Ligand-induced Degradation of the Ethylene Receptor ETR2 through a Proteasome-dependent Pathway in Arabidopsis*

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Protein degradation plays an important role in modulating ethylene signal transduction in plants. Here we show that the ethylene receptor ETR2 is one such target for degradation and that its degradation is dependent upon perception of the signaling ligand ethylene. The ETR2 protein is initially induced by ethylene treatment, consistent with an increase in transcript ing ligand ethylene. The ETR2 protein is initially induced by ethylene receptor ETR2 is one such target for degradation and ethylene signal transduction in plants. Here we show that the ETR2 protein is initially induced by ethylene treatment, consistent with an increase in transcript.

In plants, the gaseous hormone ethylene regulates seed germination, seedling growth, leaf and petal abscission, organ senescence, ripening, stress responses, and pathogen responses (4). Plant responses to ethylene have been found to occur over a range of ethylene concentrations from 0.2 nl/liter to 1000 μl/liter (4 – 6). In Arabidopsis, ethylene is perceived by a five-member receptor family that includes ETR1, ETR2, ERS1, ERS2, and EIN4 (7, 8). The ethylene receptors have similar overall structures with transmembrane domains near their N termini and putative signaling motifs in their C-terminal halves. The transmembrane domains apparently serve two functions. First, they are responsible for membrane localization of the receptors, the receptor ETR1 having been demonstrated to localize to the endoplasmic reticulum (ER)2 (9). The ER is an unusual location for a hormone receptor but one compatible with the ready diffusion of ethylene in aqueous and lipid environments. Second, based on genetic and biochemical evidence, the transmembrane domains contain the ethylene-binding site (10, 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 as components in bacterial phosphorelays and are now known to be present in plants, fungi, and slime molds (12).

The five-member family of Arabidopsis ethylene receptors contains two subfamilies based on phylogenetic analysis and some shared structural features (8). Members of subfamily 1 (ETR1 and ERS1) contain three transmembrane domains and histidine kinase domains with all of the conserved residues required for histidine kinase activity (13, 14). Members of subfamily 2 (ETR2, ERS2, and EIN4) contain the three conserved transmembrane domains plus an additional N-terminal hydrophobic domain that is predicted to serve as a signal sequence. In addition, their histidine kinase domains lack residues essential for histidine kinase activity, and they have been proposed to act as Ser/Thr kinases (14). Members from both subfamilies (ERS1, ETR2, and ERS2) are induced by ethylene at the transcriptional level (15), indicating that the same ligand detected by the receptors also affects expression levels of the receptors.

In this paper, we report on the biochemical characterization of ETR2, a member of subfamily 2 that has not been characterized previously at the protein level. Our results are consistent with a model whereby binding of ethylene to ETR2 induces degradation of the receptor, similar to what has been observed...
following ligand binding to animal growth factor receptors. But in contrast to the paradigm established with animal growth factor receptors, turnover of ethylene receptors is mediated by the proteasome rather than the lysosome, a situation potentially arising due to localization of receptors to the endoplasmic reticulum rather than to the plasma membrane.

EXPERIMENTAL PROCEDURES

Plant Materials—Wild-type Arabidopsis (Columbia ecotype) and various ethylene response mutants were used, including ethylene-insensitive mutants (etr1-1, etr1-2, ein2-1) (16–18), a constitutive ethylene response mutant ctr1-2 (19), and receptor loss-of-function mutants (etr1-7 and etr2-3) (20). The etr1-2, ctr1-2 and the etr1-1;ctr1-2 double mutants were generated by crossing, with plants homozygous for ctr1-2 identified by the constitutive ethylene response phenotype and those homozygous for etr2-1 and etr1-1 identified by PCR-based genotyping (20).

