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Involvement of *RTE1* in conformational changes promoting ETR1 ethylene receptor signaling in Arabidopsis

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Summary

Ethylene is an important regulator of plant growth, development and responses to environmental stresses. Arabidopsis perceives ethylene through five homologous receptors that negatively regulate ethylene responses. *RTE1*, a novel gene conserved in plants, animals and some protists, was recently identified as a positive regulator of the ETR1 ethylene receptor. Here, we genetically analyze the dependence of *ETR1* on *RTE1* in order to obtain further insight into *RTE1* function. The function of *RTE1* was found to be independent and distinct from that of *RAN1*, which encodes a copper transporter required for ethylene receptor function. We tested the ability of an *rte1* loss-of-function mutation to suppress 11 *etr1* ethylene-binding domain mis-sense mutations, all of which result in dominant ethylene insensitivity due to constitutive signaling. This suppression test uncovered two classes of *etr1* mutations – *RTE1*-dependent and *RTE1*-independent. The nature of these mutations suggests that the ethylene-binding domain is a possible target of *RTE1* action. Based on these findings, we propose that *RTE1* promotes ETR1 signaling through a conformational effect on the ethylene-binding domain.

Keywords

RTE1; ETR1; ethylene; receptor; signaling; Arabidopsis

Introduction

Ethylene is a gaseous plant hormone that is responsible for many aspects of growth and development, including fruit ripening, organ abscission, senescence, seed germination, root hair formation and flowering (Abeles *et al.*, 1992). Ethylene is also involved in the induction of responses to abiotic and biotic stresses (Abeles *et al.*, 1992). In *Arabidopsis thaliana*, dark-grown seedlings display a specific ethylene response known as the triple response, which consists of inhibition of hypocotyl and root elongation, radial swelling of the hypocotyl, and exaggeration of the apical hook (Bleecker *et al.*, 1988; Guzmán and Ecker, 1990). The signaling pathway leading to the triple response and other ethylene responses has been dissected based on genetic screens for triple-response mutants (Li and Guo, 2007).

In Arabidopsis, ethylene is perceived by five homologous ethylene receptors (ETR1, ERS1, EIN4, ETR2 and ERS2) that are related to two-component histidine kinase receptors (Chang *et al.*, 1993; Hua *et al.*, 1995, 1998; Sakai *et al.*, 1998). The receptors share a similar domain

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structure, consisting of three or four N-terminal transmembrane domains containing the ethylene-binding site, followed by a GAF domain of unknown function (GAF is present in phytochromes and cGMP-specific phosphodiesterases) and a C-terminal signaling output domain(s). The five receptors exhibit functional redundancy and are negative regulators of ethylene responses (Hua and Meyerowitz, 1998; Qu *et al.*, 2007). Null mutants of the receptor genes have no detectable phenotypes, with the exception of *etr1* and *ers1*, which exhibit hypersensitivity to ethylene (Cancel and Larsen, 2002; Hua and Meyerowitz, 1998; Qu *et al.*, 2007). Removing several ethylene receptor genes at once results in a constitutive triple-response phenotype, even in the absence of ethylene (Hua and Meyerowitz, 1998).

Ethylene binding (Schaller and Bleecker, 1995) requires a copper co-factor (Rodriguez *et al.*, 1999) provided by RAN1, a homolog of the mammalian Menkes/Wilson P-type ATPase copper transporter (Hirayama *et al.*, 1999; Woeste and Kieber, 2000). RAN1 appears to be required for the proper conformation and/or activity of all the ethylene receptors; reduced RAN1 function alters the ligand specificity of the receptors (Hirayama *et al.*, 1999), and a severe *ran1* loss-of-function mutation confers a constitutive triple-response phenotype in dark-grown seedlings (Woeste and Kieber, 2000). The constitutive phenotype can be partially rescued by growing the seedlings on copper-supplemented medium (Woeste and Kieber, 2000). The molecular mechanism of ethylene receptor signaling is much less clear (Hall *et al.*, 2007). *In vitro* phosphorylation assays show that ETR1 has histidine autokinase activity (Gamble *et al.*, 1998), whereas ETR2, EIN4 and ERS2 have serine/threonine kinase activity and ERS1 has both activities (Moussatche and Klee, 2004). However, these kinase activities do not have a primary role in ethylene signaling *in vivo* (Gamble *et al.*, 2002; Moussatche and Klee, 2004; Wang *et al.*, 2003).

