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Ethylene Regulates Levels of Ethylene Receptor/CTR1 Signaling Complexes in *Arabidopsis thaliana*[*]

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Background: Plant responses to the hormone ethylene depend on ethylene receptors and the kinase CTR1.
Results: The receptors and CTR1 exist as signaling complexes whose levels change in response to ethylene.
Conclusion: A model incorporating transcriptional induction and ethylene-dependent turnover of receptor/CTR1 complexes is proposed.
Significance: Results presented here reconcile molecular responses at the receptor level with physiological changes in sensitivity to ethylene.

The plant hormone ethylene is perceived by a five-member family of receptors in *Arabidopsis thaliana*. The receptors function in conjunction with the Raf-like kinase CTR1 to negatively regulate ethylene signal transduction. CTR1 interacts with multiple members of the receptor family based on co-purification analysis, interacting more strongly with receptors containing a receiver domain. Levels of membrane-associated CTR1 vary in response to ethylene, doing so in a post-transcriptional manner that correlates with ethylene-mediated changes in levels of the ethylene receptors ERS1, ERS2, EIN4, and ETR2. Interactions between CTR1 and the receptor ETR1 protect ETR1 from ethylene-induced turnover. Kinetic and dose-response analyses support a model in which two opposing factors control levels of the ethylene receptor/CTR1 complexes. Ethylene stimulates the production of new complexes largely through transcriptional induction of the receptors. However, ethylene also induces turnover of receptors, such that levels of ethylene receptor/CTR1 complexes decrease at higher ethylene concentrations. Implications of this model for ethylene signaling are discussed.

The gaseous plant hormone ethylene (C_2H_4) regulates a variety of growth and developmental processes, including seed germination, seedling growth, leaf and petal abscission, fruit ripening, organ senescence, and pathogen responses (1). In *Arabidopsis thaliana*, ethylene is perceived by a five-member family of receptors composed of ETR1, ERS1, ETR2, ERS2, and EIN4 (2–5). 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, subfamily 2 being composed of ETR2, ERS2, and EIN4. Genetic analysis indicates that there is functional overlap among the receptors, but that the subfamily-1 receptors generally play the predominant role in ethylene signaling (6–8).

The ethylene receptors have a similar overall modular structure, each containing three conserved transmembrane domains near the N terminus, followed by a GAF domain, and then signal output motifs in the C-terminal half. The transmembrane domains contain the ethylene-binding site (9–11), and also serve in localization of the receptor to the endoplasmic reticulum (ER)[4] and possibly to the Golgi apparatus (12–15). The GAF domain has been implicated in protein-protein interactions among the receptors and may help mediate the formation of higher order receptor clusters (15–17). The signal output domains of the receptors are related to two-component signaling elements, with all five receptors containing histidine kinase-like domains and all except ERS1 and ERS2 also containing receiver domains. The subfamily-1 receptors have functional histidine-kinase domains (18, 19), but the subfamily-2 receptors lack the necessary residues for histidine-kinase activity and appear to function as serine/threonine kinases (19).

Truncation studies using ETR1 demonstrate the importance of the C-terminal half of the protein for signal output, but this importance is not primarily dependent on the enzymatic activity contained in the histidine-kinase domain (20–22). Instead the key role for the histidine-kinase domain appears to be as a docking site for the downstream Raf-like kinase CTR1. CTR1 is a negative regulator of the ethylene pathway, loss-of-function mutations in CTR1 resulting in constitutive ethylene responses, with phosphorylation of its substrates required to suppress ethylene responses (23–26). Several lines of evidence indicate that CTR1 can interact with ethylene receptors. First, CTR1 is found associated with the endoplasmic reticulum in an

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[4] The abbreviations used are: ER, endoplasmic reticulum; TAP, Tandem Affinity Purification; ACC, aminocyclopropane carboxylic acid.

ethylene receptor-dependent manner, mutations of ethylene receptors reducing the levels of membrane-associated CTR1 (27). Second, a physical association of CTR1 with the ethylene receptor ETR1 is supported by two-hybrid analysis, *in vitro* binding experiments, and co-purification analysis from *Arabidopsis* extracts (24, 27, 28). Studies on the interaction of CTR1 with other members of the receptor family are more limited than with ETR1, although weak interactions have been observed with ERS1 and ETR2 based on two-hybrid analysis (28, 29).

Current evidence suggests that members of the ethylene receptor family may be subject to transcriptional as well as post-transcriptional regulatory mechanisms (2, 13, 30). Post-transcriptional regulation of receptor levels encompasses any processes following transcription that affect the protein level and includes such processes as translational control and protein degradation. The extent that these mechanisms play in modulating receptor levels is largely unexplored, as is how such changes may affect interactions with CTR1. Here, we report on transcriptional and post-transcriptional effects of ethylene on the levels of CTR1 as well as on members of the ethylene receptor family. Our data support a model in which perception of ethylene results in the production of new ethylene receptor/CTR1 complexes largely through transcriptional induction of the receptors, but that ethylene also induces post-transcriptional turnover of receptors. As a result, the levels of ethylene receptor/CTR1 complexes decrease at higher ethylene concentrations. Our results also indicate that interactions between CTR1 and the ethylene receptor ETR1 may help protect ETR1 from ethylene-mediated turnover.

EXPERIMENTAL PROCEDURES

Constructs and Plant Transformation—The CTR1-Myc-Tap (CTR1-MT) construct has been previously described (27). For preparation of ERS1, ETR2, ERS2, and EIN4 with C-terminal Myc tags, the vector pCAMBIA1380-*Myc* was used. For this vector, the DNA encoding the Myc tag was cut from the vector 6-CMYC (*Arabidopsis* Biological Resource Center stock no. CD3–128) by SalI and HindIII, and cloned into pCAMBIA1380 (GenBank™ accession no. AF234301). The genomic region encoding the receptor along with upstream promoter sequence were amplified for *ERS1* from the *Arabidopsis* BAC clone T20B5 (GenBank™ accession no. AC002409) using primers 5′-GGATCCCAGGGATGTGCACTGAAG-3′ and 5′-GGATCCACCAGTTCCACGGTCTGG-3′, for *ERS2* from the *Arabidopsis* BAC clone F19P19 (GenBank accession no. AC000104) using primers 5′- GTCGACGGTAAGAGTCCACGTAGG-3′ and 5′-GTCGACAGTGGCTAGTAGACGGAG-3′) and for *EIN4* from the *Arabidopsis* BAC clone F7O18 (GenBank™ accession no. AC011437) using primers 5′-GTCGACGCTCTTCTCCGTTGTGGC-3′ and 5′-GTCGACACTCGCTCGCGGTCTGCA-3′. The *ERS1* PCR product was cloned into the BamHI site, while the *ERS2* and *EIN4* PCR products were cloned into the SalI site of pCAMBIA1380-*Myc*. The *ETR2* gene was constitutively expressed from the *ETR1* promoter, for which purpose the region containing the *ETR1* 5′-UTR along with 1-kb upstream promoter sequence was amplified from a 7.3-kb genomic clone of *ETR1* (31) using primers 5′-ACATGAGGATCCAGTGGTTCCAAC-3′ and 5′-GCAGACGTCGACTATGAATTTTTTACACTA-3′. The PCR product was cloned into the BamHI and SalI sites of pCAMBIA1380-*Myc*. The region encoding *ETR2* was then amplified from a cDNA clone using primers 5′-GTCGACATGGTTAAAGAAATAGCT-3′ and 5′-GTCGACAGAGAAGTTGGTCAGCTT-3′, and PCR product cloned into the SalI site.

