REVERSION-TO-ETHYLENE SENSITIVITY1, a conserved gene that regulates ethylene receptor function in *Arabidopsis*

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Arabidopsis thaliana has five ethylene hormone receptors, which bind ethylene and elicit responses critical for plant growth and development. Here we describe a negative regulator of ethylene responses, *REVERSION-TO-ETHYLENE SENSITIVITY1* (*RTE1*), which regulates the function of at least one of the receptors, *ETR1*, in *Arabidopsis*. *RTE1* was identified based on the ability of *rte1* mutations to suppress ethylene insensitivity of the dominant gain-of-function allele *etr1-2*. *rte1* loss-of-function mutants have an enhanced ethylene response that closely resembles the *etr1* null phenotype. The *etr1 rte1* double null mutant is identical to the *etr1* and *rte1* single null mutants, suggesting that the two genes act in the same pathway. *rte1* is unable to suppress the *etr1-1* gain-of-function allele, placing *RTE1* at or upstream of *ETR1*. *rte1* also fails to suppress gain-of-function mutations in each of the four other ethylene receptor genes. *RTE1* encodes a previously undescribed predicted membrane protein, which is highly conserved in plants and protists but absent in fungi and prokaryotes. Ethylene treatment induces *RTE1* expression, and overexpression of *RTE1* confers reduced ethylene sensitivity that partially depends on *ETR1*. These findings demonstrate that *RTE1* is a negative regulator of ethylene signaling and suggest that *RTE1* plays an important role in *ETR1* function.

The gaseous hormone ethylene has numerous effects on plant growth and development. Responses to ethylene include promotion of fruit ripening, seed germination, flowering, abscission, senescence, and the induction of stress responses (1). In *Arabidopsis*, dark-grown seedlings treated with ethylene exhibit the “triple response,” which consists of inhibition of hypocotyl and root elongation, radial swelling of the hypocotyl, and exaggeration of the apical hook (2, 3). The isolation of mutants having an altered triple-response phenotype has uncovered an ethylene signaling pathway that leads from ethylene binding to changes in gene expression (4). Perception of ethylene is carried out by receptors related to the two-component histidine protein kinase family primarily found in prokaryotes (5–8). The five ethylene receptors in *Arabidopsis* (*ETR1*, *ERS1*, *EIN4*, *ETR2*, and *ERS2*) exhibit redundancy and are negative regulators of ethylene responses (9–11). Null mutants of each receptor gene are phenotypically wild-type, with the exception of *etr1-7*, which displays enhanced sensitivity to ethylene (9, 12). Triple-receptor null mutant combinations display constitutive ethylene responses, and a quadruple-receptor null mutant displays a severe phenotype that includes small leaves and rosette-stage lethality (9). In contrast, dominant gain-of-function mutations in the ethylene receptor genes result in ethylene insensitivity, characterized by the absence of the triple response, slightly larger rosette leaves, and delayed senescence (2). Gain-of-function alleles encode amino acid substitutions residing in the ethylene-binding domain (5–8). An example is the *etr1-1* mutation, which blocks ethylene binding, resulting in ethylene insensitivity (13). According to the current model of ethylene receptor action, the receptors repress responses in the absence of ethylene binding,

and receptor signaling is shut off upon ethylene binding, resulting in activation of responses.

The *Arabidopsis* *ETR1* receptor is a homodimer (14) localized at the endoplasmic reticulum (15). The membrane-bound N-terminal portion of *ETR1* binds ethylene (16) with a copper cofactor Cu(I) (17), but the mechanism of ethylene receptor signaling is unclear. Although *ETR1* has histidine autokinase activity *in vitro* (18), such activity is not required for ethylene signaling *in vivo* (19–21), and other ethylene receptors have serine/threonine kinase activity (21–23). The ethylene receptors interact with the Raf-like protein kinase *CTR1* (24, 25), which is a negative regulator of the pathway (26). Inactivation of *CTR1* leads to activation of *EIN2*, which has a novel C terminus and an N-terminal transmembrane domain with similarity to the N-ramp family of metal ion transporters (27). Downstream of *EIN2*, the plant-specific *EIN3* transcription factor family induces expression of *ERF1* and other transcription factors, which in turn activate ethylene-response target genes (28). *EIN3* is regulated posttranslationally by a proteasome-mediated protein degradation pathway (29–31).