In the current model of ethylene receptor signaling, the receptors signal to repress ethylene responses in the absence of ethylene binding. When ethylene is bound, transmission of the signal occurs via a conformational change in the receptors, turning signaling off and allowing responses to proceed (Wang *et al.*, 2006). This model is supported by dominant gain-of-function mutations in the receptor genes. Importantly, all known gain-of-function mutations lie within the hydrophobic transmembrane region comprising the ethylene-binding domain. For example, the *etr1-1* mutation substitutes a tyrosine for a cysteine residue that is required for binding of the copper co-factor (Rodriguez *et al.*, 1999). Therefore, the mutant ETR1-1 receptor cannot bind ethylene (Schaller and Bleecker, 1995) and the receptor is locked into a signaling conformation that cannot be turned off, resulting in ethylene insensitivity. In addition to mutations that disrupt ethylene binding, a class of gain-of-function mutations has been identified that result in insensitivity without disrupting ethylene binding (Wang *et al.*, 2006). Such alleles are thought to specifically prevent transmission of the ethylene signal to the signaling domains of the receptor (Wang *et al.*, 2006), and include the *etr1-2* allele (Hall *et al.*, 1999).

A negative regulator of ethylene responses, *REVERSIONTO-ETHYLENE SENSITIVITY 1* (*RTE1*), was identified in Arabidopsis based on suppression of *etr1-2* (Resnick *et al.*, 2006). *RTE1* encodes a novel integral membrane protein of unknown function found in plants, animals and some protists. The RTE1 protein co-localizes with ETR1 at the Golgi apparatus and the endoplasmic reticulum in Arabidopsis (Dong *et al.*, 2008). *RTE1* is thought to be a positive regulator of ETR1 receptor function based on several lines of evidence (Resnick *et al.*, 2006; Zhou *et al.*, 2007). *rte1* loss-of-function mutants display ethylene hypersensitivity identical to that of the *etr1-7* null mutant, whereas over-expression of *RTE1* results in ethylene insensitivity that is largely dependent on *ETR1* (Resnick *et al.*, 2006; Zhou *et al.*, 2007). Similarly, over-expression of *GREEN-RIPE* (*GR*), a tomato *RTE1* homolog, prevents tomato fruit ripening (Barry and Giovannoni, 2006). Interestingly, the loss of *RTE1* function suppresses *etr1-2*, but is unable to suppress the *etr1-1* allele (Resnick *et al.*, 2006). *etr1-1* is a strong allele resulting

in complete ethylene insensitivity, whereas *etr1-2* results in a partial ethylene response (Hall *et al.*, 1999). The RTE1/GR family has conserved cysteine and histidine residues, which could potentially bind a metal, leading to speculation that RTE1 and GR might play a role in the binding or transport of copper (Barry and Giovannoni, 2006; Resnick *et al.*, 2007).

In this study, we investigated the *RTE1*-dependent nature of *ETR1*, including placement of *RTE1* in the ethylenesignaling pathway with respect to *RAN1* and *ETR1*. Our results indicate that *RTE1* acts upstream of *ETR1*, independently of and functionally distinct from *RAN1*. Suppression analysis of various dominant *etr1* alleles suggests a model in which *RTE1* is involved in promoting or stabilizing the 'on' signaling state in *ETR1* via the ethylene-binding domain.

Results

RTE1 and RAN1 have independent and distinct functions with respect to ETR1

RTE1 and *RAN1* both play a role in wild-type *ETR1* function, as a severe loss of function of either gene results in greatly reduced *ETR1* function (Hirayama *et al.*, 1999; Resnick *et al.*, 2006; Woeste and Kieber, 2000). The *ETR1* protein is still expressed in the *ran1-3* severe loss-of-function mutant (Zhao *et al.*, 2002), indicating that *ETR1* is present but nonfunctional in *ran1-3*. To determine whether the *ETR1* protein is similarly present in the *rte1-2* mutant, we stably transformed the *rte1-2 etr1-7* double mutant with a transgene construct encoding *ETR1* fused to a 5xMyc epitope at the C-terminus. Expression of the transgene was driven by the native *ETR1* promoter. This same construct is capable of rescuing the *etr1-7* null mutation (Dong *et al.*, 2008). Using Western blot analysis, we easily detected the *ETR1*–5xMyc fusion in the *etr1-7 rte1-2* mutant (Figure 1). This indicates that the *rte1-2* phenotype is not due to an absence of *ETR1*, but more likely due to disruption of *ETR1* function.