For transformation into *Arabidopsis*, constructs were introduced into *Agrobacterium tumefacians* strain GV3101 and used to transform *Arabidopsis* by the floral-dip method (32). The CTR1-MT construct was transformed into the *ctr1–2* mutant line of *Arabidopsis* that contains a loss-of-function mutation in the *CTR1* gene (27). The Myc-tagged receptors were transformed into the transgenic line containing CTR1-MT to allow for co-immunoprecipitation analysis. In addition, to test for functionality of the Myc-tagged receptors, ETR2-Myc, ERS2-Myc, and EIN4-Myc constructs were transformed into the *etr2/ers2/ein4* triple mutant (6). The ERS1-Myc construct was transformed into the *ers1–3* mutant background (8).

Seedling Ethylene Treatment and Response—Treatment and analysis of the triple response of dark-grown *Arabidopsis* seedlings to ethylene was performed as described, with seedlings grown at 24 °C (33). Aminovinylglycine, an inhibitor of ethylene biosynthesis, was included in the growth medium for dark-grown seedlings. For ethylene treatments, Petri dishes remained lidded to prevent seedling desiccation except for the short-term kinetic analysis (Fig. 2*C*) in which lids were removed to facilitate rapid equilibration of ethylene. For ethylene treatment of *Arabidopsis* seedlings grown in liquid culture (12), ~30 seeds were grown per well in 6-well microtiter plates under continuous light, blotted to remove excess liquid, and transferred to 35-mm Petri dish bottoms for an ethylene treatment of 6 h at 24 °C.

Isolation of Arabidopsis Membranes—Microsomal fractions were isolated from dark-grown *Arabidopsis* seedlings (33) or *Arabidopsis* plants grown in liquid culture under continuous light at 24 °C (12). Plant material was homogenized in a buffer containing 30 mM Tris (pH 8 at 22 °C), 150 mM NaCl, 1 mM EDTA, and 20% (*v*/*v*) glycerol with protease inhibitors (Sigma plant protease inhibitor mixture; 1 mM phenylmethylsulfonyl fluoride) and then centrifuged at 8,000 × *g* for 15 min as described (12). The supernatant was then centrifuged at 100,000 × *g* for 30 min, and the resulting membrane pellet resuspended in 10 mM Tris (pH 7.6 at 22 °C), 150 mM NaCl, 0.1 mM EDTA, and 10% (*v*/*v*) glycerol with protease inhibitors (resuspension buffer).

Purification of TAP-tagged CTR1—For affinity purification of the CTR1-MT protein, microsomes were isolated from plants grown in liquid culture. Microsomes were brought to 1 mg/ml protein and incubated with 0.5% (*w*/*v*) lysophosphatidylcholine (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine from Avanti Polar-Lipids, Inc.) for 1 h at 4 °C, then centrifuged at 100,000 × *g* for 30 min. The supernatant was diluted to 0.25% (*w*/*v*) lysophosphatidylcholine and incubated with human IgG-agarose (Sigma) for 3 h at 4 °C. The beads were washed with resuspension buffer supplemented with 0.5% Nonidet P-40