Results

Isolation of *rte1* Mutants. To uncover additional components in the ethylene-response pathway, we carried out a genetic screen for suppressors of the weak ethylene-insensitive mutant *etr1-2*, which lacks the triple-response phenotype when grown in the presence of ethylene. Mutants that displayed the triple-response phenotype were isolated from an *etr1-2* M₂-mutagenized population germinated on medium containing the ethylene precursor compound 1-aminocyclopropane-1-carboxylic acid (ACC). Recessive extragenic suppressor mutations were identified, and two of these, *rte1-1* and *rte1-2* (derived from ethylmethanesulfonate and fast neutron mutagenesis, respectively), were found to be allelic by complementation testing. Based on the *RTE1* gene sequence (described below), we obtained a third allele, *rte1-3*, which is likely to be a null mutation. Because all of the *rte1* mutants displayed similar phenotypes (described below), we deduced that *rte1-1* and *rte1-2* are loss-of-function alleles.

Requirement of *RTE1* for Ethylene Insensitivity of *etr1-2*. In an ethylene dose–response assay to verify the suppression, both of the mutant lines isolated above (*etr1-2 rte1-1*, and *etr1-2 rte1-2*) exhibited ethylene responses similar to the wild type (Fig. 1A). Both lines,

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Abbreviation: ACC, 1-aminocyclopropane-1-carboxylic acid.

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Fig. 1. Mutant alleles of *rte1* suppress *etr1-2* ethylene insensitivity. (A) Ethylene dose-response of hypocotyl length in 4-day-old dark-grown seedlings. The two mutant lines isolated in the genetic screen (*etr1-2 rte1-1* and *etr1-2 rte1-2*) show suppression of *etr1-2* ethylene insensitivity. WT, wild-type Col-0. The mean $\pm$ SE is shown for 10 seedlings per genotype at each dose. (B) Comparison of 4-day-old dark-grown seedlings germinated in the presence or absence of the ethylene precursor ACC (100 μ M). Representative seedlings of three suppressor lines (*etr1-2 rte1-1*, *etr1-2 rte1-2*, and *etr1-2 rte1-3*) and two single mutants (*rte1-2* and *rte1-3*) all show similarity to the *etr1-7* null mutant. (C) Comparison of 4-day-old light-grown seedlings (no ethylene treatment) for the same genotypes as labeled in B. Representative seedlings of the three suppressor lines (*etr1-2 rte1-1*, *etr1-2 rte1-2*, and *etr1-2 rte1-3*) and two single mutants (*rte1-2* and *rte1-3*) all show similarity to the *etr1-7* null mutant, which has shorter roots and smaller cotyledons than the wild type. (D) Ethylene-induced leaf senescence in 5-week-old plants treated with or without 100 ppm of ethylene for 4 days. Senescence is observed in the wild type and the three suppressor lines (*etr1-2 rte1-1*, *etr1-2 rte1-2*, and *etr1-2 rte1-3*), whereas *etr1-2* is largely insensitive.

as well as the constructed *etr1-2 rte1-3* double mutant, displayed a triple-response phenotype comparable to that of the wild type (Fig. 1B). The hypocotyl and root, however, appeared to be slightly shorter, similar to the *etr1-7* null mutant (9), both with and without ethylene treatment (Fig. 1B). The suppressed mutant seedlings were also comparable to *etr1-7* when germinated in the light, with smaller cotyledons and a slightly shorter root than either the wild type or *etr1-2* (Fig. 1C). At the adult stage, the suppressed mutant lines exhibited ethylene-induced senescence, whereas the *etr1-2* single mutant remained largely resistant (Fig. 1D). The suppression of distinct ethylene-insensitive phenotypes in seedlings and adult plants confirmed that *RTE1* is required for ethylene insensitivity of *etr1-2* and suggested that *RTE1* acts at an early step in the ethylene signaling pathway.

