ABSTRACT

Title of Dissertation: *REVERSION-TO-ETHYLENE-SENSITIVITY1:* A NOVEL REGULATOR OF ETHYLENE RECEPTOR FUNCTION IN *ARABIDOPSIS THALIANA*

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Ethylene is a plant hormone that has profound effects on plant growth and development. Genetic analysis has been central in the elucidation of the ethylene-signaling pathway, made possible through the isolation of ethylene-response mutants in *Arabidopsis.* This thesis focuses on elucidating the function of the *Arabidopsis REVERSION-TO-ETHYLENE–SENSITIVITY1* (*RTE1)* locus, which was identified in a genetic screen for suppressors of the ethylene-insensitive receptor mutant *etr1-2.*

The *RTE1* gene was cloned by positional cloning and found to encode a novel integral membrane protein with homologs in plants and animals, but with no known molecular function. Our studies show that *RTE1* is a negative regulator of the ethylene-response pathway, specifically acting as a positive regulator of the ETR1 ethylene receptor. Loss-of-function mutations in the *RTE1* gene suppress the *etr1-2* ethylene-insensitive phenotype, and genetic analysis suggests that loss of *RTE1* results in a largely non-functional ETR1-2 mutant receptor. Similarly, wild-type ETR1 function appears to be greatly reduced in the absence of *RTE1*. Overexpression of the *RTE1* gene confers weak ethylene insensitivity that is largely dependent on

ETR1. *rte1* mutations do not appear to affect the other four ethylene receptors of *Arabidopsis*, indicating that RTE1 specifically regulates ETR1. Sequence analysis revealed regions of conserved cysteine and histidine residues, and one *rte1* loss-of-function mutant contains a point mutation at Cys^{161}. Since such residues are common in metal binding proteins, we explored the possibility that RTE1 may be involved in facilitating the binding of an essential copper cofactor to the ETR1 receptor. However, experimental evidence suggests that this is not the likely role of RTE1.

Interestingly, *rte1* was unable to suppress the ethylene insensitive mutant *etr1-1*, indicating that the differences between *etr1-2* and *etr1-1* may hold a clue as to how *RTE1* regulates *ETR1*. A suppression analysis of eleven additional *etr1* insensitive mutants suggests that *RTE1* plays a role in regulating signaling by the transmitter domain of ETR1. A possible role for RTE homologs in non-plant systems is also discussed, although more work is required to elucidate a detailed biochemical model for RTE1 action.

REVERSION-TO-ETHYLENE-SENSITIVITY1: A NOVEL REGULATOR OF
ETHYLENE RECEPTOR FUNCTION IN *ARABIDOPSIS THALIANA*

by

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Dedication

This dissertation is dedicated to my husband Jonathan, who regularly tells me that

nothing is beyond reach.

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Firstly, I would like to thank my advisor and mentor, Dr. Caren Chang, who gave 110% to ensure not only that my project was progressing but also that I was developing as a Scientist. Her availability 24 hours a day, 7 days a week is something for which I would like to express deep gratitude.

I would like to acknowledge my committee, for their continued support of my project, and for their advice, help and encouragement given over the last five years.

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I would also like to thank the other members of the Chang lab, for the enduring team spirit that provided an open forum for discussion, debate and troubleshooting.

Finally I would like to thank my family and friends, who have given me endless support, love and encouragement.