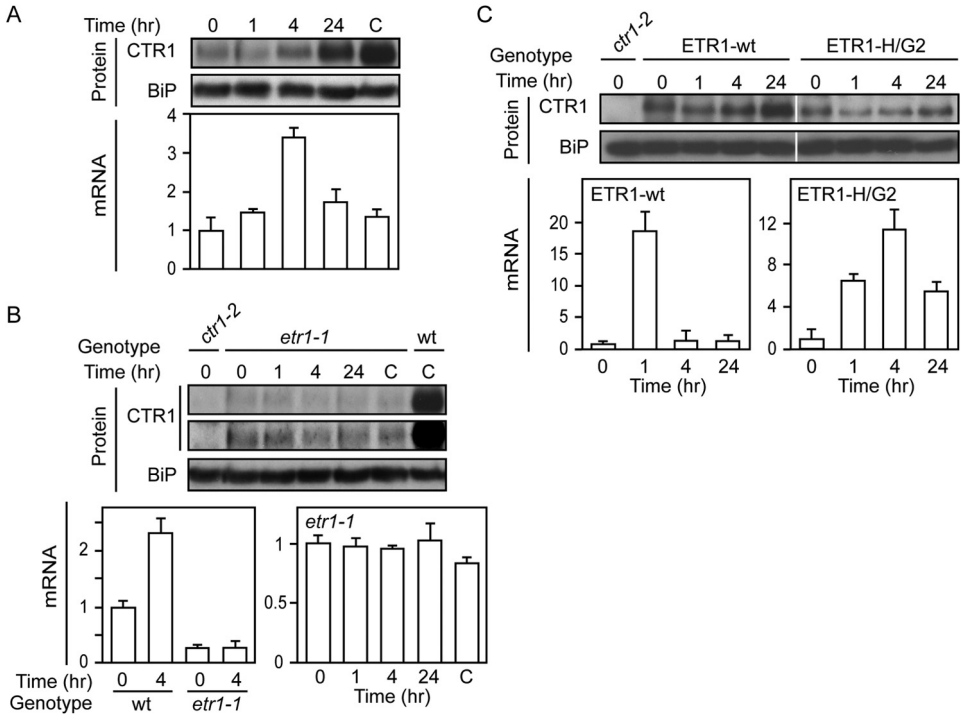

FIGURE 1. **Kinetic analysis of CTR1 protein levels in response to ethylene.** Dark-grown seedlings were treated with ethylene at concentrations and times indicated prior to harvest at 4 days. CTR1 protein levels (*Protein*) in the membrane fraction were determined by immunoblot analysis with an anti-CTR1 antibody, with BiP detected with an anti-BiP antibody as a loading control. Message levels for *CTR1* (*mRNA*) were determined by real time PCR; expression was normalized to a tubulin control and is presented as relative to the untreated wild-type control. Error bars are ± S.E. *A*, time course for CTR1 expression in response to 1 μl/liter ethylene. Wild-type seedlings were treated for the times indicated or with constant (*C*) ethylene. *B*, ethylene induction of CTR1 protein is blocked in the *etr1-1* mutant background. The *ctr1-2*-null mutant serves as a negative control for CTR1 protein expression, and wild-type (*wt*) treated with constant ethylene serves as a positive control for maximal CTR1 protein expression. Two immunoblot exposures are shown for CTR1 to aid in comparisons. *C*, time course for CTR1 expression in wild-type (ETR1-wt) and kinase-inactive (ETR1-H/G2) versions of ETR1. *Boxed regions* of immunoblots are from the same exposure. Message levels of *CTR1* are presented as relative to the untreated controls.

(Sigma) to remove unbound proteins, and the bound proteins eluted with 1% SDS and analyzed by immunoblot.

Antibodies and Immunoblot Analysis—ETR1 was identified by use of a polyclonal anti-ETR1 antibody generated against amino acids 401–738 of ETR1 (12). CTR1 was identified by use of a polyclonal anti-CTR1 antibody (27). Transgenic proteins with the c-Myc epitope tag were detected with a monoclonal anti-Myc antibody conjugated to horseradish peroxidase (monoclonal 9E-10; Santa Cruz Biotechnology). Antibodies targeted against BiP or the H^+-ATPase were used for membrane-protein loading controls (27). An anti-Hsc70 antibody was used for the soluble protein loading control (27). Immunoblot analysis was performed as described (34). Protein concentration was determined by use of the bicinchoninic acid reagent according to the manufacturer (Pierce) after first adding 0.1 ml 0.5% (*w*/*v*) SDS to solubilize membrane proteins. Bovine serum albumin was used as a standard for protein assays. Prior to SDS-PAGE (35), protein samples were mixed with SDS-PAGE loading buffer and incubated at 37 °C for 1 h or ramped from 37 °C to 65 °C over 40 min using a thermocycler, so as to prevent the aggregation of integral membrane proteins that can occur with boiling (36, 37). Equal protein loading of the various samples (typically 50 μg) was used for SDS-PAGE analysis. Following SDS-PAGE, proteins were electrotransferred to Immobilon nylon membrane (Millipore) for immunoblotting. Immunodecorated proteins were visualized by enhanced chemiluminescence detection according to the manufacturer (Pierce). Quantifications of blot intensities were determined using the program ImageJ by comparison to a dilution series and normalized to the loading control (33).

Quantitative Real-time PCR—Real-time PCR was performed as described (13), using primer sets specific for *CTR1* (5′-GGTTGTAGCTGCGGTTGGTTTCAA-3′ and 5′-TCGATTATGGCTGCAACCTGAGGA-3′), *ETR1* (5′-GAAGTGACCACGGTGAGTTCAA-3′ and 5′-ACACGTCCATGAAGACCACTTTG-3′), *ERS1-Myc* (5′-TCTCTGTAAACGGTTTGTCGGGCT-3′ and 5′-CGCCCAAGCTCTCCATTTCATTCA-3′), *ERS2-Myc* (5′-TTTCAACTCCGGCGATCGATGATG-3′ and 5′-ATCGATACCGTCGACACAAATCCG-3′), *EIN4-Myc* (5′-GGAGATGGTTGGTTTCGAAGTAGC-3′ and 5′-CGCCCAAGCTCTCCATTTCATTCA-3′), *CTR1-MT* (5′-GGTTGTAGCTGCGGTTGGTTTCAA-3′ and 5′-CGCCCAAGCTCTCCATTTCATTCA-3′), and a β-tubulin (At5g62700) control (5′-TGGTGGAGCCTTACAACGCTACTT-3′ and 5′-TTCACAGCAAGCTTACGGAGGTCA-3′).

RESULTS

Ethylene Regulates Transcript and Protein Levels of CTR1—We performed kinetic and dose analyses on the role of ethylene in regulating *CTR1* at the transcript and protein levels (Figs. 1 and 2). Dark-grown seedlings were used for these analyses due to their well-characterized ethylene response (38). A kinetic analysis on seedlings treated with 1 μl/liter ethylene revealed a transient increase in the *CTR1* transcript levels, maximum

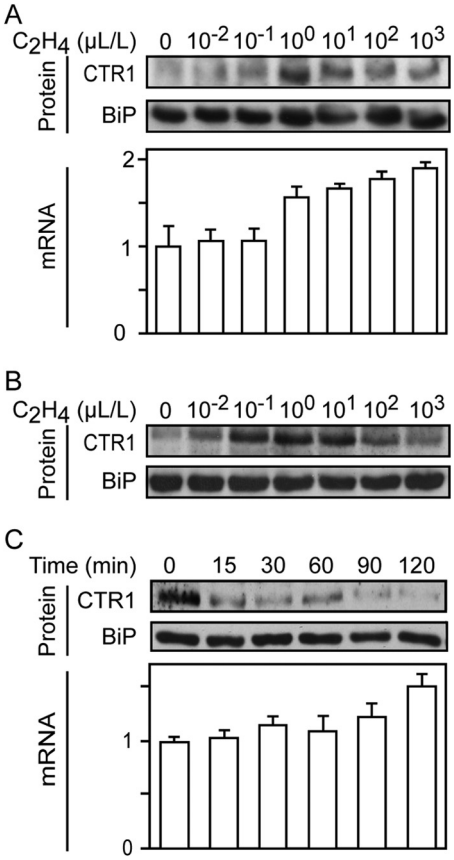

FIGURE 2. **Effect of ethylene concentration on *CTR1* expression and protein levels.** Dark-grown seedlings were treated with ethylene at concentrations and times indicated prior to harvest at 4 days. CTR1 protein levels (*Protein*) in the membrane fraction were determined by immunoblot analysis with an anti-CTR1 antibody, with BiP detected with an anti-BiP antibody as a loading control. Message levels for *CTR1* (*mRNA*) were determined by real time PCR and are presented as relative to the untreated wild-type control. Error bars are ± S.E. *A*, dose-response analysis, with seedlings treated for 4 h with air and 0.01, 0.1, 1, 10, 100, and 1000 μl/liter ethylene. *B*, dose-response analysis with seedlings treated for 24 h at the indicated ethylene concentrations. *C*, time course for CTR1 expression in response to 100 μl/liter ethylene, following initial growth on 1 μM ACC to induce CTR1.

expression being observed at the 4-h time point, and a decrease toward basal expression levels being observed at the 24-h and constant ethylene time points (Fig. 1*A*). In contrast, the levels of CTR1 protein associated with microsomes exhibited a continuous increase over time, maximal levels being observed when seedlings were continuously exposed to ethylene (Fig. 1*A*). The lack of correlation between message and protein levels supports post-transcriptional regulation of membrane-associated CTR1 levels.