Plant Growth Conditions—For growth of etiolated seedlings, sterile seeds were placed on Petri dishes with half-strength Murashige and Skoog basal medium with Gamborg’s vitamins (pH 5.75; Sigma) and 0.8% (w/v) agar. Aminoethylvinyl-Gly (5 μM) was included in the growth medium to inhibit ethylene biosynthesis by the seedlings. After a 2-day cold treatment at 4 °C, plates were exposed to light at 22 °C for 0.5 days. Plates were then placed in sealed chambers, and seedlings were grown for 3.5 days in the dark at 24 °C in the presence of ethylene at the indicated 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. For short term treatments with inhibitors, seedlings were grown on filter paper placed on the solid plate medium and then moved to the same medium without agar containing the inhibitor and incubated for the indicated times. In cases where prolonged exposure to inhibitors in aqueous solution was required, plants were grown under constant light in liquid culture (21) to allow for consistency between growth and treatment conditions. Sterile seeds were grown in full-strength Murashige and Skoog basal medium with Gamborg’s vitamins supplied with 2% (w/v) sucrose for ~2 weeks. Ethylene biosynthesis was induced in liquid culture seedlings by the addition of the biosynthetic precursor 1-aminoacyclopropane-1-carboxylate (ACC) at 1 mM for 24 h. Growth in the light increases ACC oxidase activity (22) and therefore the levels of ethylene capable of being produced by the addition of ACC. The proteasome inhibitors and brefeldin A were added directly to the liquid growth medium at the times and concentrations indicated.

Preparation of the ETR2 Antibody—Fusion proteins were made of ETR2 with either a His6 tag (ETR2-His6) or a GST tag (ETR2-GST). A portion of the ETR2 gene (17) representing amino acids 390–570 was amplified by PCR using a 5’ primer containing a BamHI site (GGATCTCCTGATTCTACGAGAGA) and a 3’ primer containing a Xhol site (CTCGAGTTGTCCCTAACCCTATG). The PCR product was cloned into the vector pET-28a (Novagen, Madison, WI) to make ETR2-His6 and into the vector pGEX-KG (23) to make ETR2-GST. The ETR2-His6 protein was expressed in Escherichia coli, and inclusion bodies were isolated according to the manufacturer by solubilizing with 6 M urea and affinity-purifying on His-bind resin (Novagen). The ETR2-GST protein was expressed in E. coli and purified by binding to glutathione-agarose (Sigma) (24). Polyclonal antiserum was prepared from the ETR2-His6 recombinant protein by Cocalico Biologicals, Inc. (Reamstown, PA). The anti-ETR2 antibodies were affinity-puriﬁed with glutathione-agarose columns cross-linked with ETR2-GST (25). Antibodies were eluted with 100 mM glycine, pH 2.5, and the pH was neutralized with 1 M Tris, pH 8.0.

Membrane Fractionation and Immunoblot Analysis—Microsomal and soluble fractions were isolated from dark-grown seedlings or plants grown in liquid culture using a homogenization buffer containing 30 mM Tris (pH 8.2 at 22 °C), 150 mM NaCl, 10 mM EDTA, and 20% (v/v) glycerol with protease inhibitors as described (9, 26). Briefly, the plant material was homogenized and then centrifuged at 5,000 × g for 5 min. The supernatant was then centrifuged at 100,000 × g for 30 min, and the resulting membrane pellet was 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 (9). Specific membranes were identified by use of antibodies against the ER markers ETR1 and ACA2 (9, 27), the plasma membrane (PM) marker H+-ATPase (9, 27), the tonoplast marker VM23 (28), the mitochondrial inner membrane marker F1-ATPase (29), and the Golgi marker α-mannosidase I (30). Thylakoid membranes were identified by spectrophotometric analysis of chlorophyll levels (21). Immunodecorated proteins were visualized and quantified as described (26).

In Vitro Assay for ETR2 Degradation—Microsomes from seedlings grown in liquid culture were isolated using an homogenization buffer containing 25 mM Tris-HCl (pH 7.6 at 22 °C), 10 mM MgCl2, 10 mM NaCl, 5 mM dithiorthioëitol, 20% (v/v) glycerol, and resuspended at 10 mg/ml in the same buffer but with 10% (v/v) glycerol. In vitro assays were performed in 50 μl of resuspension buffer, containing 25 μg of microsomal protein, 5 mM ATP, and inhibitors as indicated. Following 1 h of incubation at 22 °C, the reaction was terminated by addition of 6× SDS-PAGE loading buffer.

