Localization of the Raf-like Kinase CTR1 to the Endoplasmic Reticulum of *Arabidopsis* through Participation in Ethylene Receptor Signaling Complexes*

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The plant hormone ethylene is perceived by a five-member family of receptors related to the bacterial histidine kinases. The Raf-like kinase CTR1 functions downstream of the ethylene receptors as a negative regulator of ethylene signal transduction. CTR1 is shown here to be associated with membranes of the endoplasmic reticulum in *Arabidopsis* as a result of its interactions with ethylene receptors. Membrane association of CTR1 is reduced by mutations that eliminate ethylene receptors and by a mutation in CTR1 that reduces its ability to bind to the ethylene receptor ETR1. Direct evidence that CTR1 is part of an ethylene receptor signaling complex was obtained by co-purification of the ethylene receptor ETR1 with a tagged version of CTR1 from an *Arabidopsis* membrane extract. The histidine kinase activity of ETR1 is not required for its association with CTR1, based on co-purification of tagged ETR1 mutants and CTR1 after expression in a transgenic yeast system. These data demonstrate that CTR1 is part of an ethylene receptor signaling complex in *Arabidopsis* and support a model in which localization of CTR1 to the endoplasmic reticulum is necessary for its function. Additional data that demonstrate a post-transcriptional effect of ethylene upon the expression of CTR1 suggest that production of ethylene receptor signaling complexes may be coordinately regulated.

The simple gas ethylene serves as a diffusible hormone in plants (1, 2). Ethylene regulates seed germination, seedling growth, leaf and petal abscission, organ senescence, ripening, and responses to stress and pathogens. Mutants affecting ethylene responses have been isolated in the plant *Arabidopsis*, and characterization of these mutants has led to the identification of ethylene receptors and several downstream components in the ethylene signal transduction pathway (3–6).

The ethylene receptor family of *Arabidopsis* consists of five members: ETR1, ERS1, ETR2, ERS2, and EIN4 (6, 7). ETR1 was the first member of the receptor family identified and has been characterized in the most detail. The ethylene receptors have similar overall structures with transmembrane domains near their N termini and putative signaling motifs in their C-terminal halves. The receptors have three conserved transmembrane domains that based on genetic and biochemical evidence contain the ethylene binding site (8–10). Recent studies of ETR1 indicate that the transmembrane domains also serve in localization of the receptor to the endoplasmic reticulum (ER),[1] an unusual location for a hormone receptor but one compatible with the ready diffusion of ethylene in aqueous and lipid environments (11). In the C-terminal half of each receptor are domains with similarity to histidine kinases and in some cases the receiver domains of response regulators. Histidine kinases and receiver domains are signaling elements originally identified in bacterial two-component phosphorelays and are now known to be present in plants, fungi, and slime molds (12).

The ethylene receptors form two subfamilies based on phylogenetic analysis and some shared structural features (6, 7, 13). ETR1 and ERS1 belong to subfamily 1 and contain histidine kinase domains with all the conserved residues required for enzymatic activity. Histidine kinase activity has been demonstrated for ETR1 (14), but the function of this activity in signal output is still not clear (15, 16). ETR2, ERS2, and EIN4 belong to subfamily 2 and, although they contain histidine kinase-like domains, these domains lack residues considered essential for histidine kinase activity. Each member of subfamily 2 also has an additional hydrophobic domain at the N terminus that is predicted to function as a cleaved signal sequence for targeting to the secretory pathway (6). Some of the receptors (ERS1, ERS2, and ETR2) are induced by ethylene at the transcriptional level (17), but this induction is not specific to either receptor subfamily.

CTR1 is a protein kinase that functions downstream of the ethylene receptors based on genetic analysis (18). The kinase domain of CTR1 is in the C-terminal half of the protein and shows the greatest similarity to the Raf family of serine/threonine protein kinases (18, 19). CTR1 may function as a mitogen-activated protein kinase kinase kinase (MAPKKK) in analogous fashion to Raf, and regulate signaling through a MAPK cascade in *Arabidopsis*. Consistent with this possibility, ethylene stimulates MAPK activity in *Arabidopsis* (20, 21). Loss-of-function mutations in *CTR1* result in constitutive ethylene responses, thereby indicating that *CTR1* is a negative regulator, and that phosphorylation of substrates by CTR1 is apparently required to suppress ethylene responses (18, 19).

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[1] The abbreviations used are: ER, endoplasmic reticulum; ACC, 1-aminocyclopropane-1-carboxylic acid; GST, glutathione *S*-transferase; PM, plasma membrane.

Thus, the early steps of ethylene signal transduction combine signaling elements of disparate evolutionary origin, the receptors being related to the histidine kinases of prokaryotes but CTR1 being related to a serine/threonine kinase family found only in eukaryotes. Interestingly, CTR1 has been found capable of directly interacting with the ethylene receptors ETR1, ERS1, and ETR2 based on two-hybrid analysis and *in vitro* binding experiments (19, 22, 23). The region of CTR1 involved in the interaction lies within the N-terminal half of the protein, a region that, based on the Raf kinase model, could be involved in regulation of kinase activity (19, 22). CTR1 was found capable of interacting with both the histidine kinase and receiver domains of ETR1, and the histidine kinase domain of ERS1 (which lacks a receiver domain) (22). However, the physiological relevance of these interactions has remained unclear as no interaction between CTR1 and the ethylene receptors has been demonstrated in plants. In this study, we demonstrate that native CTR1 is localized to the endoplasmic reticulum of *Arabidopsis* and that this localization arises due to interactions with ethylene receptors. Our data support the existence of a signaling complex involved in the initial steps of ethylene signal transduction and suggest that assembly of the signaling complex may be coordinately regulated.

EXPERIMENTAL PROCEDURES

Membrane Fractionation—Microsomal and soluble fractions were isolated from either dark-grown *Arabidopsis* seedlings (24) or *Arabidopsis* plants grown in liquid culture with an 18-h light period (11), using a homogenization buffer containing 30 mM Tris (pH 8.3 at 4 °C), 150 mM NaCl, 10 mM EDTA, and 20% (v/v) glycerol with protease inhibitors as described (11, 24). Briefly, plant material was homogenized and then centrifuged at $8,000 \times g$ for 15 min. The supernatant was then centrifuged at $100,000 \times g$ for 30 min, and the resulting membrane pellet resuspended in 10 mM Tris (pH 7.6 at 22 °C), 150 mM NaCl, 1 mM EDTA, and 10% (v/v) glycerol with protease inhibitors.