Induction of CTR1 was affected by receptor mutations that inhibited perception and transmission of the ethylene signal. As shown in Fig. 1*B*, ethylene induction of CTR1 was abolished in the dominant ethylene-insensitive mutant *etr1-1* (31, 39), indicating that the post-transcriptional effect on protein levels is dependent on ethylene perception by the receptors. Levels of *CTR1* transcript were also reduced in the *etr1-1* background compared with wild type, suggesting a role for ethylene in maintaining basal levels of *CTR1* expression. The decreased expression of *CTR1* can account in part for the low level of membrane-associated CTR1 (~25% of the basal wild-type level) detected in *etr1-1*.

As shown in Fig. 1*C*, the kinetics of ethylene induction of membrane-associated CTR1 were altered in a background containing a kinase-inactive version of ETR1. Wild-type (ETR1-wt) and kinase-inactive (ETR1-H/G2) versions of ETR1 transgenically expressed in an *etr1-9 ers1-3* mutant background were used for this analysis (22). Although both versions of ETR1 rescue growth of the mutant and can interact with CTR1 (22, 27), the line containing kinase-inactive ETR1 exhibits reduced ethylene sensitivity resulting in a decreased ability to induce CTR1 in response to 1 μl/liter ethylene (Fig. 1*C*) (22). Expression of *CTR1* transcript is strongly induced by ethylene in both lines, but with substantially differing kinetics. In the line containing wild-type ETR1, the *CTR1* transcript peaks at the 1 h and then returns to basal levels at the later time points, whereas the CTR1 protein continues to increase throughout the time course (Fig. 1*C*). In contrast, in the line containing kinase-inactive ETR1, induction of the *CTR1* transcript is delayed, peaking at the 4 h time point and remaining at an elevated level compared with untreated samples throughout the time course (Fig. 1*C*). Even with the elevated levels of *CTR1* transcript, levels of CTR1 protein in the kinase-inactive ETR1 line never achieve those found in the wild-type ETR1 line. These data also demonstrate a lack of correlation between transcript and membrane-associated protein levels of CTR1.

A dose-response analysis, following 4-h ethylene treatment of wild-type seedlings, also supports a role for post-transcriptional regulation in controlling levels of CTR1. CTR1 was induced at low ethylene concentrations in a manner consistent with the increase in *CTR1* transcript levels (Fig. 2*A*). But at ethylene concentrations above 1 μl/liter, the CTR1 protein levels decreased in a manner independent of the message levels, which remained essentially unchanged. A dose-response analysis following 24-h ethylene treatment revealed a similar effect of ethylene concentration on CTR1 protein levels (Fig. 2*B*).

A kinetic analysis also revealed that dynamic changes in membrane-associated CTR1 occur in response to 100 μl/liter ethylene that cannot be explained by transcriptional changes (Fig. 2*C*). For this experiment, we initially induced CTR1 by growing seedlings on the ethylene biosynthetic precursor aminocyclopropane carboxylic acid (ACC; 1 μM), and then treated for varying times with 100 μl/liter ethylene. Ethylene treatment resulted in a rapid decrease in CTR1 levels, observable within 15 min of exposure and persisting throughout the 2-h time period examined. This decrease in CTR1 protein levels was independent of the mRNA expression levels, which increased slightly over the course of the experiment. Both the kinetic and dose-response studies are thus consistent with post-transcriptional regulation playing a substantial role in the control of CTR1 protein levels.

Interactions between CTR1 and the Ethylene Receptors—Based on amino acid sequence, CTR1 is a soluble protein with no transmembrane segments (23). However, CTR1 is found associated with membranes in a manner that is dependent on the presence of ethylene receptors (27). Thus, the post-transcriptional effects on CTR1 protein levels could reflect limitations on the ability of CTR1 to participate in receptor/CTR1 signaling complexes, such as changes in the abundance of the receptor(s) with which CTR1 interacts. We therefore examined

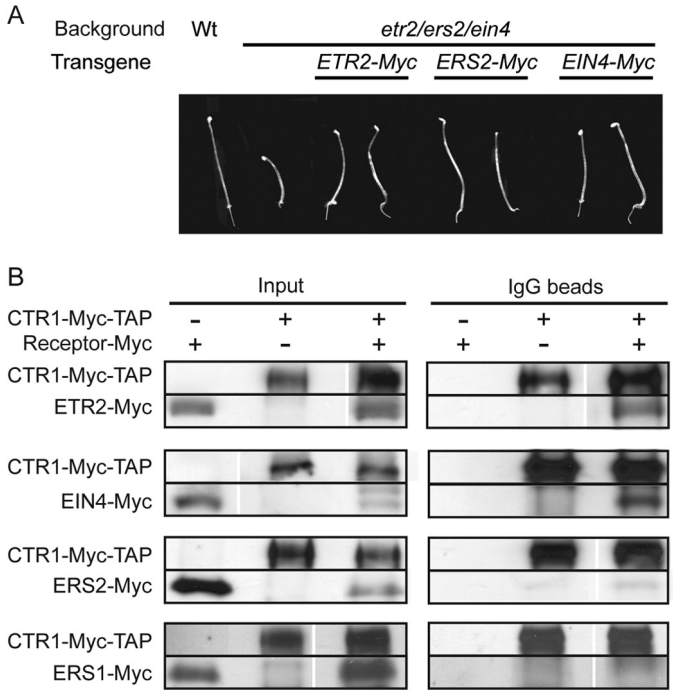

FIGURE 3. **Interaction of CTR1 with ethylene receptors.** *A*, functionality of Myc-tagged ethylene receptors. Expression of ETR2-Myc, ERS2-Myc, and EIN4-Myc complement the *etr2 ers2 ein4* constitutive-ethylene response phenotype. Phenotypes of dark-grown seedlings grown in air are shown. Two independent transgenic lines are shown for each Myc-tagged ethylene receptor. *B*, co-purification of ethylene receptors with CTR1-Myc-TAP. Microsomes from transgenic plants grown in liquid culture were solubilized with 0.5% (*w/v*) lysophosphatidylcholine, and the soluble supernatants incubated with IgG beads. The constructs expressed in the transgenic plants are indicated by +. The amounts of CTR1-Myc-TAP and the Myc-tagged ethylene receptors before IgG binding (*INPUT*) and on the IgG beads (*IgG Beads*) were detected by immunoblot analysis using an anti-Myc antibody. *Boxed regions* of the immunoblots are from the same exposure.

the ability of CTR1 to form *in planta* associations with the ethylene receptors.