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List of Abbreviations

ACC, 1-aminocyclopropane 1-carboxylic acid
ACS, ACC synthase
ARR, *Arabidopsis* Response Regulator
ATX, Antioxidant Protein 1
AVG, 1-aminoethoxyvinylglycine
BAT, Brown Adipose Tissue
CAPS, Cleaved Amplified Polymorphic Sequence
CCC, Cytosolic Copper Chaperone
CCH, Copper Chaperone
Cer, Ceruloplasmin
cGMP, Cyclic guanosine monophosphate
Col, Columbia
COPT, Copper Transporter
CTR, Constitutive Triple Response
dCAPS, Derived Cleaved Amplified Polymorphic Sequence
DUB, Deubiquitinating
EBD, Ethylene Binding Domain
EBF, EIN3-binding F-box
EIL, EIN3-Like
EIN, Ethylene Insensitive
ERE, Ethylene Responsive Element
ERF, Ethylene Response Factor
ERS, Ethylene Response Sensor
EREBP, Ethylene Response Element Binding Protein
ETR, Ethylene Resistant
Fet3, Ferrous Transporter 3
GAF, cGMP-specific and Stimulated Phosphodiesterases, *Anabaena* Adenylate cyclases and *E-coli* Fhl1A
GFP, Green Fluorescent Protein
GR, Green ripe
HAH, Human ATX1 Homolog
HK, Histidine Kinase
HOG, High Osmolarity Glycol
JA, Jasmonic Acid
JAMM, Jab1/Pad1/MPN-domain metallo-enzyme
LTP, Lipid Transfer Protein
MAPK, Mitogen Activated Protein Kinase
MAPKK, MAPK Kinase
MAPKKK, MAPKK Kinase
MMK, Medicago MAP Kinase
MNK, Menkes Copper-Translocating P-type ATPase
MS, Murashige and Skoog Medium
MT, Metallothioneins

NC, Neocuproine
NR, Never ripe
NRAMP, Natural Resistance-Associated Macrophage Protein
PAG, Proliferation Associated Gene
PERE, Primary Ethylene Response Element
PCR, Polymerase Chain Reaction
RAN, Responsive To Antagonist
RER, Retrieval to the ER
RFP, Red Fluorescent Protein
RR, Response Regulator Protein
RTE, Reversion To Ethylene Sensitivity
RTH, RTE1 Homolog
RT-PCR, Reverse Transcriptase PCR
SA, Salicylic Acid
SCF, Skp1-Cullin-F-box Ubuquitin Ligase
SH3, Src Homology 3
SIMK, Stress Activated MAP Kinase
SIMKK, SIMK Kinase
SOD, Super Oxide Dismutase
TILLING, Targeting Induced Local Lesions In Genomes
Ub, Ubiquitin
UBA, Ub-Associated
UBC, Ub Conjugating
UCH, Ub C-terminal Hydrolases
UBP, Ub-Specific Processing Proteases
UIM, Ub-Interaction Motif

Chapter 1: The ethylene-signaling pathway

The gaseous plant hormone ethylene is a simple hydrocarbon that was one of the first plant hormones to be identified (Abeles et al., 1992). The properties of this hormone have been utilized unwittingly for thousands of years. For example, in ancient Egypt, people would wound figs knowing it could speed up the ripening process. The wounding of fruit causes it to produce ethylene, which promotes fruit ripening. The saying "One bad apple spoils the barrel" is commonly used now in reference to one person who can spoil everything around them through their actions, but it's origins go back to the fact that one rotten apple in a barrel of apples can cause them all to go bad. The reason behind this is that the rotting apple produces ethylene (as a result of wounding and senescence), and since ethylene is a gas, it can result in both premature ripening and premature senescence of the surrounding fruit.

The use of ethylene in agriculture dates back to before its identification as an active compound. Pineapple farmers in Puerto Rico would light fires near the pineapple plants to promote and synchronize flowering. Lemon farmers in the U.S. stored lemons in warm warehouses to ripen the fruit. These warehouses were heated by kerosene heaters, but when they were replaced by more modern heaters, the farmers no longer got the desired effect.

In the early twentieth century, it was noticed that leaking illuminating coal gas (carried through pipelines) caused vegetation to senesce much more than in surrounding areas. Dimitry Neljubov published a paper in 1901 that identified

ethylene as the active component in illuminating gas that was causing such damage to plants (Neljubov, 1901).

Ethylene has profound effects on plant growth and development. Responses are evident during all stages of a plant's life cycle: from early stages, in processes such as promotion of seed germination and root hair formation; through mid-life stages such as ripening of climacteric fruits, and promotion or inhibition of flowering; and finally in the end life stages where it plays an important role abscission of organs and senescence (Abeles et al., 1992). Ethylene also plays a part in plant defense responses: a plant may endogenously produce ethylene in response to environmental stresses such as water stress (i.e. flooding or drought) or pathogen attack and wounding, which may lead to responses such as abscission of affected organs, premature senescence, and/or activation of defense-response pathways (Abeles et al., 1992).