Similarity of the *rte1* Single Mutant to the *etr1-7* Null Mutant. The *rte1* single mutant has an enhanced ethylene-response phenotype that largely phenocopies the *etr1-7* null mutant. Seedlings of

Fig. 2. The *rte1-2* mutant has enhanced ethylene sensitivity resembling that of the *etr1-7* null mutant (9). (A) Ethylene dose-response of hypocotyl length in 4-day-old dark-grown seedlings reveals similarity of enhanced ethylene sensitivity in *rte1-2*, *rte1-3*, and *etr1-7* in contrast to the wild type (WT). The mean $\pm$ SE is shown for 17–30 seedlings per genotype at each dose. (B) Four-day-old dark-grown seedlings of *etr1-7* and *rte1-2* display the triple-response phenotype when grown on a low concentration of ACC (0.5 μ M), whereas the wild type does not. (C) Treatment with the ethylene biosynthesis inhibitor 1-aminoethoxyvinylglycine (10 μ M) partially alleviates hypocotyl shortening ($P < 0.0001$) in *rte1-2* and *etr1-7* in the absence of ethylene treatment. The mean $\pm$ SE is shown for 10–12 seedlings per genotype. (D) Treatment with the ethylene-response inhibitor AgNO₃ (100 μ M) alleviates the enhanced ethylene response of *rte1-2* and *etr1-7*. The wild type similarly does not respond to ACC in the presence of AgNO₃. The *ctr1-1* control is not alleviated, because *CTR1* acts downstream of the ethylene receptors, which are the likely targets of AgNO₃ inhibition. For each treatment, the mean $\pm$ SE is shown for 10–12 seedlings per genotype.

rte1-2 and *rte1-3* single mutants resembled *etr1-7* seedlings, both with and without ethylene treatment (Fig. 1B and C). An ethylene dose-response of hypocotyl length in dark-grown seedlings showed that, like *etr1-7*, both *rte1-2* and *rte1-3* are slightly shorter than the wild type at each dose (Fig. 2A). As shown in Fig. 2B, a low concentration of ACC (0.5 μ M) elicited the triple-response phenotype in both *etr1-7* and *rte1-2* but not in the wild type. The shortened hypocotyl in *etr1-7* in the absence of ethylene treatment is known to be primarily due to enhanced sensitivity to endogenously produced ethylene, because the shortening is partially alleviated when ethylene biosynthesis is blocked (12). When *rte1-2* seedlings were germinated on medium containing the ethylene biosynthesis inhibitor 1-aminoethoxyvinylglycine, hypocotyl shortening was alleviated to nearly the same extent as in *etr1-7* (Fig. 2C), indicating that the shortened hypocotyl in the absence of ethylene treatment could be at least partially attributed to an enhanced sensitivity to endogenously produced ethylene. No other phenotypes were detected for *rte1* plants under normal growth conditions.

Reversal of hypocotyl shortening was also obtained by treatment with silver nitrate (32). Silver is thought to bind to the copper-binding site of the receptors causing conformational changes that result in ethylene insensitivity. In the presence of silver nitrate, both *rte1-2* and *etr1-7* were insensitive to a high concentration of ACC (100 μ M) (Fig. 2D). These striking similarities between *rte1* and *etr1-7* indicated that, like *ETR1*, *RTE1* is a negative regulator of ethylene responses. Moreover,

Fig. 3. Ethylene dose-response of double mutants suggests that *RTE1* acts in the same pathway as *ETR1*. (A) The *etr1-7 rte1-2* double mutant does not display an enhanced phenotype in comparison to the *etr1-7* and *rte1-2* single mutants, suggesting that *RTE1* acts in the same pathway as *ETR1*. (B) The *ers1-3 rte1-3* double mutant appears to have an enhanced response, which is distinct from the *ers1-3* single null mutant, suggesting that *RTE1* action is either partially or fully independent of *ERS1*. (C) *rte1-2* does not suppress the gain-of-function ethylene-insensitive mutant *etr1-1*. (D) *rte1-3* does not suppress *ers1-10*, which is a weak gain-of-function ethylene-insensitive mutation in the *ERS1* ethylene receptor gene. For each graph, the mean $\pm$ SE is shown for 11–30 seedlings per genotype for each dose.

the data supported the hypothesis that *RTE1* is required for the function of both the *etr1-2* and wild-type *ETR1* genes.