We previously demonstrated that CTR1 could interact with ETR1 to form a signaling complex *in planta* (27). For this purpose, we generated a tagged version of CTR1, called CTR1-MT, driven by its native promoter and which contains a c-Myc epitope tag to aid in immunological detection and a Tandem Affinity Purification (TAP) tag to aid in affinity purification. Affinity purification of CTR1-MT resulted in co-purification of ETR1, thereby demonstrating their association in the same protein complex. To gain information about the *in planta* association of CTR1 with other members of the ethylene receptor family, c-Myc-tagged versions of the subfamily-2 receptors ETR2, EIN4, and ERS2, as well as the subfamily-1 receptor ERS1, were generated to allow for their immunological detection. Expression of each of the tagged receptors was driven by its native promoter, except for *ETR2* which was driven by the *ETR1* promoter to increase basal expression. Functionality of ETR2-Myc, EIN4-Myc, and ERS2-Myc was confirmed by transforming each construct into an *etr2/ers2/ein4* triple mutant (Fig. 3*A*). The tagged receptors rescued the constitutive ethylene-response phenotype of the mutant, indicating that the C-terminal c-Myc tag does not disrupt receptor function.

The Myc-tagged receptors were transformed into the transgenic line containing CTR1-MT to allow for co-purification analysis. The CTR1-MT protein was affinity purified by incubating lysophosphatidylcholine-solubilized membrane proteins with IgG beads. The IgG beads bind the protein-A portion of the TAP tag, resulting in affinity purification of CTR1-MT. These pull-down experiments demonstrated that ETR2 and EIN4 co-purified with CTR1-MT (Fig. 3*B*). The IgG beads did not pull-down ETR2 or EIN4 in the absence of CTR1-MT, indicating that the co-purification of ETR2 and EIN4 was mediated by CTR1-MT. Significantly, ETR2 and EIN4 are both subfamily-2 members of the receptor family, and these results thus indicate that CTR1 is able to interact with members of subfamily-2 in addition to its ability to interact with the subfamily-1 member ETR1. In contrast, we did not observe co-purification of ERS1 or ERS2 with CTR1-MT (Fig. 3*B*). Interestingly, these are the two receptors that lack a receiver domain, suggesting that the receiver domain may play a role in strengthening the interaction between the ethylene receptors and CTR1.

Post-transcriptional Effects of Ethylene on CTR1 Correlate with Effects on the Ethylene Receptors—The reduction in membrane-associated CTR1 at higher ethylene concentrations (Fig. 1*C*) is similar to what we previously observed for the ethylene receptor ETR2, where ethylene binding to the receptor induces its proteasome-mediated degradation (13). We hypothesized that the decrease in CTR1 levels could arise due to ligand-induced degradation of the associated receptor. We therefore examined ethylene-induced turnover for other members of the ethylene receptor family. We took advantage of our Myc-tagged lines to examine the effects of ethylene on the receptors ERS1, ERS2, and EIN4, expression of each of these receptors being driven by its native promoter. We also examined the tagged version of CTR1 (CTR1-MT), present in the ERS2-Myc line, to confirm that it exhibited similar post-transcriptional regulation to that observed for wild-type CTR1. The effects of ethylene on ETR1 levels are described in the section following this one.

Seedlings were treated with 1 μl/liter ethylene, a concentration that resulted in maximal induction of CTR1 protein, and with 100 μl/liter ethylene, a concentration that induced a post-transcriptional reduction in CTR1 protein levels (Fig. 1). We examined responses following 4-h ethylene treatment (Fig. 4*A*), a time point at which we had observed transcriptional induction of *CTR1*, and following 24-h ethylene treatment (Fig. 4*B*), a time point by which the *CTR1* transcript had returned to basal levels. As shown in Fig. 4, the effects of ethylene on CTR1-MT message and protein are similar to those observed for wild-type *CTR1*. We observe a transcriptional induction of the *CTR1-MT* message at 4-h but not at 24-h ethylene treatment. Furthermore, regardless of the transcript level, we observe the maximal level of membrane-associated CTR1-MT protein at 1 μl/liter ethylene, treatment with 100 μl/liter ethylene resulting in a reduction from this maximum.

The effects of ethylene on protein levels of the receptors ERS1-Myc, ERS2-Myc, and EIN4-Myc (Fig. 4) correlated with what we observed for CTR1 (Figs. 1 and 2) and CTR1-MT (Fig. 4). For all three receptors, we observed maximal protein levels of the receptor at 1 μl/liter ethylene, treatment with 100 μl/liter ethylene resulting in a reduction from this maximum. The ethylene receptors ERS1 and ERS2 are transcriptionally induced by