Because of the important roles ethylene plays in a plant's life cycle, its production is tightly regulated in response to these developmental stages or external stimuli. The ethylene biosynthetic pathway is largely understood and has been well documented (Yang and Hoffman, 1984; Kende, 1993; Johnson and Ecker, 1998; Wang et al., 2002). It is actually more clearly understood than the signaling cascade that it instigates. In brief, ethylene is produced from the amino acid methionine, which is converted to S-adenosyl methionine (AdoMet) by AdoMet synthetase. The next step is the rate-limiting step in ethylene biosynthesis and involves the conversion of AdoMet to a-aminocyclopropane-1-carboxylic acid (ACC), from which the conversion to ethylene can easily occur via the action of the enzyme ACC oxidase.

The synthesis of ethylene is regulated in a cyclic fashion, where perception of ethylene leads to a signaling cascade and ultimately the induction of ethylene-response genes. These genes (involved in various responses to physical, environmental or stress stimuli) are themselves able to regulate the enzymes involved in ethylene biosynthesis – i.e. ACC synthase and ACC oxidase (Johnson and Ecker, 1998), and consequently, ethylene biosynthesis is a self-regulated system in higher plants.

Arabidopsis seedlings grown in the dark in the presence of ethylene exhibit a characteristic "triple response", which consists of a shortening of the hypocotyl and root (caused by an inhibition of cell elongation), radial swelling of the hypocotyl, and an exaggeration in the curvature of the apical hook (Fig. 1-1). Screening for mutants exhibiting an altered triple response has been central in elucidating components of the ethylene-signaling pathway, which begins with the binding of ethylene and culminates with changes in gene expression. Triple-response mutants can be divided into two main categories: ethylene-insensitive mutants – those that are unable to respond to ethylene and do not exhibit the triple response even under high concentrations of ethylene (although some weaker insensitive mutants may display slight hypocotyl shortening); and constitutive-response mutants, which act as though ethylene is 'constitutively' present, exhibiting the triple response even when no ethylene is present. Close analysis of these mutants has enabled a detailed dissection of the ethylene-signaling pathway, and the development of a mostly linear response-pathway model (Guzman and Ecker, 1990; Bleecker and Kende, 2000; Hirayama and Alonso, 2000; Stepanova and Ecker, 2000) (Fig. 1-2).

- ethylene   + ethylene

Figure 1-1. The *Arabidopsis* triple response.

Dark-grown *Arabidopsis* grown in the presence of ethylene or the ethylene precursor ACC exhibits a characteristic triple response phenotype. Defining features of this response include inhibition of cell elongation in the hypocotyl and root, shortening of the hypocotyl and root, radial swelling of the hypocotyl, exaggerated curvature of the apical hook, and a proliferation of root hairs.

Figure 1-2. Known components of the ethylene-signaling pathway.