Sucrose density gradient centrifugation was performed as described (11) using 20–50% (w/w) sucrose gradients in 10 mM Tris (pH 7.6), 1 mM dithiothreitol, 2 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. For analyses performed in the presence of Mg^{2+}, 5 mM $MgCl_2$ was added to homogenization, resuspension, and centrifugation buffers. Gradient fractions were analyzed for the presence of the ER, PM, mitochondrial inner membrane, and tonoplast by immunoblot using antibodies that recognized specific membrane markers. Thylakoid membranes were identified by spectrophotometric analysis of chlorophyll levels, and Golgi membranes were identified based on Triton X-100-stimulated UDPase activity (25).

Antibodies and Immunoblot Analysis—For preparation of the CTR1 antibody, a fusion protein was made of CTR1 with a His$_6$ tag (CTR1–6His). A *Hin*dIII to *Bgl*II fragment of the *CTR1* cDNA (18), encoding Lys^{310} to Leu^{678} of CTR1, was cloned into the *Hin*dIII and *Bam*H I sites of the expression vector pET15b (Novagen). The CTR1–6His protein was expressed in *Escherichia coli*, and inclusion bodies isolated according to the manufacturer (Novagen) and used to prepare polyclonal antisera by Cocalico Biologicals, Inc. (Reamstown, PA). The anti-CTR1 antibodies were affinity purified on Affi-Gel-15 columns (Bio-Rad) cross-linked with CTR1–6His that had been solubilized from inclusion bodies with 1.5% (w/v) Sarkosyl, 2% (w/v) Triton X-100. Antibodies were eluted with 100 mM glycine, pH 2.5, and neutralized with 1 M Tris, pH 8.0.

Specific *Arabidopsis* membranes were identified by use of antibodies against the ER-markers ETR1, ACA2, and BiP (11, 26–28), the PM-marker H$^+$-ATPase (29), the mitochondrial inner membrane marker F1-ATPase (30), and the tonoplast-marker VM23 (31). An antibody against the cytosolic heat shock protein Hsc-70 (StressGen) was used as a marker for soluble proteins in *Arabidopsis*. GST fusion proteins were identified by use of a polyclonal anti-GST antibody (Santa Cruz Biotechnology).

Immunoblot analysis was performed as described (15). Protein concentration was determined by use of the BCA reagent (Pierce) according to the manufacturer after first adding 0.2 ml 0.4% (w/v) deoxycholate to solubilize membrane proteins. Bovine serum albumin was used as a standard for protein assays. Prior to SDS-PAGE (32), 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 thermocyler, so as to prevent the aggregation of integral membrane proteins that can occur with boiling (25, 33). 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 Chemical).

RNA Isolation and Northern Blotting—Northern blot analysis was performed with *Arabidopsis* mRNA isolated as previously described (24).

Preparation and Purification of TAP-tagged CTR1—A binary vector (pCAMBIA2380-myc-TAP) was prepared for expression of affinity-tagged proteins in *Arabidopsis*. Initially, the vector pCAMBIA1380 (GenBank™ accession no. AF234301) was modified by replacing the gene for hygromycin resistance with the NPT II gene for kanamycin resistance and the resultant vector designated pCAMBIA2380. The TAP tag was amplified from the vector pBS1479 (34) and cloned into the *Kpn*I and *Hin*dIII restriction sites of the vector 6-CMYC (*Arabidopsis* Biological Resource Center stock no. CD3–128) that contains a cassette encoding 6 copies of the c-Myc epitope tag. The region encoding the c-Myc and TAP tags was then amplified with primers that contained *Xho*I restriction sites and cloned into the *Xho*I site of pCAMBIA2380 to make pCAMBIA2380-myc-TAP. The region encoding CTR1 along with upstream promoter sequence was amplified from the *Arabidopsis* BAC clone F17C15 (GenBank™ accession no. AL162506) using 5′-primer GTCGACGGAGAAGTAGAAAAGAAAAC and 3′-primer GTCGACA-CAAATCCGAGCGGTTGG. The PCR product was cloned into the *Sal*I site of pCAMBIA2380-myc-TAP to make pCAMBIA-CTR1-MT. Transformation and selection of *Arabidopsis* plants with pCAMBIA-CTR1-MT was performed as described (15).

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 2 h at 4 °C, then centrifuged at $100,000 \times g$ for 30 min. The supernatant was diluted to 0.25% LPC and incubated with Rabbit IgG-agarose (Sigma) for 4 h at 4 °C. The beads were washed with resuspension buffer to remove unbound proteins, and the bound proteins analyzed by immunoblot.

Analysis of CTR1 Interaction with GST-ETR1 Fusion Proteins—For expression of GST and GST fusion proteins in yeast, the vector pEG(KT) was used (35). This vector contains the GST domain under control of a Gal-inducible promoter and allows for uracil selection in yeast. The GST-ETR1 fusion proteins have been previously described (14, 15). For expression of CTR1 in yeast, the vector pYcDE-2 was used (36). This vector has a constitutive *ADH1* promoter and allows for Trp selection. A fragment of the *CTR1* gene representing amino acids 1–698 was cloned into the EcoR I site of pYcDE-2. The pYcDE-2 construct and the pEG(KT) constructs were transformed together into yeast (*Saccharomyces cerevisiae*) strain EGY188 (*MATa ura3 his3 trp1 LexA-LEU2*) (37). Standard media and procedures were used for growth (38). For induction of GST fusion proteins, 0.5% galactose was used. GST fusion proteins were isolated from mid-log phase cultures by affinity purification with glutathione-agarose beads as previously described (14). GST fusion proteins were visualized by immunoblotting using an anti-GST antibody. Co-purification of CTR1-(1–698) with the GST fusion proteins was assessed by using the anti-CTR1 antibody.

RESULTS

Analysis of CTR1 Expression in Arabidopsis—To characterize the CTR1 protein in plants, we prepared a polyclonal antibody directed against CTR1. This antibody recognizes a protein of 92 kDa in *Arabidopsis* membranes, consistent with the calculated molecular mass of 90 kDa for CTR1 (Fig. 1A). As would be predicted, this protein is absent in the loss-of-function *ctr1-2* mutant line. The *ctr1-2* mutant has a 17-bp deletion that results in a frameshift within the coding region (18).