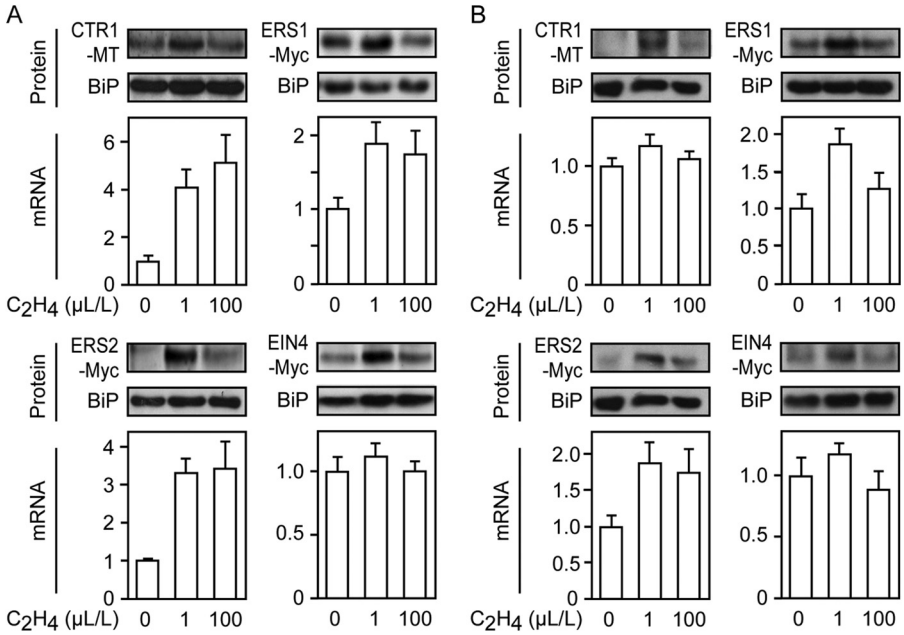

FIGURE 4. **Effect of ethylene on the expression of ethylene receptors and CTR1.** Transgenic plants expressing CTR1-Myc-TAP (*CTR1-MT*), ERS1-Myc, ERS2-Myc, or EIN4-Myc were examined. Dark-grown seedlings were treated with 0, 1, and 100 μl/liter ethylene for 4 h (*A*) or 24 h (*B*). Microsomes were isolated, and protein levels of tagged receptors and CTR1 immunologically determined with an anti-Myc antibody (*Protein*). Immunologically-detected BiP served as the protein loading control. Message levels (*mRNA*) of the transgenes were determined based on real-time PCR.

ethylene (Fig. 4), which can account for the increase in protein levels at 1 μl/L ethylene but does not account for reduction observed at 100 μl/liter. Interestingly, although ethylene does not transcriptionally regulate expression of *EIN4*, we still observed an increase in protein levels of this receptor at 1 μl/liter ethylene (Fig. 4), suggesting that a post-transcriptional mechanism such as translational control may play a role in induction of this ethylene receptor. These data, combined with those from our previous study on ETR2 (13), indicate that ethylene post-transcriptionally regulates the protein levels of four members of the receptor family in *Arabidopsis*: ERS1, ERS2, ETR2, and EIN4. Furthermore, the effects of ethylene on these receptor family members correlate with the effects of ethylene on the levels of membrane-associated CTR1.

Ethylene-induced Turnover of ETR1 Is Modulated by the Presence of CTR1—In wild-type seedlings ethylene had only a modest effect on ETR1 protein levels (Fig. 5*A*), unlike what we had observed for other members of the receptor family. In contrast, we observed substantially higher turnover of ETR1 when examined in a *ctr1–2* mutant background (Fig. 5*A*). The *ctr1-2* mutation arises from a 17-bp deletion in *CTR1*, resulting in a frameshift and a lack of immuno-detectable CTR1 protein (27). Ethylene dose-response analysis revealed a continuous decline in ETR1 protein levels in the *ctr1-2* mutant background, the reduction first being detectable at 0.1 μl/liter ethylene and, at its maximum, corresponding to a 60% decrease compared with the untreated control.

We considered two potential explanations for the increased sensitivity of ETR1 turnover to ethylene in the *ctr1-2* mutant background. It could be related to the constitutive ethylene response phenotype observed in *ctr1* mutant backgrounds (23). Alternatively, it could be related to the physical interaction of CTR1 with ETR1 (27), an interaction that would be lost in the *ctr1-2* mutant background. To differentiate between these possibilities, we compared ETR1 turnover in the *ctr1-2* background (lacks CTR1 protein) to that in the *ctr1-4* background (a missense mutation that produces an enzymatically inactive protein) (23, 24). Greater ETR1 turnover was observed in the *ctr1-2* background than in the *ctr1-4* background (Fig. 5*B*), indicating that the presence of CTR1 protein, whether active or inactive, can serve to protect ETR1 from ligand-induced turnover. In contrast, ETR2 protein exhibited ligand-induced turnover in all three backgrounds tested (wild-type, *ctr1-2*, and *ctr1-4*) (Fig. 5*B*).

Effect of Ethylene on Levels of Soluble CTR1—We previously observed that CTR1 was detectable in the membrane but not the soluble fraction of dark-grown seedlings, potentially due to rapid turnover of free CTR1 (27). In contrast, we detected CTR1 in both membrane and soluble fractions of seedlings grown in liquid culture under lights. Furthermore, we found that loss-of-function mutations that eliminated receptors resulted in a redistribution of CTR1 from the membrane to the soluble fraction of plants grown in liquid culture (27). We therefore explored the effects of ethylene on the distribution of CTR1 between membrane and soluble fractions, making use of both dark-grown and light-grown seedlings (Fig. 6). To increase the sensitivity of the assay, we made use of an *etr1-7* loss-of-function mutant background because this mutant eliminates ETR1 and so association of CTR1 with membranes would arise from those receptors that exhibit the most pronounced ethylene-induced turnover.

In dark-grown seedlings (Fig. 6*A*), we observed a dose-dependent effect of ethylene on the levels of membrane-associated CTR1 similar to that observed in Fig. 2. No soluble CTR1 was detected at any of the ethylene concentrations (Fig. 6*A*), consistent with what we have previously observed for dark-grown