To examine the relationship between *RTE1* and *ETR1* further, we constructed an *etr1-7 rte1-2* double mutant and compared its phenotype to the *etr1-7* and *rte1-2* single mutants. The *etr1-7 rte1-2* double mutant displayed virtually the same response as the *etr1-7* and *rte1-2* single mutants, consistent with *RTE1* acting in the same pathway as *ETR1* (Fig. 3A). In contrast, a double mutant of *rte1-3* and *ers1-3* (a null allele of the *ERS1* ethylene receptor gene) exhibited a response that was clearly distinct from that of the *ers1-3* single mutant (Fig. 3B). The *ers1-3 rte1-3* phenotype was not as severe as would be expected for an *etr1 ers1* double null (11), indicating that *rte1-3* does not fully phenocopy the *etr1* null. Nevertheless, the results are consistent with the hypothesis that *RTE1* is largely required for the function of wild-type *ETR1*.

Inability of *rte1* to Suppress Other Dominant Ethylene Receptor Mutations. We next tested whether *rte1-2* could suppress *etr1-1*, which is another gain-of-function allele conferring ethylene insensitivity. Interestingly, the *etr1-1 rte1-2* double mutant phenotype was indistinguishable from the *etr1-1* single mutant in both root and hypocotyl length in the triple-response assay, demonstrating that *rte1-2* could not suppress *etr1-1* (Fig. 3C and Table 1, which is published as supporting information on the PNAS web site). Similarly, dominant ethylene-insensitive mutations in each of the four other ethylene receptor genes were not suppressed by *rte1-2* (or *rte1-3*) (Table 1). Notably, *rte1-3* did not suppress *ers1-10* (33), which is possibly the weakest known ethylene receptor gain-of-function allele (Fig. 3D and Table 1). Thus, suppression by *rte1* appeared to be specific for *ETR1*

and/or particular ethylene receptor mutations. Finally, *rte1-2* did not suppress the recessive ethylene-insensitive mutation *ein2-1*, which acts downstream of the receptors in the ethylene-response pathway (data not shown).

Molecular Cloning and Sequence Analysis of the *RTE1* Gene. We mapped the *RTE1* locus to a 32-kb interval of chromosome 2 near the *erecta* locus (Fig. 4A). Upon sequencing the corresponding DNA segments from *rte1-1* and *rte1-2*, we found that each mutant carries a single-nucleotide mutation in the ORF of gene At2g26070 (GenBank NP_180177); *rte1-1* carries a G-to-A missense mutation, and *rte1-2* lacks a single nucleotide near the 3' end of the coding sequence. We confirmed that this was the *RTE1* gene by rescuing the suppressed phenotype of the *etr1-2 rte1-2* double mutant with a 4.33-kb genomic DNA fragment of wild-type At2g26070 (comprising 2.66 kb upstream and 0.80 kb downstream of the predicted start and stop codons, respectively) (Fig. 4B). The deduced *RTE1* protein sequence contains 250 amino acids with a predicted molecular mass of 28 kDa (Fig. 4C). The *rte1-3* allele, which was identified through TILLING (35), introduces a stop codon at residue 57. Transcript levels of *RTE1* in the *rte1-3* mutant were reduced compared to the wild type and *rte1-2* (data not shown), possibly because of nonsense-mediated mRNA decay (36).