Real Time PCR—Total RNA was isolated from etiolated seedlings by using the RNase-free plant kit followed by treatment with RNase-free DNase I according to the manufacturer’s instructions (Qiagen, Germantown, MD). RNA (1 μg) pooled from three biological replicates was used as template for first-strand cDNA synthesis using SuperScript III (Invitrogen). Real time PCR was performed using a mix containing 10 μl of 2× SYBR Premix Ex Taq (TaKaRa Bio Inc., Otsu, Shiga, Japan); 0.8 μl of forward and reverse primer mix (0.2 μM final concentration), 0.4 μl of 50× ROX Reference Dye II, 5 μl of cDNA, and 3.8 μl of deionized water. PCR was run on an ABI 7500 fast real time PCR system (Applied Biosystems) using a 10-min initial denaturation at 95 °C, followed by 40 cycles of 95 °C (15 s) and 56 °C (1 min). Three experimental replicates of each reaction were performed using primer pairs specific for ETR2 (At3g23150) (5’-tgctgtcgttatgccggtg-3’ and 5’-agagaaacctttgctgatggt-3’) and β-tubulin (At5g62700) (5’-ctaaattcctcgttcaagt-3’ and 5’-agagttcggctgtctc-3’). Average Ct values were generated and analyzed by SDS software, version.
**Ligand-induced Degradation of ETR2**

![Image of a table showing the effects of ethylene on ETR2 protein expression]

**FIGURE 1. Induction of ETR2 protein expression by ethylene.** Membranes were isolated from wild type (wt), loss-of-function mutants in ETR2 (etr2-3) and ETR1 (etr1-7), ethylene-insensitive mutants (etr2-1, etr1-1, ein2-1), and a constitutive ethylene response mutant (ctr1-2). The ethylene receptors ETR2 and ETR1 were detected by immunoblot analysis using 20 μg of protein, with immunodetectable H+ -ATPase serving as an internal loading control. Ethylene induction of proteins was tested by treating the 2.5-day-old dark-grown seedlings for 24 h with either air (-) or 1 μl/liter ethylene (+).

1.4, which uses the comparative cycle threshold method (31). -Fold change compared with the untreated wild-type control was calculated after normalization to the β-tubulin Ct values. All primer sets generated a single distinct peak during melting curve analysis.

**RESULTS**

**Biochemical Characterization of ETR2**—Previous analysis has centered on ETR1, a member of subfamily I that appears to be constitutively expressed based on prior analysis of RNA and protein levels. ETR2 is a member of subfamily II and, based on Northern blot analysis, is induced at the transcriptional level by ethylene (15). To study ETR2 at the protein level, we raised and affinity-purified a polyclonal antibody against ETR2. The anti-ETR2 antibody was used to probe membranes from wild-type Arabidopsis that had been treated with either air or ethylene (1 μl/liter for 24 h) (Fig. 1). The anti-ETR2 antibody recognizes a protein of 83 kDa in the membrane fraction of Arabidopsis, consistent with the calculated molecular mass of 86 kDa for ETR2. The ETR2 receptor is induced by ethylene in wild type and absent in the loss-of-function etr2-3 mutant. Ethylene induction of ETR2 is not affected by a loss-of-function mutant in another ethylene receptor (etr1-7). The ability of ethylene to induce ETR2 is curtailed in the ethylene-insensitive mutants etr1-1, etr2-1, and ein2. In contrast, ETR2 is present without ethylene treatment in the constitutive ethylene response mutant ctr1-2. These data indicate that protein levels of the ethylene receptor ETR2 are tightly controlled by ethylene in plants and are consistent with transcriptional regulation previously reported (15). For comparison, protein levels of the subfamily-1 ethylene receptor ETR1 are shown, a receptor whose expression is not induced by ethylene. As previously found, the ethylene-insensitive mutant etr1-1 is present at increased levels relative to wild-type ETR1 (26).