Because of the parallels between *RTE1* and *RAN1*, we were interested in positioning *RTE1* in the ethylenesignaling pathway in relation to *RAN1* and *ETR1*. We found that over-expression of *RTE1* in *ran1-3* could not compensate for the loss of *ran1* either in the dark (with and without copper) or in the light (Figure S1). Thus, *RTE1* does not appear to be functionally redundant with *RAN1*. Overexpression of *RTE1* is known to confer ethylene insensitivity in the wild-type (Resnick *et al.*, 2006), but transgenic *ran1-3* seedlings carrying the *RTE1* over-expression construct did not show reduced sensitivity to ethylene. Instead, the seedlings exhibited essentially the same phenotype as the untransformed *ran1-3* mutant, with and without ethylene treatment (data not shown). The absence of ethylene insensitivity suggests that the *RTE1* over-expression phenotype is dependent on *RAN1*, raising the possibility that *RTE1* acts in the same pathway as *RAN1*. Alternatively, the absence of ethylene insensitivity may have been a consequence of the effect of *ran1* on ethylene receptor function, such that the *RTE1* over-expression phenotype was largely blocked by the loss of *ETR1* function. This possibility is consistent with the dependence of the *RTE1* over-expression phenotype on *ETR1* (Resnick *et al.*, 2006; Zhou *et al.*, 2007; J.S.R. and C.C., unpublished data). Moreover, when we constructed a double mutant of *ran1-3* and *rte1-2*, the double mutant displayed an ethylene-response phenotype that appeared to be additive (Figure 2a), which suggests that *RAN1* and *RTE1* act in independent pathways rather than in the same pathway. *rte1-2* is a severe loss-of-function mutant, phenotypically equivalent to the *rte1-3* null mutant and closely resembling the *etr1-7* null mutant (Resnick *et al.*, 2006). It is also possible that *ran1-3* and *rte1-2* have minor inhibitory effects on hypocotyl elongation unrelated to ethylene signaling.

In order to determine whether *RAN1* and *RTE1* act through similar mechanisms to regulate *ETR1*, we tested whether *ran1-3*, like *rte1*, could suppress the dominant *etr1-2* mutation. We found that the *ran1-3 etr1-2* double mutant displayed an ethylene-insensitive phenotype identical to that of the *etr1-2* single mutant, demonstrating that *ran1-3* does not suppress

etr1-2 (Figure 2b). This result is in sharp contrast to the ability of *rte1* mutations to suppress *etr1-2* (Resnick *et al.*, 2006), and indicated that *RTE1* and *RAN1* have distinct effects on *ETR1*. The results also revealed that the *etr1-2* mutation prevents inactivation of the signaling domain independently of copper availability (and therefore of ethylene binding), even though the *ETR1-2* mutant protein is known to bind wild-type levels of ethylene (Hall *et al.*, 1999). Thus, *etr1-2* behaves differently from wild-type *ETR1*, whose function is substantially reduced in *ran1-3* (Woeste and Kieber, 2000).

We next tested whether *rte1-3* suppression of *etr1-2* is independent of *RAN1*, by determining whether *rte1-3* is able to suppress the ethylene insensitivity of the *ran1-3 etr1-2* double mutant. We constructed a *ran1-3 etr1-2 rte1-3* triple mutant, and observed that it was identical in phenotype to the *etr1-2 rte1-3* double mutant when both were treated with ethylene (using the ethylene precursor 1-aminocyclopropane-1-carboxylic acid, ACC), thus demonstrating that *etr1-2* was suppressed (Figure 2b). In the absence of ACC treatment, the triple mutant displayed a constitutive ethylenesensitivity phenotype as expected for the *ran1-3* mutation (Figure 2b). As the constitutive ethylene-response phenotype of *ran1-3* was not exhibited by the *ran1-3 etr1-2* double mutant, the appearance of the constitutive-response phenotype in the triple mutant was probably due to suppression of *etr1-2* by *rte1-3* (Figure 2b). The fact that *rte1-3* was able to suppress *etr1-2* to the same extent in the presence and absence of *RAN1* provided further evidence that *RTE1* function is independent and distinct from *RAN1* function.

The above findings are summarized in the genetic model in Figure 2(c). In the case of wild-type *ETR1*, both *RTE1* and *RAN1* are required, and when either gene is absent, *ETR1* is largely non-functional. In contrast, the dominant *etr1-2* mutation depends on *RTE1* but not *RAN1* to confer insensitivity (i.e. maintain the signaling state). Interestingly, the strong *etr1-1* allele is independent of *RTE1* (Resnick *et al.*, 2006). *etr1-1* is also presumably independent of *RAN1*, as the *etr1-1* mutation blocks the binding of copper. In addition, *ran1-3* does not suppress *etr1-3* (Woeste and Kieber, 2000), another dominant allele that disrupts ethylene binding (similar to *etr1-1*) (Hall *et al.*, 1999).