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Ethylene is perceived by a family of five ethylene receptors, ETR1, ETR2, EIN4, ERS1 and ERS2, one of which (ETR1) has been localized to the endoplasmic reticulum (Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998; Chen et al., 2002). These receptors form homodimers and require the association of a copper cofactor in order to bind ethylene (Rodriguez et al., 1999; Hirayama and Alonso, 2000). The delivery of copper is dependent on the transporter RAN1, which is probably localized to the trans-Golgi (Hirayama et al., 1999; Woeste and Kieber, 2000). Under wild-type conditions, the receptors functionally repress downstream ethylene responses through CTR1, a raf-like MAPKKK that also acts to negatively regulate the pathway (possibly through a MAPK cascade) (Kieber et al., 1993). Upon ethylene binding, the receptor is inactivated, and therefore no longer signals to CTR1. With CTR1 no longer repressing downstream signaling, EIN2 becomes activated. EIN2 is an integral membrane protein with some similarity to the NRAMP family of metal-ion transporters (Alonso et al., 1999). The function of EIN2 is unknown, but signaling is believed to occur through its novel C-terminal domain (Alonso et al., 1999; Hirayama and Alonso, 2000). EIN2 is a positive regulator of ethylene responses, and acts to activate EIN3, which is a member of the EIN3/EIL1 family of transcription factors (Chao et al., 1997). EIN3 is regulated by two F-box proteins, which in the absence of ethylene target EIN3 for degradation through the Ub-proteasomal pathway (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). Upon ethylene binding, EIN3 is stabilized, enabling it to activate a transcriptional cascade and the expression of ethylene-response genes.
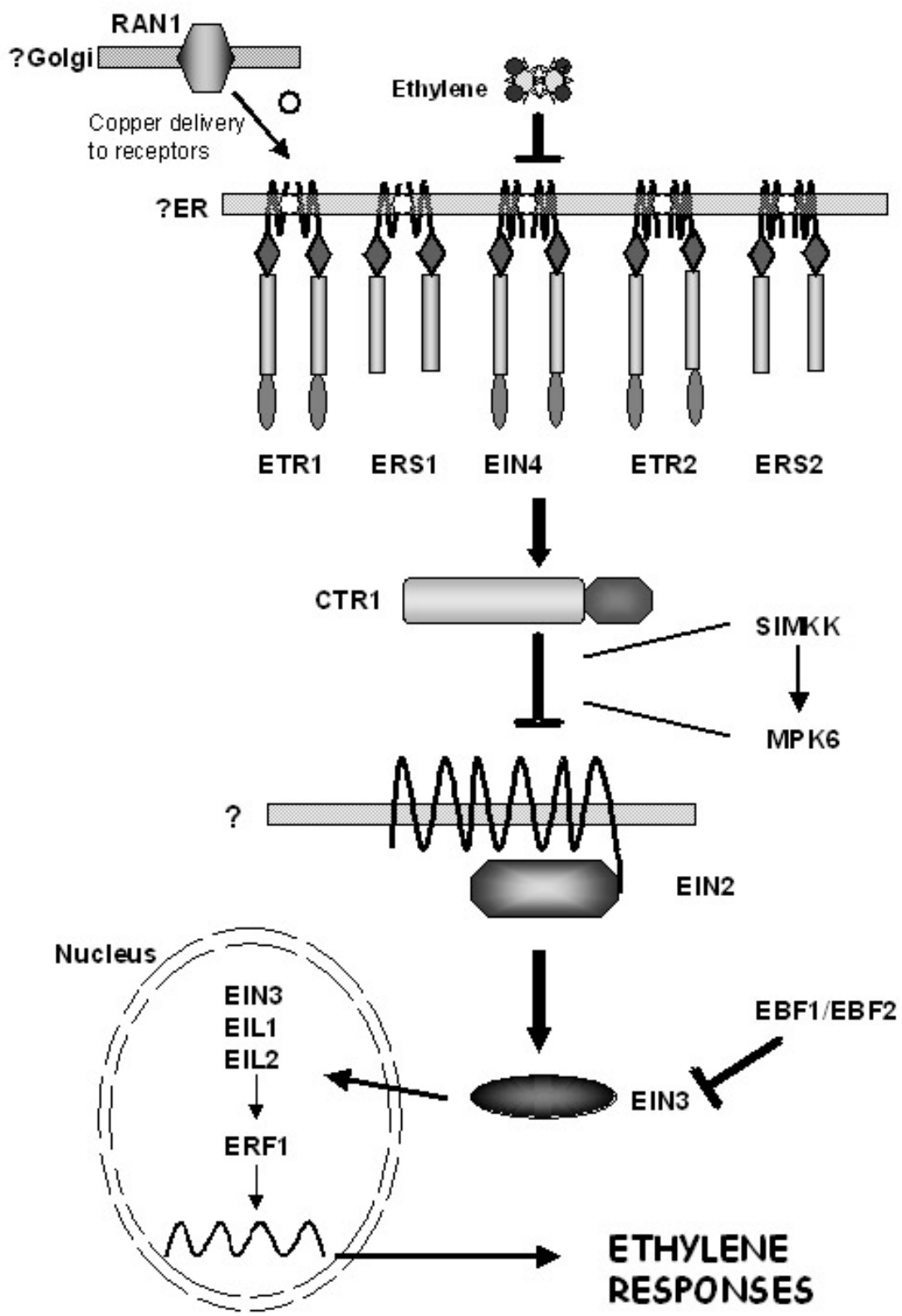
Ethylene is perceived by a family of receptors that are related to prokaryotic two-component histidine kinase proteins. These receptors signal to CTR1 – a Raf-like protein kinase, which is a negative regulator of the pathway (Kieber et al., 1993). Inactivation of CTR1 leads to the activation of the next downstream component, EIN2: a membrane bound protein with similarity to the NRAMP family of metal ion transporters (Alonso et al., 1999). *EIN2* is a positive regulator of downstream transcription factors such as EIN3 and EIL1, which are the first in a series of transcription factors involved in a transcriptional cascade that ultimately regulate the expression of ethylene-response genes (Chao et al., 1997)