We previously found that the ethylene receptor ETR1 is localized to the ER membrane (9). To determine whether ETR2 is localized to the ER, we fractionated Arabidopsis membranes by sucrose density gradient centrifugation (Fig. 2). For this purpose, we used the ctr1-2 mutant line in which ETR2 is constitutively expressed (Fig. 2A) as well as wild-type plants in which ETR2 was induced by treatment with the ethylene precursor ACC (Fig. 2B). ER vesicles migrate to different densities dependent on whether they are associated with ribosomes or not. Ribosomes can be stripped from the ER by removal of Mg2+ from the medium, resulting in a diagnostic shift of the ER from higher to lower density (32). Fractions were analyzed by immunoblotting for ETR2 as well as for the membrane markers ETR1 and ACA2 (ER), H+ -ATPase, the mitochondrial inner membrane marker F1-ATPase (pM021), the Golgi marker α-mannosidase, and the vacuole marker VM23. Thylakoid membranes were identified spectrophotometrically.

**FIGURE 2. Localization of ETR2 to the endoplasmic reticulum based on sucrose density gradient centrifugation.** Membranes isolated from ctr1-2 (A) or ACC-treated wild-type (B) plants were fractionated over 20–50% (w/w) sucrose gradients. Gradients were run in the presence of magnesium (+) to stabilize membrane-associated proteins or in the absence of magnesium (−) to dissociate membrane-associated proteins. Samples (20 μl) of each fraction were analyzed by immunoblot for ETR2, the ER markers ACA2 and ETR1, the PM marker H+ -ATPase, the mitochondrial inner membrane marker F1-ATPase (pM021), the Golgi marker α-mannosidase, and the vacuole marker VM23. Thylakoid membranes were identified spectrophotometrically.
Receptor levels were determined by immunoblot analysis. (Fig. 3). But at ethylene concentrations above 1 μl/liter, the levels of ETR2 protein decreased in a manner independent of the message levels, which remained essentially unchanged. In addition, a time course revealed that dynamic changes in ETR2 expression occur in response to treatment with 100 μl/liter ethylene that cannot be explained by transcriptional changes (Fig. 3). Ethylene treatment resulted in an initial increase in ETR2 levels consistent with increased levels of mRNA expression. This was then followed by a rapid decrease in ETR2 protein levels that was independent of the mRNA expression levels. Both the dose-response and time course studies are thus consistent with post-transcriptional regulation of ETR2 expression. Under the same conditions, only a modest effect was noted on ETR1 expression at the protein and mRNA levels (data not shown).

To separate out transcriptional and post-transcriptional effects upon expression, we examined the effects of ethylene upon expression of ETR2 in the ctr1-2 mutant background. This mutation results in constitutive activation of ethylene responses in the plant (19), including constitutive expression of ETR2 (Fig. 1). Thus, one is able to examine changes in ETR2 expression that operate post-transcriptionally, without interfering changes in transcript levels of the gene. Ethylene dose-response analysis showed a reduction in ETR2 protein levels with increased levels of ethylene; a reduction was observed at ethylene concentrations of 0.1 μl/liter and is clearly apparent above 1 μl/liter (Fig. 3). In contrast, no significant differences in RNA levels were noted for the receptors, as predicted when using the ctr1 mutant background. Thus, the ethylene-induced decrease in ETR2 levels occurs post-transcriptionally. A time course analysis indicated that the ethylene effect upon ETR2 expression can be observed within 1 h of ethylene treatment (Fig. 3).

**Ethylene Perception by the Receptors Initiates Ligand-induced Turnover of ETR2**—We used both chemical and genetic approaches to confirm that the ligand-induced turnover of ETR2 results from ethylene perception by the receptors. Silver replaces a copper cofactor required by the receptors for binding ethylene, resulting in ethylene insensitivity (11). The etr-1 and the etr-1-1 mutants are both missense mutations within the predicted ethylene-binding regions of the receptors that result in dominant ethylene insensitivity (17, 33). Treatment of ctr1 plants with silver or introduction of either the etr-2-1 or the etr-1-1 mutations into the ctr1 background abolished ethylene-induced turnover of ETR2 protein (Fig. 4). Thus, ethylene perception by the receptors initiates the ethylene-induced decrease in ETR2 protein levels. These data also indicate that even at the highest ethylene level tested (1000 μl/liter) that this is not a generic effect of ethylene upon protein expression but