BLAST searches revealed a previously unreported family of highly conserved *RTE1* homologs widely distributed in eukaryotes, including *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Danio rerio*, *Plasmodium*, and *Trypanosoma* (Fig. 4C and Fig. 6, which is published as supporting information on the PNAS web site). The human sequence has 40.5% identity with *RTE1* over 156 amino acids. *RTE1* is not found in fungi, which most likely lost the *RTE1* gene, nor is it found in prokaryotes. *RTE1* appears to be present in a single copy in each species, except in plants, in which there are two copies in *Arabidopsis* and three copies in rice. The second copy in *Arabidopsis*, which we have named *RTE1-HOMOLOG* (*RTH*) (At3g51040, GenBank NP_190673), encodes a protein of 231 amino acids with 51% identity to *RTE1* over 209 amino acids.

The *RTE1* sequences contain no known motifs, and no biological function has been assigned to this gene/protein in any species. The existence of *RTE1* in a wide range of organisms, however, suggests that it performs an essential conserved function. Sequence alignment revealed two highly conserved regions containing conserved Cys and His residues, in particular Cys⁵⁴, His⁷², and Cys¹⁶¹ (Figs. 4C and 6). The *rte1-1* missense mutation replaces Cys¹⁶¹ with tyrosine. Sequence analysis suggests that *RTE1* is an integral membrane protein. Plant homologs contain two to four likely transmembrane segments, with one close to the N terminus and two or three at the C-terminal end (Fig. 4C). The *rte1-2* mutation causes a frameshift that replaces the last 27 residues with 15 incorrect residues; this might result in mislocalization of the protein, because most of the last 27 residues comprise a strongly predicted transmembrane domain. Animal *RTE1* proteins are generally predicted to have the first three transmembrane segments, although *C. elegans* has only two. In some *RTE1* sequences, a putative glycosylation site is located immediately N-terminal to the penultimate transmembrane domain.

Ethylene Induction of *RTE1* Expression. Gene array data for *Arabidopsis* indicate that *RTE1* is expressed at detectable levels in most organs and stages but is most highly expressed in seeds and in late-stage siliques (www.cbs.umn.edu/arabidopsis and www.geneinvestigator.ethz.ch/at). Slightly higher expression is also found in carpels and the shoot apex. Array data indicate that exposure to ethylene results in a 4-fold increase in *RTE1* transcript levels (37). Using RNA blots, we observed a similar up-regulation of *RTE1* expression in whole plants after ethylene treatment (Fig. 4D).

Fig. 4. *RTE1* encodes a previously undescribed conserved protein and is up-regulated by ethylene treatment. (A) Gene structure of *Arabidopsis RTE1* (At2g26070) based on comparison of the *RTE1* cDNA with the annotated genomic DNA sequence. (B) Rescue of the *etr1-2 rte1-2* mutant phenotype, by using a wild-type 4.33-kb genomic DNA fragment "gRTE1" whose position is indicated in (A). (C) Alignment (34) of the predicted protein sequences of *Arabidopsis RTE1* "AtRTE1" (NP.180177), *Arabidopsis RTH* "AtRTH" (NP.190673), three *Oryza sativa* homologs "Rice1A" (NP.916598), "Rice1B" (AAV59409), and "Rice2" (AAO37528), the *Trypanosoma cruzi* homolog "Tcruzi" (XP.804751.1), and the *H. sapiens* homolog "Human" (NP.115501) (extended at the N terminus based on the sequence of EST B1667401). Gray bars indicate approximate positions of predicted transmembrane domains for AtRTE1 and AtRTH. Locations of *Arabidopsis RTE1* mutations [stop codon in *rte1-3* (W57*), the *rte1-1* substitution (C161Y), and the *rte1-2* frameshift, which occurs after F223] are indicated. (D) RNA blot showing induction of *RTE1* mRNA levels in wild-type plants after exposure to 10 ppm of ethylene (C_2H_4) for 2.5 and 5 h. Two isoforms are detected, possibly because of alternative splicing. *ERF1* (28) is an ethylene-inducible control. Ethidium-bromide staining of the gel shows similar loading of rRNA between samples.