The CTR1 protein was found to be ethylene-induced, based upon treatment of seedlings with the ethylene precursor aminocyclopropane carboxylic acid (ACC) or with ethylene itself (Fig. 1). The protein levels of CTR1 were 3-fold higher in ACC-treated seedlings compared with untreated seedlings (Fig. 1A). Treatment of *Arabidopsis* seedlings with 100 µl/liter ethylene resulted in an apparent increase in CTR1 levels within 15 min, with a maximal 4-fold increase in CTR1 levels
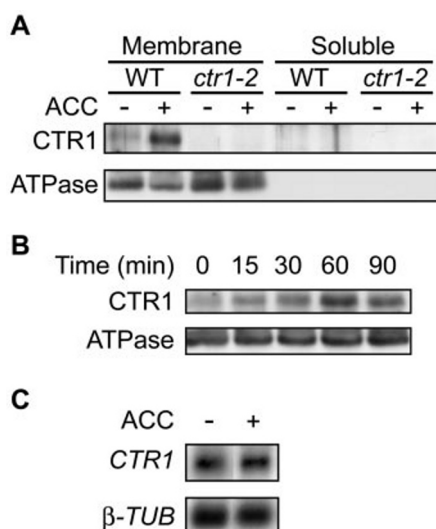
FIG. 1. **Analysis of CTR1 expression in *Arabidopsis*.** *A*, immunological detection of CTR1 in *Arabidopsis* membranes. Membrane and soluble proteins were isolated from 3-day-old wild type and *ctr1-2* seedlings grown in the dark. Ethylene induction of proteins was tested by growing plants in the presence of the biosynthetic precursor ACC (50 μM). Proteins (20 μg) were examined by immunoblot using antibodies against CTR1 and the H^+-ATPase as a loading control for membranes. The apparent molecular masses were 92 kDa for CTR1 and 98 kDa for the H^+-ATPase. *B*, time course for induction of CTR1 by ethylene. Three-day-old dark-grown seedlings were treated with 100 μl/liter ethylene for the indicated times and membrane-associated CTR1 visualized by immunoblot analysis. *C*, Northern blot analysis of mRNA isolated from dark-grown seedlings grown in the presence or absence of 50 μM ACC. Blots were probed with a *CTR1* probe and a β-tubulin gene probe as an internal control.

observed 60 min after the initiation of ethylene treatment (Fig. 1*B*).

Ethylene induction at the mRNA level has been previously noted for a *CTR1*-like gene from tomato (39, 40), but was not previously observed for *Arabidopsis* (18). To determine if ethylene induction of *CTR1* in *Arabidopsis* occurred at the transcriptional or post-transcriptional level, transcript levels of *CTR1* were determined by Northern blot in both ACC-treated and untreated plants (Fig. 1*C*). No change in transcript levels of *CTR1* was detected, consistent with previous studies (18), thereby indicating that the differences in CTR1 protein levels are likely due to post-transcriptional mechanisms of regulation.

Localization of CTR1 to the Endoplasmic Reticulum—Immunoblot analysis of microsomal and soluble protein extracts revealed that CTR1 was associated with the membrane fraction of *Arabidopsis* (Fig. 1), despite CTR1 itself lacking transmembrane domains or obvious sites for lipid modification (18). To resolve the subcellular membrane localization of CTR1, sucrose density gradient centrifugation was performed on *Arabidopsis* microsomes (Fig. 2). Centrifugation was performed under conditions that would allow for the discrimination of ER-associated proteins, the ER being a logical membrane location for CTR1 based on the previous finding that the ethylene receptor ETR1 is localized to the ER (11). Centrifugation was thus performed in the presence and absence of Mg^{2+}. Association of ribosomes with the ER is Mg^{2+}-dependent, so removal of Mg^{2+} results in dissociation of ribosomes from the ER and a diagnostic redistribution of ER from higher to lower density on the gradient (41).

Fractions from the sucrose gradient were analyzed by immunoblot for the presence of CTR1 as well as for markers specific for PM, mitochondria, tonoplast, and ER (Fig. 2). The majority of CTR1 exhibited a strong Mg^{2+}-dependent density-shift from
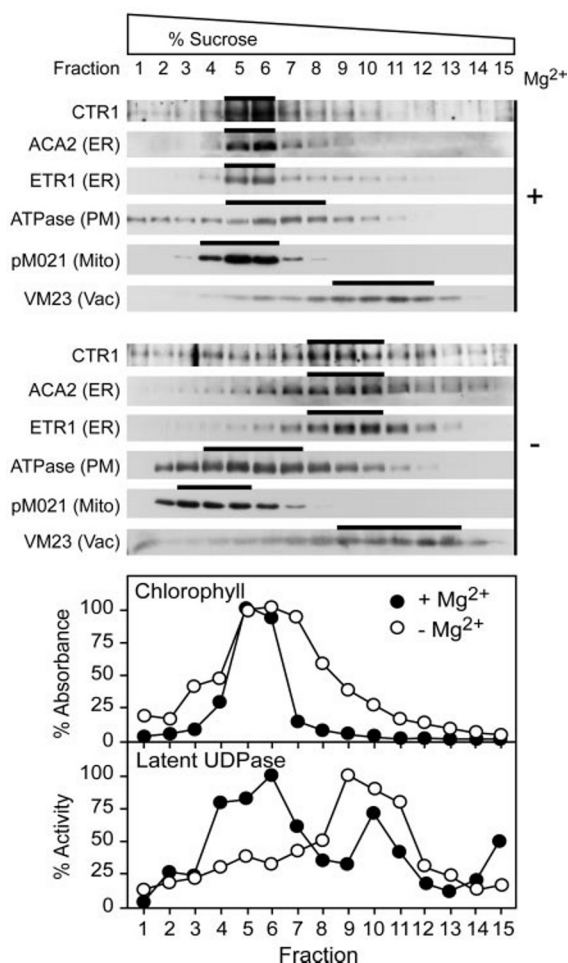

FIG. 2. **Localization of CTR1 to the endoplasmic reticulum based on analysis by sucrose density gradient centrifugation.** Membranes were isolated from *Arabidopsis* plants treated with ACC for 24 h, and the membranes were then fractionated over 20–50% (w/w) sucrose gradients. Gradients were run in the presence of Mg (+) to stabilize membrane-associated proteins or in the absence of Mg (−) to dissociate membrane-associated proteins. Samples (20 μl) of each fraction were analyzed by immunoblot for CTR1, the ER markers ACA2 and ETR1, the PM marker H^+-ATPase, the mitochondrial inner membrane marker F1-ATPase (pM021), and vacuole marker VM23. Thylakoid membranes were identified spectrophotometrically. Golgi membrane fractions were identified based on Triton X-100-stimulated UDPase activity.