### Figure 3. Dose and time dependence of ETR2 expression upon ethylene.

Dark-grown wild-type (wt) or ctr1-2 seedlings were treated with ethylene at concentrations and times indicated prior to harvest at 3.5 days. To determine the effect of ethylene concentration (Dose Response) upon expression of ETR2, seedlings were treated for 24 h with air and 0.01, 0.1, 1, 10, 100, and 1000 μl/liter ethylene. To determine the effect of incubation time (Time Course) for the effect of ethylene upon expression of ETR2, seedlings were treated with 100 μl/liter (wt) or 1000 μl/liter (ctr1-2) ethylene for the indicated times. Protein levels of ETR2 (Protein) were determined by immunoblot analysis with an anti-ETR2 antibody. Representative immunoblots are shown, with relative levels of ETR2 being based on the average of two experiments and normalized relative to the ATPase loading control (LC). Message levels for ETR2 (mRNA) were determined by real time PCR.

### Figure 4. Ethylene perception by receptors initiates ethylene-mediated turnover of ETR2.

**A**. Effect of silver treatment upon turnover of ETR2 by ethylene. Dark-grown ctr1-2 seedlings were grown in the presence or absence of 10 μg/ml AgNO3, an inhibitor of ethylene perception, and then treated with air or 1000 μl/liter ethylene for 24 h. **B**. Effect of ethylene-insensitive mutations in receptors upon ethylene-induced turnover. Seedlings containing double mutants of etr2-1;ctr1-2 or etr1-1;ctr1-2 were treated with air or the ethylene biosynthetic precursor ACC (1 μm) for 24 h in liquid culture. Receptor levels were determined by immunoblot analysis.
Ligand-induced Degradation of ETR2

arises due to interaction between ethylene and the receptors. Furthermore, these data indicate that the turnover of one member of the receptor family can be modulated by the ability of another member of the family to perceive ethylene, a situation reminiscent of the ethylene-insensitive mutant etr2-1 requiring the presence of ETR1 for full effect (34).

Ethylene Induces Proteasome-dependent Degradation of ETR2—Post-transcriptional effects upon receptor levels could be due to regulation of translational rate and/or regulation of protein degradation. To distinguish between these possibilities, we tested the effects of the protein biosynthetic inhibitor cycloheximide as well as proteasome inhibitors upon the ligand-induced decrease in ETR2 levels. Cycloheximide treatment allowed for the examination of turnover in the absence of translation, the prediction being that if the effect of ethylene was solely upon the translation rate then we should no longer observe an effect of ethylene upon ETR2 levels. We still observed the ethylene-induced decrease in ETR2 protein levels following in vivo treatment of seedlings with cycloheximide, indicating that the effect of ethylene was post-translational (Fig. 5A) (9). Consistent with a post-translational effect, we found that in vivo treatment of seedlings with two proteasome inhibitors, MG132 and ALLN (35), inhibited the ability of ethylene to down-regulate levels of ETR2 (Fig. 5B). These data support a model in which ETR2 is degraded by a proteasome-dependent pathway in response to ethylene binding.

Two lines of evidence suggest that degradation of ETR2 takes place at its subcellular location on the ER membrane. First, we found that ETR2 could be degraded in vitro in isolated microsomes (Fig. 5C). This degradation is sensitive to ALLN (Fig. 5C) and MG132 (not shown), thereby implicating the proteasome and suggesting that ETR2 degradation may occur due to proteasomes associated with the ER membrane. Additional evidence that degradation occurs at the ER was obtained by in vivo treatment of ctr1-2 seedlings with the vesicle trafficking inhibitor brefeldin A (36). Ligand-induced degradation of ETR2 was still observed following treatment with brefeldin A (Fig. 5D), suggesting that this process does not require export of ETR2 from the ER to other destinations of the secretory pathway, such as the PM or vacuole.