Reduced Ethylene Sensitivity Conferred by *RTE1* Overexpression. Consistent with *RTE1* being a negative regulator of ethylene responses, overexpression of the *RTE1* gene resulted in reduced ethylene sensitivity in the wild-type background. Transgenic lines carrying the *RTE1* coding sequence under the control of the cauliflower mosaic virus 35S promoter were germinated on ACC or in ethylene, and all 20 lines exhibited reduced ethylene sensitivity in the triple-response assay (Fig. 5 A and B and data not shown). When the same construct was transformed into *etr1-2*, all 20 lines examined showed reduced ethylene sensitivity that was enhanced relative to untransformed *etr1-2* (Fig. 5A and data not shown). When the same construct was transformed into *etr1-7*, reduced ethylene sensitivity was not observed, but there was detectable alleviation of the enhanced ethylene response of *etr1-7*, with and without ethylene, in all 20 lines examined (Fig. 5 A and C and data not shown).

Discussion

We have identified a previously undescribed conserved gene that negatively regulates ethylene responses in *Arabidopsis*. Several lines of evidence are consistent with a model in which *RTE1* is a regulator of *ETR1* function. Not only is *RTE1* required for ethylene insensitivity in the *etr1-2* gain-of-function mutant, but

loss of *rte1* function results in a phenotype that closely resembles the *etr1-7* null mutant. That the *etr1-7 rte1-2* double mutant is indistinguishable from the *etr1* single null mutant suggests that *RTE1* acts with or through *ETR1*, rather than in an *ETR1*-independent pathway. The inability of *rte1* to suppress the *etr1-1* allele (and several other receptor mutant alleles) makes it likely that *RTE1* lies at or upstream of *ETR1*, as opposed to downstream in the ethylene-response pathway. The allele specificity of suppression also raises the possibility that *RTE1* affects the function of *ETR1* at the protein level rather than at the level of DNA or transcription. The induction of *RTE1* expression by ethylene might represent a negative-feedback mechanism of the ethylene-response pathway.

We cannot, however, exclude the possibility that *RTE1* is involved in the function of more than one ethylene receptor isoform or even other ethylene pathway components, particularly because *RTE1* overexpression conferred partial rescue of the enhanced ethylene response in the *etr1-7* null mutant. It is possible that *RTE1* overexpression imparted ectopic effects that could not be suppressed by *etr1-7*, but it is also conceivable that the *etr1* null-like phenotype of *rte1-3* is the consequence of slight reductions in the function of more than one ethylene receptor. Having a weak effect on multiple receptors would be consistent

Fig. 5. Overexpression of *RTE1* confers reduced sensitivity to ethylene. (A) Representative 4-day-old dark-grown seedlings of wild-type *etr1-2*, and the null mutant *etr1-7*, either untransformed (–) or stably transformed (+) with *35S::RTE1*, germinated in the presence or absence of the ethylene precursor ACC (10 μ M). (B) Measurements of hypocotyl length for wild-type and three independent transgenic lines (15, 17, and 19) transformed with *35S::RTE1* in the wild-type background, with and without ethylene treatment. For each treatment, the mean $\pm$ SE is shown for 14–20 seedlings per line. (C) Measurements of hypocotyl length for *etr1-7* and three independent transgenic lines (4, 5, and 10) in the *etr1-7* background, with and without ethylene treatment. Differences for each transformed line compared with untransformed *etr1-7* are significant ($P < 0.0001$). For each treatment, the mean $\pm$ SE is shown for 14–19 seedlings per line.

with our finding that the phenotype of the *ers1-3 rte1-3* double null mutant is not as severe as the *etr1 ers1* double null, which has a distinct phenotype that includes rosette lethality (11). Although *rte1-2* did not suppress mutant alleles of the other receptor genes, this outcome could have been specific to the particular alleles tested. Because ETR1 is considered the most prevalent of the ethylene receptors [based on relative transcript levels (38) and/or signaling strength], this could give ETR1 the appearance of being the main target of RTE1. If there is indeed specificity for ETR1, it is perhaps based on the subcellular distribution of the receptors, their biochemical properties, and/or their relative abundance.