40–41% to 32–36% (w/w) sucrose, similar to that observed for the ER markers ACA2 and ETR1. The distribution of CTR1 could be differentiated from the plasma membrane marker (H^+-ATPase), the mitochondrial inner membrane marker (pM021), the tonoplast marker (VM23), and the chloroplast thylakoid marker (chlorophyll absorbance), which did not demonstrate the same Mg^{2+}-induced shift. CTR1 could be differentiated from the Golgi marker (latent UDPase), which had a broader distribution than CTR1 in the Mg^{2+}-containing gradient and fractionated as two peaks. Although the majority of CTR1 is associated with the ER based on sucrose density gradient centrifugation, a small amount of CTR1 did not demonstrate a Mg^{2+}-dependent density-shift and may thus represent protein that resides at another membrane.

Effect of Mutations in CTR1 upon its Membrane Association—Loss-of-function mutations have been isolated in *CTR1* (18, 19). We analyzed these mutants for their effects upon the expression of CTR1 and the ability of CTR1 to associate with membranes. For this purpose, we performed one set of experiments using dark-grown seedlings because this is a growth
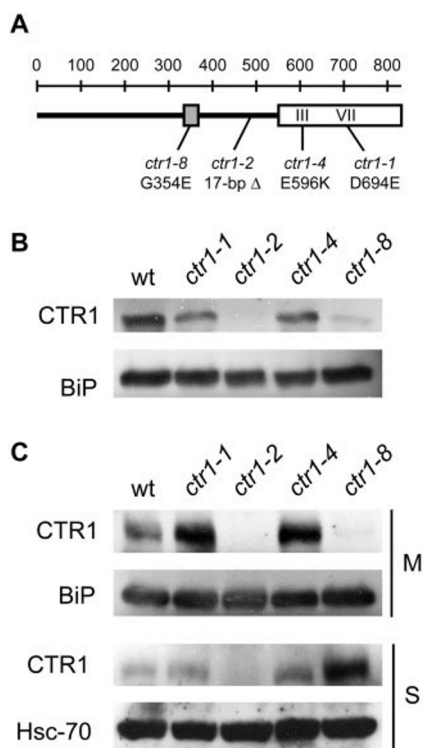
FIG. 3. **Effect of mutations in CTR1 upon expression and membrane localization of the protein.** *A*, sites of mutations within the CTR1 sequence. The *open square* represents the kinase domain of CTR1; the *gray square* represents the CN box found in other CTR1-like proteins. *B* and *C*, expression and membrane localization of CTR1 mutants. Soluble (*S*) and membrane (*M*) fractions of *Arabidopsis* were isolated from wild type (wt) and from different *ctr1* mutants (*ctr1-1*, *ctr1-2*, *ctr1-4*, and *ctr1-8*). Immunoblot analysis was performed on 20 μg of protein using antibodies against CTR1, BiP (membrane loading control), and Hsc-70 (soluble loading control). Expression was analyzed in membrane fractions from dark-grown seedlings grown in the presence of 50 μM ACC (*B*), and in membrane and soluble fractions from plants grown in liquid culture and treated for 48 h with 50 μM ACC (*C*).

condition that displays a pronounced and well-characterized ethylene response (4–6). However, we were able to detect CTR1 only in the membrane fraction of dark-grown seedlings (Fig. 1*A*), potentially due to rapid turnover of any soluble CTR1. We therefore performed a second set of experiments using plants grown in liquid culture under lights, because with this growth condition CTR1 was detectable in the soluble fraction as well as in the membrane fraction. In both sets of experiments, plants were treated with ACC to induce the ethylene receptors and CTR1 to their maximal expression levels, thus helping to equalize expression of these ethylene-induced genes in the different genetic backgrounds. Results from these experiments are discussed below.

Various *ctr1* alleles were analyzed to determine if they produced stable ctr1 protein and if the mutation affected association with membranes. All the mutants examined displayed a constitutive ethylene response phenotype in both dark-grown seedlings and plants grown in liquid culture (19 and data not shown). The *ctr1-2* is a 17-bp deletion that results in a frameshift within the first third of the coding region (Fig. 3*A*) (18). No CTR1 protein was detected in *ctr1-2* seedlings (Figs. 1 and 3), suggesting that the protein is absent or prematurely terminated. The *ctr1-1* and *ctr1-4* alleles are missense mutations affecting highly conserved residues of the kinase domain (18, 19). The ctr1-1 mutant protein expressed and purified from baculovirus has been demonstrated to lack kinase activity (19). Although there was some variability in the ctr1-1 and ctr1-4 protein expression levels, which was dependent upon the

growth condition used, both proteins were readily detected in membranes isolated from *Arabidopsis* (Fig. 3), indicating that the mutant phenotype arises from the lack of kinase activity rather than destabilization of the protein. The *ctr1-8* allele is a missense mutation is in the N-terminal half of CTR1 that alters a residue that is highly invariant in a domain (the CN box) found in CTR1-like proteins in *Arabidopsis* and other plants. The ctr1-8 allele does not affect the kinase activity of purified heterologous CTR1 protein *in vitro*, but rather has been shown to reduce the ability of CTR1 to interact with the ethylene receptor ETR1 using yeast two-hybrid analysis (19). The level of membrane-associated ctr1-8 protein was substantially reduced compared with that found in wild type (Fig. 3), in both dark-grown seedlings and plants grown in liquid culture. Analysis of plants grown in liquid culture revealed that coincident with the decrease in membrane-associated ctr1-8 was an increase in the level of soluble ctr1-8 (Fig. 3*C*), indicating that the ctr1-8 mutation did not simply reduce expression of the mutant protein but instead reduced its ability to associate with membranes.