DISCUSSION

Recent data indicate that protein degradation plays a major role in the regulation of ethylene production and signal transduction. Levels of ACC synthase, an enzyme involved in ethylene biosynthesis, are regulated by protein degradation (37). Levels of EIN3, a key transcription factor in the ethylene signaling pathay, are also regulated by protein degradation, EIN3 levels increasing in the presence of ethylene (38–41). In both cases, a proteasome-dependent pathway has been implicated in regulating the protein levels. Our results demonstrate that levels of the ethylene receptor ETR2 are also regulated by proteasome degradation, but in this case, unlike EIN3, ethylene induces degradation of the receptor.

The post-transcriptional effect of ethylene on ETR2 levels is apparent at ethylene concentrations above 1 μl/liter, an ethylene concentration of physiological relevance, since ethylene responses occur from 0.2 nl/liter to 1000 μl/liter (4–6).

Although dark-grown Arabidopsis seedlings show a developmental response to ethylene initiated at less than 0.1 μl/liter, transcriptional changes have been shown to occur in Arabidopsis over a wide range of ethylene concentrations from 0.1 to 1000 μl/liter (6). Fruit ripening involves ethylene production that exceeds 100 μl/liter (4), an ethylene concentration likely to result in substantial decreases in the levels of ligand-bound receptors if similar mechanisms occur across plant species. We only detected a decrease in ETR2 levels in wild type at ethylene concentrations above 1 μl/liter, but turnover may still occur at

FIGURE 5. Degradation of ETR2 at the ER by a proteasome-dependent pathway. A, effect of cycloheximide (CH) on ethylene-induced turnover of ETR2. Dark-grown ctr1-2 seedlings were pretreated for 1 h with or without 300 μM CH and then treated with air or 1000 μl/liter ethylene for 8 h. B, in vivo analysis of proteasome inhibitors on ETR2 degradation. ctr1-2 seedlings were pretreated for 2 h with the proteasome inhibitors MG132 (100 μM) or ALLN (100 μM), a serine protease inhibitor phenylmethylsulfonyl fluoride (1 mM), or an MeSO (DMSO) control, followed by 12 h of treatment with the ethylene biosynthetic precursor ACC (1 mM) in liquid culture. C, in vitro analysis of proteasome inhibitors on ETR2 degradation. Membranes from wild-type or ctr1-2 plants were incubated for 1 h at 22 °C in the presence of 10 μM ALLN, 1 mM phenylmethylsulfonyl fluoride, or MeSO. D, in vivo analyses of BFA on ETR2 degradation. ctr1-2 seedlings were pretreated in liquid culture with BFA (5 or 50 μM) or an MeSO control for 2 h, followed by 1 mM ACC for 24 h. Levels of ETR2 and the ATPase loading control were determined by immunoblot analysis.
lower ethylene concentrations but not be as readily detected by our methods of analysis. For example, at the lower ethylene concentrations, turnover of ETR2 may be masked due to the smaller number of receptors turning over and/or offsetting effects of ethylene upon transcription and translation of ETR2. Our analysis of ETR2 in the ctr1 background suggests that turnover may occur at 0.1 μl/liter ethylene.

Ligand-induced degradation could conceivably occur by either direct or indirect mechanisms. In a direct mechanism, the physical act of ethylene binding would induce a change in the receptor that causes the receptor to become susceptible to degradation. In an indirect mechanism, ethylene binding would activate the signal transduction pathway to induce downstream ethylene responses, one possible response being the induction of a factor that would cause receptor turnover. Our data support the first model, with the physical association of ethylene with ETR2 being part of the process by which degradation of the receptor is initiated, potentially by inducing a conformational change in the receptor. This is based on our analysis of the ctr1-2 mutant, which is capable of inducing ethylene responses in the absence of bound ligand. Degradation of ETR2 did not occur in the ctr1-2 background except when seedlings were treated with ethylene. Furthermore, degradation of ETR2 in the ctr1-2 background could be abolished by inhibiting ethylene perception through either chemical (silver) or genetic (ethylene-insensitive receptor mutations) methods. These methods lock the receptor into the conformation that it normally has in the absence of ethylene, such that ethylene can no longer induce a conformational change in the protein (7, 8). Thus, ethylene-induced changes in conformation/activity of the receptor may be critical for initiating degradation.