Loss of *rte1* function suppresses a subset of dominant mutations in the *ETR1* ethylene-binding domain

The *etr1-2* mutation results in a copper-independent conformational defect in the *ETR1-2* receptor that is suppressed by the loss of *rte1* function. Similarly, the *etr1-1* mutation is thought to lock the *ETR1-1* receptor into a copper-independent signaling conformation (Hirayama *et al.*, 1999; Woeste and Kieber, 2000), but the loss of *rte1* has no effect on *etr1-1*. To gain insight into the basis for this difference, we examined the ability of the severe loss-of-function mutation, *rte1-2*, to suppress additional dominant ethylene-insensitive *etr1* alleles. We used existing *etr1* mutant transgenes that had been characterized with respect to the degree of ethylene insensitivity conferred in stably transformed plants and the ability of the encoded proteins to bind ethylene in a yeastbased assay (Wang *et al.*, 2006). Each transgene carries a mis-sense mutation located in the ethylene-binding region of *ETR1* (Figure 3a). Wang *et al.* (2006) showed that complete disruption of ethylene binding results in strong ethylene insensitivity (as in the case of *etr1-1*), but that some strong ethylene-insensitive alleles encode receptors that can still bind ethylene to varying degrees. The alleles that confer insensitivity without disrupting ethylene binding (such as *etr1-2*) are thought to prevent the conformational switch that occurs upon ethylene binding, and result in either strong or weak ethylene insensitivity (Wang *et al.*, 2006).

In order to investigate both types of mutants, we tested 11 mutations comprising alleles that are known to confer varying degrees of ethylene binding and signaling strengths (Table 1). The mutations are distributed among the three hydrophobic segments of the *ETR1* ethylene-binding domain (Figure 3a). Eight of the mutations result in strong ethylene insensitivity

(D25A, Y32A, E38A, F58A, T94M, T101A, L105A, I108A) and three result in weak ethylene insensitivity (F61A, L64A, M104A). Among the strong mutations, D25A, Y32A and T94M are known to abolish or nearly abolish ethylene binding (Wang *et al.*, 2006; W. Wang, University of Wisconsin, Madison, WI, personal communication), while T101A, L105A and I108A result in reduced ethylene binding (Wang *et al.*, 2006). The two remaining strong mutations (E38A and F58A) are thought to specifically block the conformational switch in the receptor, as these mutant receptors retain the ability to bind ethylene (Wang *et al.*, 2006). Among the three weak mutations, only F61A causes a reduction in ethylene binding (approximately 20% that of the wild-type), whereas the other two (L64A and M104A) are thought to disrupt the conformational switch, as neither are deficient in ethylene binding (Wang *et al.*, 2006).

We transformed each of the 11 mutant transgenes into wild-type plants to assess the degree of ethylene insensitivity conferred by each transgene. In parallel, each transgene was transformed into *rte1-2* mutant plants to test for the ability of *rte1-2* to suppress the insensitivity conferred by each transgene. In each background, we screened 5–8 independently transformed lines in the T₂ generation for each transgene, and measured hypocotyl length in the tripleresponse assay for three or four of these lines (Figure S2). In addition, hypocotyl length was measured for homozygous lines in the T₃ generation exposed to 0 or 20 μ M ACC (Figure 3b and Table S1). The strength of each mutant transgene (strong or weak) was determined on the basis of hypocotyl length in the wild-type background (Figure 3b and Table 1). We arbitrarily classified mutations as ‘strong’ if the mean hypocotyl length on ACC was greater than 86% of that seen without ACC. Mutations were classified as ‘weak’ if they had a mean hypocotyl length on ACC between 70–74% of that seen without ACC. None of the mutants fell within the 75–86% range.

An *etr1* mutation was considered to be suppressed if the mean hypocotyl length was less than 50% of that conferred by the same mutant transgene in the wild-type background on ACC, and non-suppressed if the mean hypocotyl length was 80% or more of that conferred by the same transgene in the wild-type background on ACC. None of the mutants fell within the 50–79% range. For suppressed mutations, we measured hypocotyl length in four or five independent transformed lines (Figure 3b, Figure S2 and Table S1). On the basis of these criteria, we found that the *rte1-2* mutation was able to suppress 6 of the 11 *etr1* mutant transgenes. Including previous data for the *etr1-2* and *etr1-1* mutant alleles (Resnick *et al.*, 2006), there were a total of seven suppressed and six non-suppressed *etr1* alleles, as summarized in Figure 3(b) and Table 1 (see also Figure S2 and Table S1). None of the suppressed lines were identical to the untransformed *rte1-2* mutant; instead the lines showed variation in the extent of suppression.

There was no clear pattern of features that distinguished the suppressed alleles from the non-suppressed alleles, such as ethylene-binding ability, strength of signaling (i.e. degree of insensitivity) or the location of the mutation in the predicted protein sequence. Of the seven suppressed alleles, five had been determined by Wang *et al.* (2006) to block the conformational switch between the binding and signaling domains of ETR1 (E38A, F58A, L64A, A102T (*etr1-2*) and M104A), as they result in ethylene insensitivity without affecting ethylene binding; two of these resulted in strong ethylene insensitivity (E38A and F58A) while the other three resulted in weak insensitivity. The two remaining suppressed alleles, Y32A and F61A, block ethylene binding and are thought to be located on adjacent helical faces that form the ethylene-binding pocket (Wang *et al.*, 2006). Y32A displays strong ethylene insensitivity, but F61A has a weak effect. Six alleles were not suppressed by *rte1-2*, and all are strong alleles (Table 1). Three (D25A, C65Y (*etr1-1*) and T94M) severely block ethylene binding, while three (T101A, L105A and I108A) retain a degree of ethylene-binding ability, and were therefore considered to be defective primarily in transmitting conformational changes to the ETR1 signaling domain (Wang *et al.*, 2006).