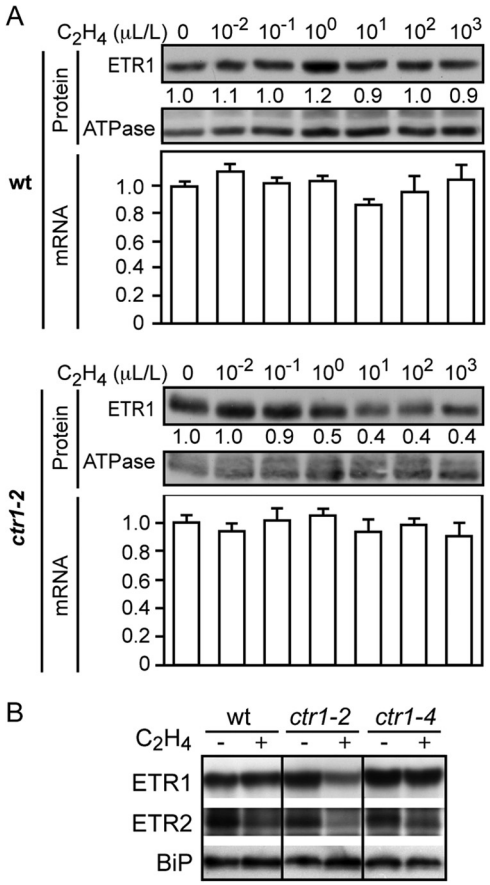

FIGURE 5. **Ethylene-induced turnover of ETR1 is modulated by CTR1.** *A*, effect of ethylene concentration upon protein levels of ETR1. Dark-grown wild-type (*wt*) or *ctr1-2* seedlings were grown for 2.5 days, then treated for 24 h with air, 0.01, 0.1, 1, 10,100, and 1000 μl/liter ethylene. Protein levels of ETR1 (*Protein*) were determined by immunoblot analysis with an anti-ETR1 antibody. Relative ETR1 protein levels were quantified densitometrically and normalized to the ATPase loading control. Message levels for *ETR1* (*mRNA*) were determined by real-time PCR. Error bars are ± S.E. *B*, effect of *ctr1* mutations on ethylene-induced turnover of ETR1 and ETR2. Dark grown wild-type (*wt*), *ctr1-2*, and *ctr1-4* seedlings were grown for 2.5 days, then treated for 24 h with 100 μl/liter ethylene. Wild-type seedlings were grown in the presence of 50 μM ACC to induce expression of ETR2, which is naturally induced in the constitutive ethylene mutants *ctr1-2* and *ctr1-4*. Protein levels of ETR1, ETR2, and the BiP loading control were determined by immunoblot analysis.

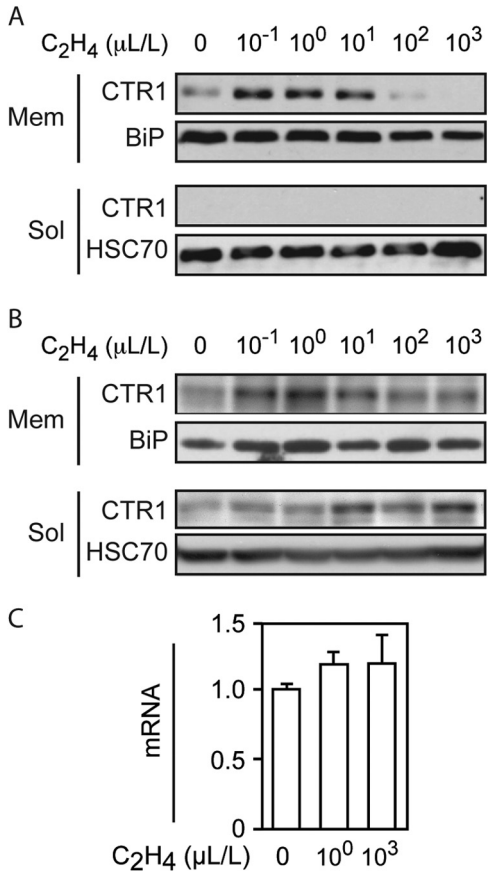

FIGURE 6. **Effect of ethylene on soluble and membrane-associated levels of CTR1.** *A*, dose-response analysis of CTR1 in dark-grown seedlings treated for 24 h with air, 0.1, 1, 10, 100, and 1000 μl/liter ethylene. Immunoblot analysis was performed using antibodies against CTR1, BiP (membrane loading control) and Hsc70 (soluble loading control). No CTR1 is detected in the soluble fractions although these are of the same immunoblot exposure as the membrane fractions. *B* and *C*, dose response analysis of CTR1 in green seedlings grown in liquid culture and treated for 6 h with the indicated concentrations of ethylene. CTR1 protein was immunologically detected in the membrane and soluble fractions, with BiP and Hsc70 as protein loading controls (*B*). Message levels for *CTR1* (*mRNA*) were determined by real time PCR and are presented as relative to the untreated wild-type control; error bars are ± S.E. (*C*).

seedlings (27). In light-grown seedlings (Fig. 6*B*), we observed a similar dose-dependence for the membrane-associated levels CTR1 to that observed in dark-grown seedlings, there being an initial increase in the membrane-associated levels, followed by a decrease at higher ethylene concentrations. CTR1 was also detected in the soluble fraction, increased levels of soluble CTR1 being detected at the higher ethylene concentrations (Fig. 6*B*). Message levels of CTR1 remained essentially unchanged at the various ethylene concentrations (Fig. 6*C*). These data demonstrate that we can observe effects on membrane-associated CTR1 with green seedlings similar to what we observe with dark-grown seedlings, and support the possibility that CTR1 redistributes from the membrane to the soluble fraction at higher ethylene concentrations.

DISCUSSION

Our results demonstrate dynamic regulation of signaling complexes involving the ethylene receptors and CTR1, this dynamic regulation arising from the interplay of three factors: 1) the physical association of the receptors with CTR1; 2) ethylene-dependent induction of new receptors; and 3) ethylene-dependent turnover of receptors. Below we consider each of these factors and how they fit into an overall model for regulation of ethylene receptor/CTR1 signaling complexes (Fig. 7).

Protein-protein interactions play a key role in signal transduction, and ethylene signaling is dependent on interactions between CTR1 and the ethylene receptors (24, 27). Interaction with the ethylene receptors serves to localize the soluble protein CTR1 to the endoplasmic reticulum (27), where CTR1 regulates activity of the downstream signaling element EIN2 by phosphorylation (25, 26). It has been unclear if CTR1 interacts with all five members or with a subset of the ethylene receptor family. Two-hybrid analysis finds the strongest interaction of CTR1 with ETR1, and only weak interactions with ERS1 and ETR2 (28, 29), suggesting that there might be specificity in interactions, and raising the question as to whether CTR1 interacts with subfamily-2 members. On the other hand, analysis of