Ligand-induced degradation of ethylene receptors is likely to play a significant role in the removal of receptor-ligand complexes, as has been found on binding of ligands to animal growth factor receptors (1–3). All five ethylene receptors bind ethylene tightly, the half-life for dissociation of ethylene from ETR2 being 10 h based on its analysis in transgenic yeast (10, 42). Thus, without ligand-induced degradation, the plant could accumulate receptors with bound ethylene that are unable to sense changes in the ethylene concentration. These receptor-ligand complexes can be considered as largely “inactive” based on the current model for ethylene signaling. According to this model, upon binding ethylene, the receptors inactivate the Raf-like kinase CTR1 (43–45). Because CTR1 functions as a negative regulator of ethylene signaling, its inactivation relieves repression of the pathway so that ethylene responses are induced. A receptor with bound ethylene is unable to stimulate CTR1 activity and thus no longer actively participates in the CTR1-dependent signaling pathway. Degradation of the ligand-receptor complex allows for its replacement by new unbound receptors that can stimulate CTR1 activity, thereby resensitizing the plant to ethylene.

The decrease in ethylene receptor levels resulting from ligand-induced turnover may also have other consequences for the propagation of the ethylene signal. Because of the negative regulation in the ethylene pathway described above, a decrease in overall ethylene receptor levels can result in increased sensitization of plants to ethylene, such that a lower concentration of ethylene is necessary for stimulating a response (34, 46). Indeed, if sufficient numbers of ethylene receptors are removed, the induction of ethylene responses can occur in the absence of ethylene (20). It is also possible that the removal of a specific subtype of receptors may serve to modulate different downstream responses. A precedent for such a possibility comes from, first, the differing roles for subfamily I and II receptors in signaling (47) and, second, the specific role for hybrid receptors (those with receiver domains) in seedling growth recovery after ethylene removal (48).

Degradation of ethylene receptors would result in release of the bound ethylene, so one would predict that the dissociation kinetics for ethylene from Arabidopsis would incorporate a more rapid ethylene dissociation component than the slow release kinetics observed when ethylene receptors are transgenically expressed in yeast (10, 42). Examination of the ethylene release kinetics in plants supports this hypothesis (49, 50). Both rapid (half-life of less than 30 min) and slow release kinetics (half-life of 12 h or more) for ethylene have been found in Arabidopsis as well as in other plants (49, 50). These differences in the release kinetics may relate to differences in the rate of turnover particular to individual subtypes of the ethylene receptor family. For example, based on their differing sensitivities to ligand-induced turnover, we would predict that the rapid release kinetics for ethylene found in Arabidopsis could be due to degradation of ETR2, whereas the slow release kinetics could be due to degradation of ETR1.

Our results show that ETR2 is subject to ligand-mediated degradation like the animal growth factor receptors but with significant mechanistic differences. Ligand-mediated degradation of animal growth factor receptors typically involves endocytosis of the ligand-bound receptor and subsequent targeting to the lysosome (2, 3). This targeting step is apparently regulated by monoubiquitination of the receptor, which serves as a sorting signal for the lysosome. Degradation of ETR2, in contrast to the paradigm established for animal growth factor receptors, is mediated by the proteasome, a situation potentially arising due to localization of ETR2 to the ER rather than to the plasma membrane (i.e. the ethylene receptors do not have the correct subcellular localization for endocytosis). Degradation of ETR2 does not require export of ETR2 to other destinations in the secretory pathway, and thus ER-associated degradation via the ubiquitin-proteasome system is likely to mediate ETR2 degradation. ER-associated degradation is primarily known for its role in the degradation of misfolded soluble and membrane proteins of the secretory system (51), but it has also been found to regulate turnover of enzymes such as 3-hydroxy-3-methylglutaryl-CoA reductase, which, like ETR2, is an integral membrane protein of the ER (51). We thus propose that ethylene binding to its receptor at the endoplasmic reticulum results in a conformational change, exposing sites for polyubiquitination on the cytosolic soluble domain of ETR2, followed by degradation at the proteasome.

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Ligand-induced Degradation of ETR2

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