Discussion

Previous work has identified *RTE1* as a novel regulator of *ETR1*. Here, we carried out detailed genetic studies to analyze the dependence of *ETR1* on *RTE1*, including the placement of *RTE1* in the ethylene-signaling pathway in relation to *ETR1* and *RAN1*. While the molecular function of *RTE1* remains unknown, it has been speculated that *RTE1* serves as a copper chaperone, perhaps acting downstream of *RAN1* (Barry and Giovannoni, 2006; Resnick *et al.*, 2007), even though the *rte1* mutant is not rescued by copper treatment (Resnick *et al.*, 2006). *RAN1* encodes a copper transporter that is thought to be essential for the formation of functional ethylene receptors (Hirayama *et al.*, 1999; Woeste and Kieber, 2000). Our genetic analyses, however, indicate that *RTE1* and *RAN1* act in independent pathways and have distinct mechanisms with respect to *ETR1* function. This is evident from phenotypic analyses of double and triple mutant combinations of severe loss-of-function mutations for *rte1* and *ran1* and the dominant ethylene-insensitive mutation *etr1-2*. In particular, the *ran1-3 rte1-2* double mutant appears to have an additive phenotype, and only *rte1-2* (not *ran1-3*) is capable of suppressing *etr1-2*. Furthermore, we found that suppression of *etr1-2* by *rte1-2* is independent of *RAN1*.

Notably, the *etr1-2*-encoded receptor retains wild-type ethylene binding ability (Hall *et al.*, 1999), but does not behave like wild-type *ETR1*, which is rendered non-functional by *ran1-3*. Our finding that the *etr1-2* phenotype is unaffected by *ran1-3*, despite the severe limitation in copper availability, suggests that the *etr1-2*-encoded receptor has a conformational defect that bypasses the requirement for copper.

For greater insight into the role of *RTE1* in *ETR1* signaling, we tested the ability of *rte1-2* to suppress a variety of dominant ethylene-insensitive *etr1* mutations, which all lie within the ethylene-binding domain of *ETR1* (defined as residues 1–128). Our results reveal a class of mutations that requires *RTE1* in order to confer ethylene insensitivity, as well as a class that is independent of *RTE1*. The underlying basis for these two classes is unclear, as there are no obvious characteristics that are exclusive to the suppressed versus non-suppressed alleles; *rte1-2* can suppress both strong and weak *etr1* alleles, independent of their effects on ethylene binding, and can suppress mutations located in any of the three *ETR1* transmembrane domains, while other mutations with a similar range of effects on ethylene binding and similar distribution in the three transmembrane domains are not suppressed.

A characteristic shared by all the *etr1* alleles tested is that they are each likely to produce a unique structural defect within the *ETR1* ethylene-binding domain that consequently inhibits, to varying degrees, the conformational transition required to turn *ETR1* signaling off (Wang *et al.*, 2006). Therefore, the *RTE1*-dependence of certain *etr1* alleles, including the wild-type *ETR1* allele, may be related to particular conformations of the ethylene-binding domain. Accordingly, we propose that *RTE1* has an effect on the conformation of the ethylene-binding domain that results in promotion or stabilization of the *ETR1* signaling 'on' state. This is based on several lines of reasoning. As proposed by Wang *et al.* (2006), a conformational shift in the *ETR1* ethylene-binding domain upon ethylene binding is responsible for transmission of the signal to the transmitter (signaling) domain in order to shut signaling off. Wang *et al.* (2006) found that a large majority of mutations in the ethylene-binding domain cause strong constitutive activation of *ETR1* transmitter signaling, suggesting that the general conserved function of the ethylene-binding domain is to control the signaling conformation of the transmitter. It appears that only subtle changes in steric structure are required for transition between the 'on' and 'off' states, because so many of the mutations that impair the signaling transition in *ETR1* do not disrupt ethylene binding (Wang *et al.*, 2006). In addition, there are no known gain-of-function mutations in the transmitter itself, consistent with the view that control of transmitter 'on'/'off' activity lies primarily in the ethylene-binding domain.