Effect of Mutations in Ethylene Receptors upon the Membrane Association of CTR1—If membrane localization of CTR1 is mediated by interactions between CTR1 and the ethylene receptors, then elimination of ethylene receptors should result in a decrease in the level of CTR1 found associated with the membranes. Loss-of-function mutations have been isolated in ethylene receptor family members *ETR1*, *ETR2*, *ERS2*, and *EIN4* (42). All of these mutations are predicted to eliminate production of full-length protein, due to premature stop codons (*etr1*, *etr2*, and *ein4* mutations) or a T-DNA insertion in the coding region (*ers2* mutation). Lack of full-length protein has been confirmed for the *etr1* loss-of-function mutations (24). In addition, a T-DNA insertion mutation has been identified in the 5′-untranslated region of the gene that encodes the ethylene receptor ERS1 (16, 24). This mutation reduces *ERS1* mRNA expression, and genetic evidence indicates that this mutant has reduced function compared with wild-type *ERS1*. Previous analyses of these ethylene-receptor mutants have indicated that whereas the single mutants have minimal effect upon ethylene responses in the plant, double and triple mutants induce progressively more pronounced ethylene-like responses in the plants (16, 24, 42). We examined single mutants as well as double and triple mutant combinations to determine if these had reduced levels of membrane-associated CTR1.

In dark-grown seedlings, only the *ers1* mutant by itself resulted in a significant reduction of membrane-associated CTR1 (Fig. 4*A*). The other single receptor mutants either had little effect or, in the case of the *etr1* mutant, actually resulted in an increased level of CTR1 at the membrane. In liquid culture, the single mutants also had little effect upon the levels of membrane associated CTR1, the *etr1* mutant again resulting in increased levels of membrane-associated CTR1. Whereas most single receptor mutants had limited effect upon the level of membrane-associated CTR1, double and triple mutants of the receptors resulted in decreased levels of membrane-associated CTR1 (Fig. 4). The triple mutants had substantially reduced levels of membrane-associated CTR1 when analyzed with either dark-grown seedlings or liquid cultures. By use of plants grown in liquid culture, a re-distribution of CTR1 from the membrane fraction to the soluble fraction could be observed in the triple mutants (Fig. 4*B*). These data indicate that ethylene receptors are required for the association of CTR1 with the membrane, because elimination of multiple receptors resulted in a re-distribution of CTR1 from the membrane to the soluble fraction. These data also demonstrate that this requirement is not strictly additive; for example, the single mutants of *etr2*
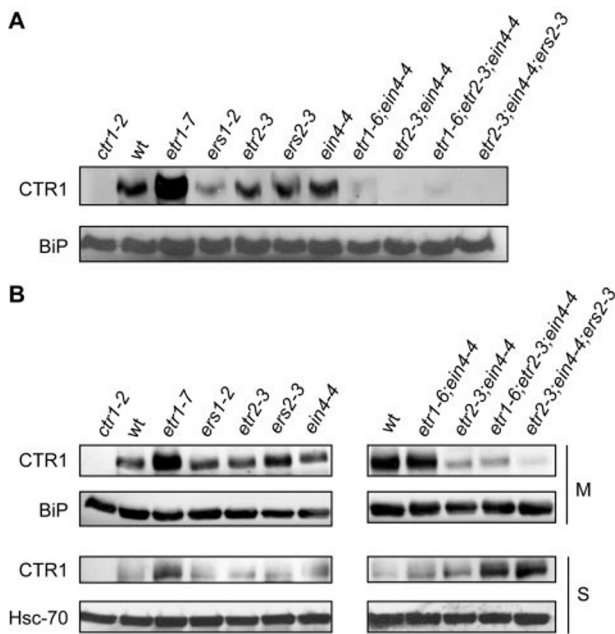
FIG. 4. **Effect of mutations in ethylene receptors upon membrane localization of CTR1.** Soluble (*S*) and membrane (*M*) fractions of *Arabidopsis* were isolated from mutant backgrounds containing single loss-of-function (*etr1-7*, *etr2-3*, *ers2-3*, and *ein4-4*), or reduction-in-function (*ers1-2*) mutations in ethylene receptors, double mutant combinations (*etr1-6/ein4-4* and *etr2-3/ein4-4*), and triple mutant combinations (*etr1-6/etr2-3/ein-4* and *etr2-3/ers2-3/ein4-4*). Each loss-of-function mutation is predicted to eliminate production of the relevant full-length receptor. Immunoblot analysis was performed on 20 μg of protein using antibodies against CTR1, BiP (membrane loading control), and Hsc-70 (soluble loading control). *A*, expression of CTR1 in membrane fractions isolated from ACC-treated dark-grown seedlings. *B*, expression of CTR1 in membrane and soluble fractions isolated from plants grown in liquid culture and treated for 48 h with 50 μM ACC.