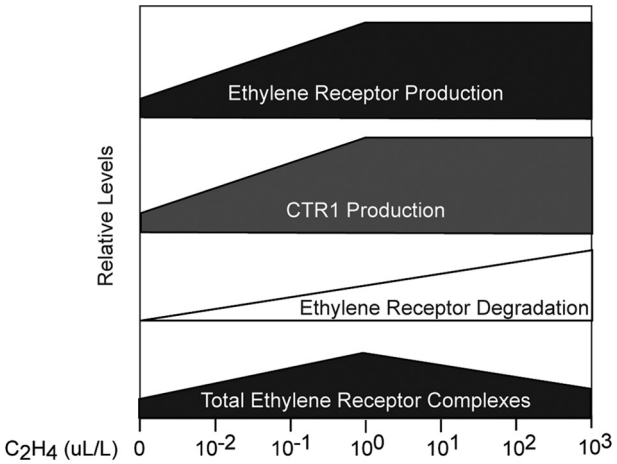

FIGURE 7. **Model for ethylene-mediated regulation of receptor/CTR1 protein complexes.** The overall quantity of ethylene receptor complexes is dependent on two opposing but ethylene-dependent functions: 1) ethylene-dependent induction of ethylene receptors; and 2) ethylene-dependent degradation of the receptors. The level of receptors determines the levels of membrane-associated CTR1 due to the physical interaction of the receptors with CTR1. At ethylene concentrations up to 1 μl/liter, the production of new receptors and associated CTR1 is greater than the loss of receptors due to degradation. At ethylene concentrations above 1 μl/liter, receptor degradation is greater than the production of new receptors, resulting in a decrease in the total levels of the receptor complexes.

loss-of-function mutations that eliminate receptors suggest that multiple receptors are required for membrane-localization of CTR1 (27). In particular, a triple receptor mutant involving only subfamily-2 members (*etr2 ers2 ein4*) results in a substantial loss of CTR1 from the membrane. As reported here, our data support the ability of CTR1 to interact with the subfamily-2 members ETR2 and EIN4 *in planta*, based on an approach previously used to demonstrate interaction of CTR1 with the subfamily-1 member ETR1 (27).

Interestingly, we did not observe co-purification of ERS1 or ERS2 with CTR1 in our pull-down analysis. These are the two receptors that lack a receiver domain, suggesting that the receiver domain may play a role in strengthening the interaction between the ethylene receptors and CTR1. This hypothesis is consistent with the finding that the receiver domain of ETR1 by itself can interact with CTR1 based on two-hybrid analysis (28). We think it likely that CTR1 does interact with these two receptors *in planta* because ERS1 and ERS2 both play roles in the membrane-association of CTR1 based on the analysis of receptor mutants (27), but that the weaker interaction is not preserved during the membrane solubilization step necessary for pull-down analysis. Taken together, these data indicate that CTR1 interacts with all five members of the receptor family but does so with varying strength.

The levels of membrane-associated CTR1 vary in response to ethylene, doing so in a post-transcriptional manner that correlates with the ethylene-mediated changes in levels of the receptors. One factor influencing receptor levels is ethylene-mediated induction, the receptors ERS1, ETR2, and ERS1 being transcriptionally induced in response to ethylene, the receptor EIN4 exhibiting a post-transcriptional increase in response to ethylene. The second and opposing factor influencing receptor levels is ethylene-mediated turnover. We previously identified ETR2 as a target for ethylene-induced degradation through a proteasome dependent pathway (13). Here we demonstrate that ERS1, ERS2, and EIN4 also turn over with a similar sensitivity to ethylene, suggesting that the same proteasome-dependent mechanism targeting ETR2 also regulates levels of these other receptors. Ethylene-induced turnover is also exhibited by tomato ethylene receptors (30), suggesting that this is a common regulatory mechanism for ethylene signaling in plants.

ETR1 did not exhibit the same degree of ethylene-dependent turnover as the other members of the receptor family, owed in part to its physical interaction with CTR1. ETR1 has a higher affinity for CTR1 than either ERS1 or ETR2 based on two-hybrid analysis (28, 29), which may explain the difference among family members. The protective effect of CTR1 against degradation suggests that the site regulating receptor turnover might overlap with the CTR1 binding site. These data also raise the possibility that the interaction of receptors with CTR1 may be reversible, with release of CTR1 from the receptors facilitating receptor turnover. Interestingly, we observed a potential ethylene-induced redistribution of CTR1 from membrane to soluble fraction in light-grown seedlings, whereas we do not detect CTR1 in the soluble fraction of dark-grown seedlings. Such a redistribution raises the possibility that CTR1 could perform additional regulatory functions in response to ethylene.

Our data support the model for regulation of ethylene-receptor/CTR1 signaling complexes depicted in Fig. 7. Ethylene perception and signal transduction induce the production of new ethylene receptors, *ERS1*, *ETR2*, and *ERS2* all being primary response genes whose transcription is rapidly induced in response to ethylene (40, 41). The increase in overall ethylene receptor levels at the membrane results in a concurrent increase in membrane-associated CTR1 levels due to the physical association of CTR1 with the receptors. The low level of CTR1 associated with membranes of the ethylene-insensitive mutant *etr1-1* is likely due in part to the reduced overall levels of receptors present in the mutant as revealed by expression and ethylene binding analyses (2). Ethylene also induces post-transcriptional degradation of receptors (13, 30), such that the levels of ethylene receptor/CTR1 complexes decrease at higher ethylene concentrations, when the receptor degradation rate exceeds the synthesis rate. Under our experimental conditions we observed maximal levels of the receptor/CTR1 signaling complex at 1 μl/liter ethylene. However, since the maximal level of the signaling complex is dependent on two opposing factors, conditions that affect the efficacy of either factor would shift this maximum. For instance, we have observed greater receptor degradation at elevated temperatures. Such a dependence on growth conditions may explain why turnover was not previously observed for ERS1 (42).

Changes in the levels of the ethylene receptor/CTR1 signaling complexes are predicted to have effects on the sensitivity of the plant to ethylene, since the signaling complex negatively regulates ethylene responses (6, 23, 29). The production of new receptors in response to ethylene has been proposed as a mechanism to reset the ethylene sensitivity of the plant (3, 40). Thus, the increase in levels of receptor/CTR1 complexes with ethylene concentrations up to 1 μl/liter would serve to desensitize the plant to ethylene, thereby facilitating the adaptation response to ethylene. Adaptation is typified by the sensitivity to

a signal being altered in response to changes in the level of the signal, thereby allowing the organism to sense the signal over as wide a range as possible. *Arabidopsis* can sense changes in ethylene concentration over six orders of magnitude (43, 44), consistent with an adaptation response. The degradation of receptors is a common mechanism employed to limit ligand action, since ligand release is often too slow to allow for responses in a physiological time frame (*e.g.* the half-life for ethylene release from ETR1 and ETR2 is 12 and 10 h, respectively) (2, 9). Thus the decrease in receptor levels we observe above 1 μl/L ethylene may restore sensitivity. However, additional factors have been identified that contribute to desensitization of the ethylene response (45–47), and these may play a role at higher ethylene concentrations and compensate for the reduction in levels of the receptor/CTR1 signaling complex.

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