It is unclear why *rte1* is capable of suppressing the *etr1-2* allele but not *etr1-1*. *etr1-2* encodes an Ala¹⁰²-to-Thr substitution in the third putative transmembrane segment of the ethylene-binding domain. Although *etr1-2* displays a gain-of-function ethylene-insensitive phenotype, the *etr1-2* protein differs from several other mutant ethylene-insensitive receptors in that it binds the same level of ethylene as wild-type ETR1 (13). The *etr1-2* mutation also confers partial ethylene insensitivity (13), suggesting that the signaling domain of *etr1-2* is capable of being turned off. In contrast, the *etr1-1* mutation fully blocks ethylene binding (13, 16), because of a Cys⁶⁵-to-Tyr substitution that prevents association with the copper cofactor (17). In addition, *etr1-1* confers strong ethylene insensitivity, indicating that the signaling domain is essentially locked in an active conformation. Therefore, *rte1* mutations might suppress alleles that are weak in terms of signaling strength and/or that do not disrupt the receptor's ability to bind ethylene. In either case, the mutant *etr1-2* receptor

might be in a conformation that causes it to be particularly susceptible to whatever process RTE1 is regulating.

One other protein known to affect ethylene receptor function is RAN1, a homolog of the human Menkes/Wilson P-type ATPase copper transporters (39, 40). Defects in RAN1 are thought to severely disrupt the formation of functional wild-type ethylene receptors, presumably because of improper copper loading. Similar to *rte1*, mutations in *ran1* are suppressed by *etr1-1* (39, 40). In the case of *ran1*, this implies that the *etr1-1* receptor is stable in the absence of copper and locked into the repressive signaling state, unlike wild-type ethylene receptors. In contrast to *rte1*, however, a *ran1* null mutation failed to suppress ethylene insensitivity in *etr1-2* seedlings (data not shown). In addition, treatment of *rte1* mutants with copper or copper chelators did not rescue the *rte1* phenotype (data not shown).

The presence of *RTE1* in plant, animal, and protist genomes suggests a conserved function of broad importance. Because ethylene receptors are not known in these other organisms, *RTE1* might either have a specialized function in plants or act on the ethylene receptor(s) through an indirect mechanism, such as changes to the membrane or altered metal ion availability. Interestingly, no phenotypes other than those in ethylene response have been detected in the *rte1* mutants. In the accompanying paper, ectopic expression of a tomato *RTE1* homolog is shown to be the basis for inhibition of fruit ripening (on ethylene-dependent phenotype) in the tomato GR mutant (41). It remains to be seen whether the *Arabidopsis RTE1* homolog, *RTH*, is redundant with *RTE1*, and whether a double mutant of the two genes will reveal additional phenotypes. Conceivably *RTH* could have a function that is closer to that in animals. Our genetic analyses are consistent with a number of possible cellular functions for the RTE1 protein, including involvement in the maturation, subcellular localization, or turnover of the ETR1 receptor, or having a role as a molecular chaperone facilitating ETR1 signaling. The connection established between RTE1 and ETR1 provides a valuable framework for the investigation of RTE1 function, as well as understanding the regulation of ethylene receptor action.

Materials and Methods

Plant Strains and Growth Conditions. The *Arabidopsis thaliana* Columbia (Col-0) ecotype was used, unless otherwise stated. The *ers1-3* T-DNA mutant (kindly provided by G. Eric Schaller, Dartmouth College, Hanover, NH) is ecotype Wassilewskija. Plants were grown in soil under 16-h light/8-h dark in a controlled environment chamber at 20°C under fluorescent light. For seedling analyses, seeds were sown on Murashige and Skoog medium containing 0.9% agar. After a 3-day stratification at 4°C, the seeds were incubated 4 days at 20°C in either complete darkness or 24-h light.

For the triple-response assay, seedlings were germinated in the presence of ethylene gas as described (42) or on medium containing ACC (Sigma Aldrich) at the stated concentrations. To inhibit ethylene biosynthesis, 10 μ M L- α -1-aminoethoxyvinyl glycine hydrochloride (Sigma Aldrich) was added to the medium. To inhibit ethylene response, 100 μ M AgNO₃ (Sigma Aldrich) was added to the medium. Measurements were made by photographing the seedlings with a digital camera and using IMAGEJ software (<http://rsb.info.nih.gov/ij/>).