FIG. 5. **Stability of CTR1 association with membranes.** Microsomal membranes (1 mg/ml) were treated with 0.5 M NaCl, 1% (w/v) Triton X-100, or 0.5% (w/v) lysophosphatidylcholine (LPC), then centrifuged at 100,000 × *g* for 30 min. The different lanes represent the protein from total membranes prior to extraction (*T*), and from the soluble (*S*) and pellet (*P*) fractions after extraction. The relative amounts of CTR1, ETR1, and BiP present in each fraction were determined by immunoblot analysis. BiP served as an internal control for extraction of membranes by Triton X-100.
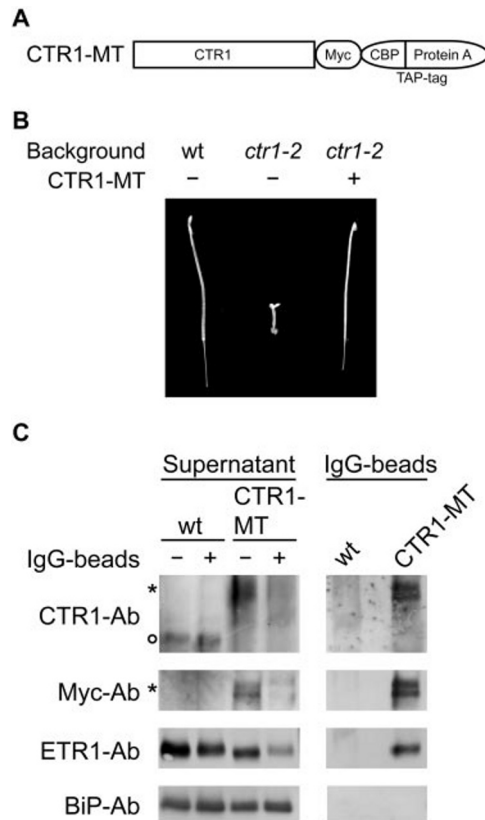
FIG. 6. **Affinity purification of CTR1 from *Arabidopsis* results in co-purification of ETR1.** *A*, structural features of CTR1-MT. The tagged version of CTR1 contains a c-Myc epitope tag and a TAP tag with calmodulin binding peptide (CBP) and a protein A peptide. Features are not drawn to scale. *B*, expression of CTR1-MT complements the *ctr1-2* mutation of *Arabidopsis*. Phenotypes of dark grown seedlings in air are shown. *C*, co-purification of ETR1 with CTR1-MT. *Arabidopsis* membranes from wild type and transgenic *ctr1-2* plants containing CTR1-MT were solubilized with 0.5% (w/v) lysophosphatidylcholine. The soluble supernatant obtained after centrifugation at 100,000 × *g* for 30 min was incubated with IgG beads. CTR1-MT bound to the IgG beads and resulted in co-purification of ETR1. CTR1-MT (indicated by *) was detected by CTR1-Ab and Myc-Ab, and is observed as a doublet presumably due to limited proteolysis. Native CTR1 (indicated by o) was detected in wild-type plants with the CTR1-Ab. ETR1 was detected with ETR1-Ab. BiP was detected with BiP-Ab, and served as an internal control that should not bind to the IgG beads.

and *ein4* have only a minimal effect upon CTR1 association with the membrane, but the double *etr2/ein4* mutant substantially reduces the level of membrane-associated CTR1.

Participation of CTR1 in Ethylene Receptor Signaling Complexes—The membrane association of CTR1 was examined by treatment of membranes with NaCl and detergents (Fig. 5). Treatment of membranes with NaCl was not effective for extraction of CTR1, indicating that the interaction of CTR1 with membranes does not rely solely upon ionic interactions. Treatment of membranes with the non-ionic detergent Triton X-100 was also not effective for extraction of CTR1 from membranes. Triton X-100 is capable of solubilizing peripheral and single-pass transmembrane proteins from plants (43–45), but is not effective for solubilizing the multi-pass transmembrane receptor ETR1 (Fig. 5). We found that CTR1, like ETR1, could be solubilized by treatment of *Arabidopsis* membranes with lysophosphatidylcholine (Fig. 5), an ionic phospholipid containing a single fatty acid chain. Thus, CTR1 is extracted from membranes under the same conditions that solubilize the transmembrane receptor ETR1, although CTR1 has no transmembrane domains itself, consistent with CTR1 being part of an ethylene receptor signaling complex.

To obtain direct evidence that CTR1 is part of an ethylene-receptor signaling complex in plants, we made a tagged version of CTR1. The tagged version of CTR1, called CTR1-MT, contains a c-Myc epitope tag to aid in immunological detection and a Tandem Affinity Purification (TAP) tag to aid in affinity purification (Fig. 6*A*). 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 (18). The CTR1-MT construct rescued the *ctr1-2* mutant phenotype (con-
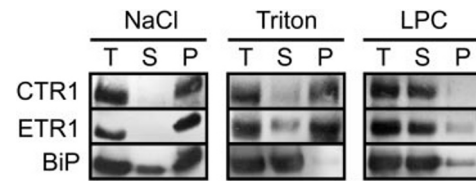
stitutive ethylene response) indicating that the tagged version of CTR1 is functional (Fig. 6*B*). The tagged version of CTR1 could be detected by immunoblot analysis using either an anti-c-Myc antibody or an anti-CTR1 antibody, and was typically observed as a doublet with molecular masses of 129 and 118 kDa (Fig. 6*C*). The 129-kDa polypeptide presumably represents the full-length CTR1-MT protein that has a predicted molecu-

Participation of CTR1 in ETR1 Signaling Complexes

lar mass of 123 kDa. The 118-kDa polypeptide may arise due to limited proteolysis of the full-length polypeptide.

The CTR1-MT protein was affinity purified by incubating lysophosphatidylcholine-solublized membrane proteins with IgG beads. The IgG beads bind the protein-A portion of the TAP tag, resulting in affinity purification of CTR1-MT (Fig. 6*C*). Significantly, we found that the ethylene receptor ETR1 co-purified with CTR1-MT on the IgG beads (Fig. 6*C*). Controls confirmed that IgG beads did not bind native CTR1 solubilized from wild-type plants; nor did IgG beads bind BiP a resident protein of the plant endoplasmic reticulum; nor did IgG beads bind ETR1 in a background that lacks CTR1-MT. Our results thus support the association of both ETR1 and CTR1 within the same protein complex.

Interaction of CTR1 with ETR1 Does Not Require the Histidine Kinase Activity of ETR1—Evidence obtained from two-hybrid analysis and *in vitro* binding studies indicates that CTR1 can directly interact with the histidine kinase domain of ETR1 and other ethylene receptors (19, 22, 23). Whereas ETR1 has histidine kinase activity (14), some members of the ethylene receptor family contain diverged histidine kinase domains predicted to lack this enzymatic activity, thereby calling into question the role of histidine kinase activity in the interactions between ethylene receptors and CTR1. We previously demonstrated that the histidine kinase domain of ETR1 has enzymatic activity when expressed in yeast, in contrast to what we observed when the same domain is expressed in *E. coli* (14). We therefore used the yeast expression system to assess the ability of CTR1 to interact with functional ETR1. Various GST fusions of ETR1 were co-expressed in yeast with a portion of CTR1 predicted to interact with ETR1 based on the two-hybrid analysis (22). The GST fusions of ETR1 were affinity-purified by binding to glutathione-agarose, and the presence of associated CTR1 determined immunologically.