It is unlikely that RTE1 acts as a co-factor for signaling output by the transmitter, because, as we have shown, some *etr1* alleles have a strong signal output even in the absence of *RTE1*. The fact that certain *etr1* alleles are susceptible to *rte1*, while others are not, is more likely to be related to the particular mutant conformation of the ethylene-binding domain, which in turn dictates the signaling output, i.e. certain mutant conformations would be stabilized by RTE1, while other mutant steric structures might be stable enough to be independent of RTE1. Consistent with this hypothesis, over-expression of *RTE1* enhances ethylene insensitivity in *etr1-2* (Resnick *et al.*, 2006) but has no effect on *etr1-1* (J.S.R. and C.C., unpublished results). Moreover, Zhou *et al.* (2007) showed that loss of the *RTE1* over-expression phenotype in the *etr1-7* null mutant background is rescued by co-expressing a truncated form of ETR1 consisting of residues 1–349 (Zhou *et al.*, 2007), which confers signaling in *etr1-7* through interactions with the ERS1 ethylene receptor (Xie *et al.*, 2006). Thus, it appears that RTE1 acts through the N-terminal portion of ETR1.

The ETR1 receptor normally exists in several different states, including the ‘on’/‘off’ states and a transitional state that lies between the ‘on’/‘off’ states, as shown in Figure 4. *RTE1* may promote or stabilize the ETR1 signaling ‘on’ state at either of two transitions. One involves the formation of functional ETR1 (‘A’ in Figure 4), while the other inhibits transition from the signaling ‘on’ state to the signaling ‘off’ state (‘B’ in Figure 4). We propose that, in the wild-type situation, RTE1 helps to promote the ETR1 signaling ‘on’ state from an undefined ground state that is phenotypically equivalent to the *etr1* null mutant (Figure 4); ethylene binding turns ETR1 signaling off, which is a state that is known to be phenotypically different from the *etr1* null mutant. We favor the model in which *RTE1* acts at ‘A’ in Figure 4, based on the finding that *rte1* loss-of-function results in an *etr1* null-like phenotype (ethylene hypersensitivity), although we cannot rule out that this null-like phenotype includes non-ethylene-related effects, as the *rte1-2* and *rte1-3* mutants do not phenocopy the *etr1* null mutant in every respect (Resnick *et al.*, 2006; Zhou *et al.*, 2007). Involvement of *RTE1* in ETR1 biogenesis would be consistent with the ability of *rte1-2* to suppress the Y32A mutant receptor. The *RTE1*-dependent Y32A mutant binds a negligible amount of ethylene, and so would rarely achieve the ethylene-bound, signaling ‘off’ state in the absence of RTE1. Similarly, the *etr1-2* mutation in the *ran1-3* mutant background is unable to bind ethylene in the absence of copper, but is still suppressed by *rte1-2* in the presence of *ran1-3*. Presumably, the *RTE1*-independent *etr1* alleles are capable of bypassing the requirement for *RTE1* in step ‘A’ of Figure 4 in order to reach the signaling ‘on’ state.

A notable aspect of our findings is the quantitative nature of *rte1* suppression. None of the *RTE1*-dependent alleles appears to be completely suppressed by *rte1-2*, as the suppressed phenotypes are not identical to the *rte1-2* single mutant. Instead, varying degrees of incomplete suppression are observed (Figure 3b). This might reflect allele differences with respect to rates of transition between ETR1 signaling ‘on’/‘off’ states or between the ETR1 ground state (‘i’ in Figure 4) and the signaling ‘on’ state. Similarly, weak or strong ethylene insensitivity in the dominant mutants might result from equilibrium shifts between the ‘on’/‘off’ signaling states influenced by RTE1 acting on the ethylene-binding domain. RTE1 may act at both points ‘A’ and ‘B’ in Figure 4, promoting the ETR1 ‘on’ state by altering the equilibrium between the various states.

The molecular mechanism by which RTE1 promotes ETR1 signaling remains unknown. RTE1 may play an indirect role by exerting an effect on the membrane environment, which in turn affects the conformation of the ethylene-binding domain within the membrane. Alternatively, RTE1 may play a more direct role in the folding of the ethylene-binding domain, perhaps acting as a molecular chaperone for ETR1. RTE1 does not have any detectable effect on ethylene binding, nor does RTE1 itself bind ethylene (A. Michiels and C.C., unpublished results). The specificity of *rte1* for certain *etr1* alleles suggests a close physical association between the

RTE1 and ETR1 proteins. RTE1 and ETR1 co-localize in the endomembrane system of Arabidopsis root cells (Dong *et al.*, 2008), but it has yet to be shown whether the two proteins physically interact. The basis for the apparent specificity of RTE1 for ETR1 versus the other ethylene receptor family members in Arabidopsis is also not yet understood.

The role of RTE1 in promoting ETR1 signaling is interesting in light of the fact that the *RTE1* gene is highly conserved in organisms that do not possess ethylene receptors. Animals carry a single copy of the *RTE1* gene, but the function of RTE1 in animals is unknown. The extensive genetic tools provided by the ethylene-signaling pathway in Arabidopsis may help to elucidate the conserved function of RTE1 in other organisms.