Ethylene treatment of adult plants was carried out in airtight clear acrylic chambers, into which ethylene gas (Specialty Gases of America, Toledo, OH) was injected. A control chamber was injected with air. For senescence assays, both chambers were placed at 20°C for 3 days under 24-h light.

Mutagenesis, Suppressor Screen, and Genetic Analysis. Seeds of *etr1-2* (5) were mutagenized with ethylmethylsulfonate (EMS) as described (3) or by fast neutron irradiation (60 Gy) (Internation-

tional Atomic Energy Agency, Vienna). Seedlings were screened for the triple-response phenotype on medium containing 100 μ M ACC. Approximately 4,000 and 30,000 M₂ seeds were screened from the EMS- and fast neutron-mutagenized populations, respectively.

When *etr1-2 rte1-1* and *etr1-2 rte1-2* were each crossed to either *etr1-2* or wild type, all F₁ were ethylene-insensitive, demonstrating that *rte1-1* and *rte1-2* are recessive and extragenic. When *etr1-2 rte1-1* and *etr1-2 rte1-2* were crossed to each other, all F₁ showed the suppressed phenotype, indicating that *rte1-1* and *rte1-2* are allelic.

The null mutant *rte1-3* was obtained through TILLING (<http://tilling.fhcrc.org:9366/home.html>) as a Col-0 *erecta* line. The *erecta* mutation was removed by backcrossing to wild-type Col-0.

Double mutants with *rte1-2* or *rte1-3* were obtained by genetic crosses described in *Supporting Text*, which is published as supporting information on the PNAS web site. Transgene constructs and transformant analysis are also described in *Supporting Text*.

Map-Based Cloning and Sequence Analysis of RTE1. The *rte1* locus was mapped in a homozygous *etr1* mutant background, by crossing *etr1-2 rte1-2* (Col-0) to a dominant ethylene insensitive *etr1* allele in the Landsberg *erecta* background (gift of A. Stepanova and J. R. Ecker, The Salk Institute for Biological Studies, San Diego), which encodes an Ile³⁵ to Phe in the first putative transmembrane domain (A. Stepanova, personal communication). The F₂ mapping population consisted of 384 individuals scored as homozygous for *rte1-2* on the basis of the triple-response phenotype. The suppressed phenotype segregated 1:16, indicating that *rte1-2* could not suppress the Landsberg *etr1* allele. For mapping, CAPS markers were designed by using a single-nucleotide polymorphism database (43).

For sequencing, genomic DNA was isolated from *rte1-2* plants, and overlapping 4-kb fragments were amplified by PCR for the entire region (32 kb). The same region was PCR-amplified from bacterial artificial chromosome T19L18 [*Arabidopsis* Biological Resource Center (ABRC), Ohio State University, Columbus]. The fragments were sequenced by using multiple internal primers, and the sequences were examined for mismatches.

BLAST was performed by using www.ncbi.nlm.nih.gov/BLAST. For fungal genomes, BLAST was carried out by using www.broad.mit.edu/annotation/cgi. Transmembrane domain predictions were obtained from the Aramemnon database (<http://crombec.botanik.uni-koeln.de>). Sequence alignments were carried out by using CLUSTALW (<http://align.genome.jp>).

To analyze mRNA expression, 4-week-old soil-grown plants were exposed to air or 100 ppm of ethylene, and total RNA was isolated according to Wen *et al.* (44). RNA was size-fractionated in formaldehyde/formamide agarose gels in 4-morpholinepropanesulfonic acid buffer, then blotted to a nylon membrane. Radiolabeled probes were synthesized by random priming by using cDNA clone templates. Hybridization and washes were carried out at 68°C for 20 h according to Church and Gilbert (45), and hybridization signals were detected by using a PhosphorImager (Molecular Dynamics).

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