CTR1 co-purified with affinity-purified GST-ETR1-(164–738), but not with affinity-purified GST alone, indicating that binding to the fusion protein was specific for the ETR1 domain (Fig. 7). A GST fusion containing just the histidine kinase domain of ETR1 (GST-ETR1-(333–609)) also bound CTR1, indicating that the GAF and receiver domains of ETR1 are not required for the interaction between ETR1 and CTR1. Site-directed mutations were used to test the necessity of the ETR1 histidine kinase activity for binding to CTR1. The glycine-containing G1 and G2 boxes are both part of the ATP binding site of histidine kinases, and mutations within these boxes eliminate autophosphorylation of ETR1 (14, 15). His-353 is the presumptive autophosphorylation site of ETR1 and mutation of this residue also abolishes autophosphorylation of ETR1 (14). None of these site-directed mutations eliminated the interaction between GST-ETR1 and CTR1, and thus indicate that the histidine kinase activity of ETR1 is not required for its interaction with CTR1.

DISCUSSION

Signal transduction involves protein-protein interactions and thus receptors are frequently found in protein complexes with their signaling partners. Our data demonstrate that the Raf-like kinase CTR1 is part of signaling complex with the ethylene receptor ETR1 in plants. Direct evidence for participation of CTR1 and ETR1 in the same protein complex comes from the finding that affinity purification of TAP-tagged CTR1 results in co-purification of ETR1. Additional correlative data are consistent with an association between CTR1 and ETR1 in plants. First, CTR1 is localized to the same subcellular membrane (the ER) as ETR1, even though CTR1 lacks transmembrane domains and known acylation motifs. Second, both CTR1 and ETR1 show a similar sensitivity to detergents when solu-
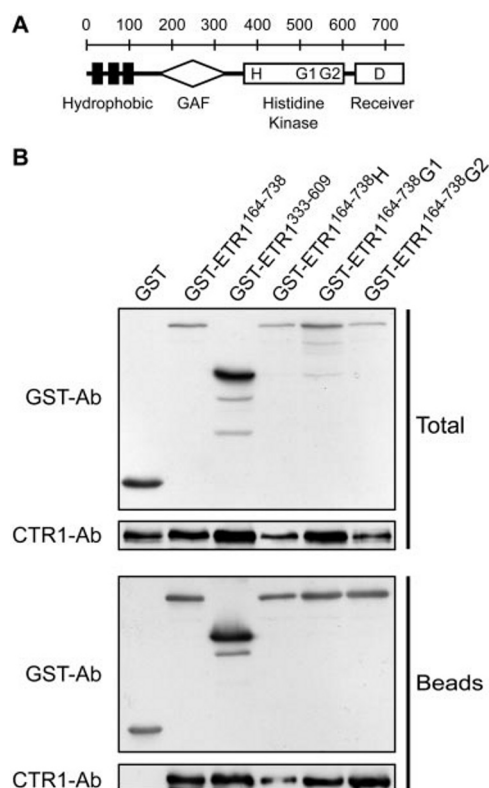

FIG. 7. **Interaction of CTR1 with the histidine kinase domain of ETR1 when transgenically expressed in yeast.** *A*, structural features of ETR1 and location of site-directed mutations. For the mutations, *H* refers to H353Q, *G1* refers to G515A/G517A, and *G2* refers to G545A/ G547A. *B*, co-purification of CTR1 with GST-ETR1 fusions in yeast. Soluble proteins were isolated from transgenic yeast expressing a portion of CTR1 representing amino acids 1–698, with which GST or various GST-ETR1 fusions were co-expressed. Immunoblot analysis is shown for the total souble fraction, and after affinity purfication of GST proteins by binding to glutathione agarose beads. GST and the GST fusions were detected with an anti-GST antibody. CTR1 was detected with the anti-CTR1 antibody.

bilized from membranes. Thus, two signaling components of disparate evolutionary origin, one from prokaryotes and one from eukaryotes, function within the same protein complex in plants.

Biochemical analysis of CTR1 from plants is limited to the demonstration that CTR1 and ETR1 are present within the same protein complex. However, several lines of evidence support a direct interaction between CTR1 and ETR1. Previous experiments with the yeast two-hybrid system and with *in vitro* binding assays on proteins expressed and purified from *E. coli* both support an interaction between CTR1 and ETR1 (22). Data reported here demonstrate an interaction between CTR1 and GST fusions of ETR1 using affinity purification after co-expression in yeast. These data indicate that the histidine kinase domain of ETR1 by itself is sufficient for the interaction with CTR1, results consistent with those obtained from the two-hybrid system (22). These data extend previous analyses by revealing that the enzymatic activity of ETR1 is not required for its interaction with CTR1. The specific sequence requirements within the histidine kinase domain of ETR1 responsible for binding CTR1 have not been resolved.

Analysis of interactions between CTR1 and the ethylene receptors using the yeast two-hybrid system finds the strongest interaction with ETR1, and only weak interactions with ERS1 and ETR2 (22, 23). Our data support the ability of CTR1 to interact with other members of the ethylene receptor family, not just ETR1. Analysis of loss-of-function mutations that elim-
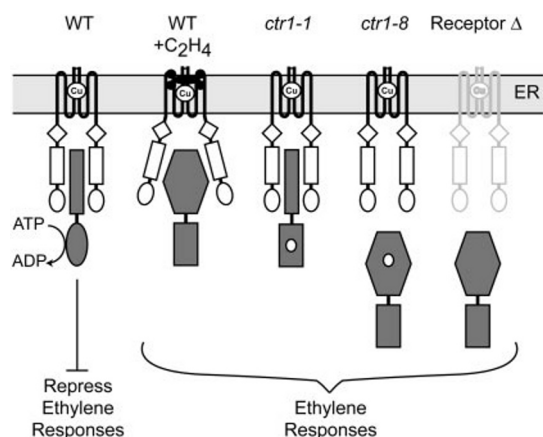
FIG. 8. **A model for signaling by the ethylene receptor·CTR1 complex.** The ethylene receptor contains one ethylene-binding site per homodimer, with ethylene binding mediated by a single copper ion (Cu) present in the ethylene-binding site (9). CTR1 (shown in *gray*) interacts with the histidine kinase domain of the receptor and as a result of this interaction is localized to the ER. 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. Mutations in CTR1 (indicated by a *white circle*) can result in an ethylene-like response in air by two different mechanisms. Mutations such as *ctr1-1* eliminate the kinase activity of CTR1 so that CTR1 is unable to repress the ethylene responses. Mutations such as *ctr1-8* disrupt the interaction of CTR1 with the receptor, resulting in mis-localization of CTR1 to the cytosol. Loss-of-function mutations that eliminate multiple members of the ethylene receptor family (receptor Δ) also result in mis-localization of CTR1 to the cytosol. In the cytosol, CTR1 may adapt a kinase-inactive conformation (as shown here) or may not be proximate to the appropriate phosphorylation substrate. Under some growth conditions, cytosolic CTR1 may be rapidly turned over.