Experimental procedures

Plant strains and growth conditions

Arabidopsis thaliana wild-type ecotype Columbia (Col-0) was used throughout this study. For all seedling analyses, seeds were sown on Murashige and Skoog (MS) medium containing 0.9% agar. Following 3-day stratification at 4°C, seeds were incubated at 20°C for 4 days in either complete darkness or 24 h light. Plants were grown in soil under a 16 h light/8 h dark cycle in controlled environment chambers under fluorescent lights. For the triple-response assay, seedlings were germinated on medium containing ACC (Sigma- Aldrich, <http://www.sigmaaldrich.com/>) at the stated concentrations. To measure hypocotyl lengths, seedlings were digitally photographed and measurements were made using ImageJ software (<http://rsb.info.nih.gov/ij/>). For copper-response assays, seedlings were germinated on medium containing 50 µM CuSO₄.

Genetic analysis

To create double mutants with *ran1-3*, a *ran1-3/+* heterozygous single mutant plant was crossed separately with *rte1-2* and *etr1-2* single mutants. F₁ plants were confirmed to be heterozygous for *ran1-3* by genotyping and allowed to self-pollinate. Double mutants were identified in the F₂ generation by genotyping. The *rte1-2 ran1-3* and *etr1-2 ran1-3* double mutant lines were maintained as heterozygotes for the *ran1-3* locus, due to the adult lethality of homozygous *ran1-3*.

To create the *ran1-3/+ rte1-3 etr1-2* triple mutant, the *ran1-3/+ etr1-2* double mutant was crossed with the *rte1-3 etr1-2* double mutant. F₁ plants confirmed by genotyping to be heterozygous for *ran1-3* were allowed to self-fertilize, and *rte1-3 etr1-2 ran1-3/+* plants were identified in the F₂ generation.

For genotyping, the *rte1-2*, *rte1-3* and *etr1-2* alleles were detected using CAPS markers (Konieczny and Ausubel, 1993) as previously described (Resnick *et al.*, 2006). The *ran1-3* allele was detected by CAPS using primers 5'-CTTTGACAAAACAGGCACCTT-3' and 5'-ACCATTATGGGTCTGAACACC-3' to amplify a DNA fragment that is cleaved by the restriction enzyme *HphI* if the fragment is from the mutant *ran1-3* allele, but not if it is from wild-type *RAN1*.

Transgenic constructs and plant transformation

For *RTE1* over-expression in *ran1-3*, a construct containing the *RTE1* coding sequence driven by the CaMV 35S promoter (described by Resnick *et al.*, 2006) was transformed by the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* strain GV3101. Homozygous *ran1-3* plants are lethal at the rosette stage, so are not viable for transformation. Therefore, *ran1-3* heterozygous plants were identified by genotyping. Transformants were selected using the herbicide FinaleTM (Bayer, <http://www.bayercropscience.com>) and were

genotyped for the *ran1-3* allele. Five independent transformants, which were homozygous for *35S:RTE1* and heterozygous for *ran1-3*, were analyzed in the T₃ generation. T₃ seedlings showing a *ran1-3* phenotype were examined and selected using Finale™ to confirm the presence of the *35S:RTE1* transgene.

All mutant *etr1* transgenes (in plasmid pPZP211) were kindly provided by the laboratory of Anthony Bleecker (University of Wisconsin, Madison, WI, USA). The *etr1* mutant transgenes were transformed into wild-type and *rte1-2* plants using the floral dip method. Transformed T₁ individuals were selected on MS medium containing 100 mg l⁻¹ kanamycin. Five to eight independent transgenic lines were screened in the T₂ generation on MS plates with and without 20 µM ACC, with a total of 100–200 seedlings observed per line. For each transgene, measurements were made of 15–25 seedlings for three or four segregating T₂ populations, as well as for one or two homozygous T₃ lines.

Western blotting

The ETR1–5xMyc construct described by Dong *et al.* (2008) was transformed into both the *etr1-7* null mutant and the *etr1-7 rte1-2* double mutant described by Resnick *et al.* (2006) using the floral dip method. Membrane proteins were isolated from 8–10-day-old lightgrown seedlings, followed by SDS–PAGE and Western blotting as described by Dong *et al.* (2008).

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Figure 1.

Presence of the ETR1 protein in the *rte1-2* loss-of-function mutant. Western blot showing the ETR1-5xMyc monomer isolated from the microsomal membrane fraction of Arabidopsis seedlings run on denaturing PAGE and detected by an anti-*c-myc* antibody (MYC-Ab). The ETR1-5xMyc construct transformed into *etr1-7* and the *etr1-7 rte1-2* double mutant gives a band of approximately 80 kDa (left and center lanes) that is absent in the untransformed wild-type (WT, right lane). ECA1, an ER membrane protein detected by the ECA1-Ab antibody (Liang *et al.*, 1997), was used as a loading control.

