skoog basal media with Gamborg's vitamins (pH 5.75; Sigma, St. Louis) and 0.8% (w/v) agar. Aminoethylvinyl-Gly (5 μM) was included in the growth media to inhibit ethylene biosynthesis by the seedlings. After a 2-d cold treatment at 4°C, plates were brought to 22°C and exposed to light for 10 h. Plates were then placed in 4-L chambers and seedlings grown in the dark in the presence of ethylene at the desired concentration. To examine the growth of seedlings in the absence of ethylene, hydrocarbon-free air was passed through the chamber to remove trace amounts of ethylene synthesized by the seedlings. Seedlings were examined after 4 d, time 0 corresponding to when the plates were removed from 4°C and brought to 22°C. To measure hypocotyl length, seedlings were grown on vertically oriented plates. Seedlings on the plates were scanned using Photoshop (Adobe Systems, Mountain View, CA) and an Epson 1240U scanner and measurements made using NIH Image version 1.6 (developed at the United States National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

Membrane Protein Isolation

To isolate membrane proteins, etiolated seedlings were homogenized at 4°C in extraction buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, and 20% [v/v] glycerol) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg mL^{-1} pepstatin, 10 μg mL^{-1} leupeptin, and 10 μg mL^{-1} aprotinin as protease inhibitors. The homogenate was strained through Miracloth (Calbiochem-Novobiochem, San Diego) and then centrifuged at 8,000g for 15 min. The supernatant was centrifuged at 100,000g for 30 min and the membrane pellet then resuspended in resuspension buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 10% [v/v] glycerol) with protease inhibitors. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL), with bovine serum albumin as the protein standard.

Immunoblot Analysis

Proteins were resuspended in SDS-PAGE loading buffer with or without 100 mM dithiothreitol (DTT; Schaller et al., 1995). The reductant DTT was not included in the loading buffer when it was desired to preserve the disulfide-linked dimer of ETR1 (Schaller et al., 1995). Membrane proteins were incubated at 50°C for 1 h and then fractionated by SDS-PAGE using either 8% or 10% (w/v) polyacrylamide gels (Laemmli, 1970). After electrophoresis, proteins were electroblotted to Immobilon nylon membrane (Millipore, Bedford, MA). Two antibodies were used to visualize ETR1. The anti-ETR1(401-738) antibody was generated against the C-terminal half of ETR1 from amino acids 401 to 738 (Schaller et al., 1995). The anti-ETR1(165-400) antibody was generated against amino acids 165 through 400 of ETR1 (Schaller et al., 1995) and was used to identify truncated ETR1 receptors. The anti-ETR1(165-400) antibody was affinity purified as previously described (Gamble et al., 2002). Immunodecorated ETR1 was visualized by enhanced chemoluminescence detection according to the manufacturer (Pierce Chemical). Densitometric analysis of immunodecorated bands was performed using NIH Image version 1.6 after first scanning the exposed film. The relative expression level for ETR1 was quantified by comparison to a dilution series of ETR1.

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