inate expression of full-length ethylene receptors is consistent with multiple members of the receptor family playing roles in membrane localization of CTR1. A single mutant in ETR1 did not result in loss of CTR1 from the membrane, as would be predicted if it were primarily responsible for membrane localization of CTR1. Although most single mutants did not result in significant loss of CTR1 from the membrane, double and triple mutant combinations did, thereby implicating multiple receptors in the membrane localization of CTR1. Of particular significance, a triple mutant involving solely members of subfamily 2 (*etr2/ers2/ein4*) resulted in a substantial loss of CTR1 from the membrane. Subfamily 2 members lack conserved residues required for histidine kinase activity (6, 7, 13), but we show here that histidine kinase activity is not a requirement for interaction with CTR1 by mutation of conserved residues in ETR1. Thus members of both subfamily 1 and subfamily 2 play roles in the membrane localization of CTR1, the simplest explanation for this being that all of the receptors are able to interact with CTR1.

Results reported here clarify the mechanism of action of CTR1 and its requirements for suppression of ethylene responses. A model incorporating these results is shown in Fig. 8. Based on genetic analysis, CTR1 is a negative regulator of ethylene responses in *Arabidopsis*, with loss-of-function mutations in CTR1 resulting in a constitutive ethylene response phenotype (18, 19). We find that kinase-inactive mutants of CTR1 still make full-length CTR1 protein that associates with the membrane, results consistent with the mutant phenotype arising from the lack of kinase activity. Although kinase activity of CTR1 is required, it is not sufficient to repress ethylene responses. Correct subcellular localization is also required. Disruption of the ability of CTR1 to interact with ethylene receptors (*ctr1-8* mutation) resulted in a loss of CTR1 protein from

the membrane. However, even though the ctr1-8 protein is still present as a soluble protein in plants grown in liquid culture, these plants exhibit a constitutive ethylene response phenotype. Similarly, elimination of multiple ethylene receptors also results in a loss of CTR1 protein from the membrane, and a constitutive ethylene response phenotype. Soluble CTR1 may be unable to repress ethylene responses due to: 1) a proposed autoinhibition of its kinase activity by its N-terminal domain, inhibition that could be relieved by interaction of its N-terminal domain with the ethylene receptors (19); or 2) no longer being proximate to a required substrate presumably located at the ER. The lack of activity found when CTR1 no longer is associated with the membrane suggests one model by which ethylene could conceivably regulate downstream signaling: ethylene binding to the receptor could result in release of CTR1. However, our data do not support such a model. In contrast to what this model would predict, we found increased levels of CTR1 at the membrane in the presence of ethylene, indicating instead that CTR1 may form a stable complex with the ethylene receptors at the endoplasmic reticulum. Changes in activity of CTR1 may thus be the result of conformational changes taking place within the signaling complex, rather than release of CTR1 from the signaling complex.

The loss of CTR1 from the membrane in the various receptor loss-of-function mutant backgrounds is similar to the physiological effects of these mutants upon ethylene responses. Single receptor mutants have little effect upon the plant, but double and triple mutant combinations demonstrate a progressively more pronounced ethylene response-like phenotype (42). Similarly, less CTR1 was found at the membrane in the double and triple receptor mutants than in the single mutants. The one exception to this trend is the *etr1-7* loss-of-function mutant, which of the single mutants is the only one to show a small but consistent ethylene response phenotype (23, 42). Paradoxically, we found 2–4-fold higher levels of CTR1 at the membrane in the *etr1-7* mutant, potentially due to a compensatory effect in the plant for the loss of ETR1. For example, another member of the ethylene receptor family might be induced in the *etr1-7* mutant to functionally compensate for the loss of ETR1, and this would presumably result in additional CTR1 being brought to the membrane in association with the induced receptor. Evidence for functional compensation between members of the ethylene receptor family has been found in tomato (46). However, the *etr1-7* mutant still exhibits a partial ethylene-response phenotype even with the higher levels of CTR1 present at the membrane, and thus ETR1 may play a specific role in activation of CTR1 that cannot be substituted for by other members of the receptor family.

Analysis of the ethylene receptor loss-of-function mutants did not reveal a clear additive effect upon the membrane localization of CTR1. For example, the *etr2* and *ein4* mutants individually had little effect upon the level of membrane-associated CTR1 compared with wild type, but an *etr2/ein4* double mutant resulted in a substantial loss of CTR1 from the membrane. One possible explanation for this phenomenon is that the interaction with CTR1 could involve multiple receptors, the stoichiometry of CTR1 interaction with the receptors being unknown. Alternatively, as discussed above, expression of another member of the ethylene receptor family could be induced to functionally compensate for the loss of a single receptor.

The assembly of protein complexes typically involves mechanisms to coordinately regulate production of the proteins within the complex. Our results indicate that the production of new ethylene receptor signaling complexes in *Arabidopsis* involves the interplay of transcriptional and post-transcriptional regulatory mechanisms. Previous work has shown that ethyl-

ene induces the expression of some ethylene receptor family members in plants at the transcriptional level (17, 47–49). Thus, one might expect to find ethylene-induced changes in the expression of other members of the receptor complex. Consistent with this possibility, ethylene resulted in a post-transcriptional increase in the levels of CTR1 at the *Arabidopsis* membrane. The ethylene-induced receptors may bind to and stabilize CTR1, thereby resulting in the increase of CTR1 levels at the membrane. Unbound CTR1 may be rapidly turned over in dark-grown seedlings, where we found no evidence for CTR1 in the soluble fraction. In tomato, a CTR1-like gene has also been identified that is regulated by ethylene, but this regulation occurs at the transcriptional level (39, 40). Thus, assembly of the receptor complexes may involve both transcriptional and post-transcriptional mechanisms, and these mechanisms may vary with the plant species.

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