ETR1 and the ethylene receptor family

A genetic screen to identify ethylene-insensitive mutants resulted in the isolation of an *etr1* mutant, and subsequent cloning of the *ETR1* receptor gene (Chang et al., 1993). This receptor has striking similarity to prokaryotic two-component regulators and was the first protein of its kind to be identified in a eukaryotic system (Schaller, 2002). The identification of four paralogous receptor genes in *Arabidopsis* soon followed: *ETR2, EIN4, ERS1* and *ERS2* all have similarity to *ETR1*, and combined they comprise the ethylene receptor family. All of these receptors are important for ethylene signaling, although *ETR1* has been characterized to the largest degree.

All receptors contain an amino-terminal membrane-bound region, which encompasses the ethylene-binding pocket. Following this is a GAF-like domain, which has unknown functional significance for ethylene signaling. GAF domains are

commonly found in both prokaryotic and eukaryotic photoreceptors, and are sometimes associated with cGMP-dependent signaling events (Aravind and Ponting, 1997), although this has not been demonstrated in the ethylene-signaling pathway. The plant photoreceptor phytochrome is believed to have evolved from prokaryotic two-component photoreceptors, and so it is possible that the ethylene receptors share a common ancestor with photoreceptors before it gained an ethylene-binding domain. ETR1 lacks a conserved cysteine residue that is necessary for chromophore attachment to phytochromes, so it is unlikely to be involved in light sensing. It is thought that GAF domains comprise one of the largest families of small-molecule-binding regulatory domains (Zoraghi et al., 2004), but it is believed that not all GAF domains bind small molecules, and may actually carry out diverse functions. One difference between plant ethylene receptors and their *Synechocystis* counterpart is the presence of a PAS/PAC (PP) domain in place of the GAF domain (Mount and Chang, 2002). PP domains are commonly found in signaling proteins, acting as signal sensory domains, and have a three-dimensional structure similar to the GAF domain (Studholme and Dixon, 2003). The conformation of some PAS domains changes upon ligand binding (Amezcua et al., 2002), perhaps suggesting that PP and/or GAF domains are able to faciliate conformational changes within their contained protein. PAS domains have also been implicated in mediating the formation of receptor heterodimers (Lindebro et al., 1995). It is therefore feasible that the GAF domain in ethylene receptors is important for conformational changes within the protein, or for its ability to interact with other receptors or proteins.

Downstream of the GAF domain is a histidine kinase-like domain (Bleecker, 1999), followed by a carboxyl-terminal receiver domain in three of the five receptors (Fig. 1-3). Based on structural similarities, the receptors are divided into two subfamilies. *ETR1* and *ERS1* comprise subfamily I (Chang et al., 1993; Hua et al., 1995), both containing three transmembrane domains followed by a conserved histidine kinase domain. *ETR2, ERS2* and *EIN4* comprise subfamily II (Hua et al., 1998; Sakai et al., 1998), all of which have 4 transmembrane domains and a degenerate histidine kinase domain that lacks one or all of the functional motifs necessary for catalytic activity (Fig. 1-3). One member of each family (*ERS1* and *ERS2*) lacks a receiver domain.