Figure 2. RTE1 action is independent and distinct from that of RAN1. Comparisons of 4-day-old dark-grown seedlings

(a) The *ran1-3 rte1-2* double mutant has an additive phenotype, suggesting that RTE1 and RAN1 act in separate pathways. Shown are representative seedlings of the indicated mutant genotypes grown in the presence and absence of 50 μM CuSO_4 , with wild-type (WT) as a control.

(b) Double and triple mutant phenotypes indicate that *ran1-3* cannot suppress *etr1-2*, and suppression of *etr1-2* by *rte1-3* is independent of RAN1. Shown are representative seedlings of the indicated genotypes grown in the presence and absence of the ethylene precursor ACC.

(c) Genetic model of RTE1 and RAN1 action with respect to *ETR1* and *etr1-2* in ethylene signaling. RTE1 and RAN1 are both required for wild-type *ETR1* signaling, which represses ethylene responses, but only RTE1 is required for *etr1-2*.

Figure 3. Effect of *etr1* dominant mutant transgenes in wild-type and *rte1-2* seedlings

(a) The three transmembrane regions of the ETR1 ethylene-binding domain showing the relative positions of *etr1* mutations tested for suppression in the *rte1-2* background. Vertical lines indicate the approximate locations of the specified amino acid substitutions carried by *etr1* transgenes transformed into the wild-type and *rte1-2* to test for ethylene insensitivity and suppression of ethylene insensitivity, respectively. In place of transgenes, the *etr1-1* (C65Y) and *etr1-2* (A102T) mutations were tested for suppression using the *etr1-1 rte1-2* and *etr1-2 rte1-2* double mutants respectively.

(b) Measurements of hypocotyl length conferred by the mutant *etr1* transgenes in wild-type versus *rte1-2* seedlings showing the degree of ethylene insensitivity and suppression, respectively. Hypocotyl lengths (mean $\pm$ SE for 15–20 seedlings) were measured in representative homozygous lines of 4-day-old dark-grown seedlings on 20 μ M ACC. Untransformed seedlings are indicated as 'no transgene'.

Figure 4. Model for the promotion of ETR1 signaling by RTE1

The three-state model of ethylene receptor signaling proposed by Wang *et al.* (2006) is adapted here for the ETR1 receptor by incorporating RTE1 at two possible points, 'A' and 'B', and including an additional state '*i*'. At both 'A' and 'B', RTE1 acts on the ethylene-binding domain to promote the signaling 'on' state. In state '*i*', the nascent non-functional ETR1 protein requires the action of RTE1 at 'A' to allow transition to the functional 'on' state. In state 'I', ethylene is not yet bound and the signaling state of ETR1 is 'on'; when ethylene binds, the receptor moves into a quasi-stable state ('II'), where ethylene is bound but the receptor is not yet off (Wang *et al.*, 2006). To enter state 'III', the ethylene-binding domain undergoes a conformational shift as a result of binding ethylene, and this conformational change is transmitted to the signaling domain to turn signaling off (Wang *et al.*, 2006). RTE1 at 'B' inhibits transition of ETR1 from the signaling 'on' state (II) to the signaling 'off' state (III). The RTE1-dependent mutant forms of ETR1 are held in either state I ('on') or state II ('on'), depending on whether they bind ethylene or not.

Table 1Summary of the ability of *rte1-2* to suppress a variety of dominant *etr1* mutant alleles

Mutation	Ethylene binding (%) ^a	Signaling strength (%) ^b	Suppressed by <i>rte1-2</i> ? (%) ^c
<i>etr1-2</i> (A102T)	150	Weak (74)	Yes (36)
Y32A	<5	Strong (100)	Yes (42)
F61A	20	Weak (73)	Yes (43)
E38A	155	Strong (99)	Yes (45)
L64A	110	Weak (70)	Yes (45)
M104A	90	Weak (74)	Yes (45)
F58A	110	Strong (87)	Yes (46)
I108A	70	Strong (98)	No (80)
D25A	0	Strong (100)	No (82)
<i>etr1-1</i> (C65Y)	0	Strong (97)	No (93)
T101A	50	Strong (99)	No (95)
T94M	1.6 ^d	Strong (97)	No (98)
L105A	50	Strong (100)	No (98)

^a Data on percentage ethylene binding with respect to wild-type obtained by Wang *et al.* (2006).^b Values are the percentage hypocotyl length on 20 μ M ACC with respect to that on no ACC in the wild-type background.^c Values are the percentage hypocotyl length in the *rte1-2* background with respect to that in the wild-type background on 20 μ M ACC (Figure 3b).^d Result provided by W. Wang (personal communication).