The amino-terminal domain is the most conserved portion of the receptors, and has been shown to be the ethylene-binding region in both ETR1 and ERS1 (Schaller and Bleecker, 1995; Hall et al., 1999; Rodriguez et al., 1999; Hall et al., 2000). Although ethylene binding has not been demonstrated for the other receptors, the high degree of sequence similarity implies that they are also able to functionally bind ethylene. It is believed that the receptors form homodimers, with one ethylene-binding site created upon dimerization (Rodriguez et al., 1999). It has long been predicted that ethylene binding would require the presence of a transition metal (Burg and Burg, 1967), and it is now known that high affinity ethylene binding is dependent on the presence of a copper cofactor (Hirayama et al., 1999; Rodriguez et al., 1999; Woeste and Kieber, 2000). ETR1 is the only receptor thus far to be shown to bind copper (Rodriguez et al., 1999), although it is predicted that all receptors are functionally dependent on its presence. Candidate ligating side chains for copper
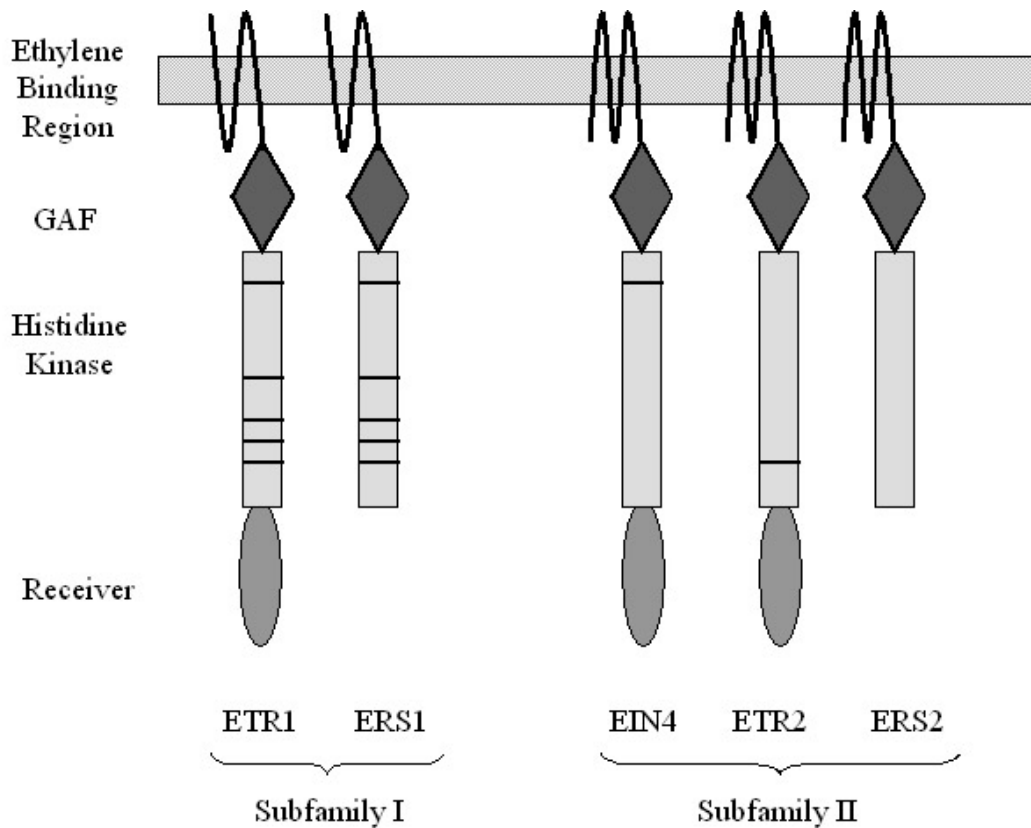
Figure 1-3. The ethylene receptor family in *Arabidopsis*.

The five ethylene receptors in *Arabidopsis* are divided into two subfamilies based primarily on sequence similarity. Type I receptors contain all motifs necessary to for a functional histidine kinase activity, where type II receptors do not. The conserved histidine kinase motifs are indicated by bars. However, the receptors are similar in most regards. All receptors contain a highly conserved transmembrane ethylene binding domain, with three membrane spanning regions in subfamily I receptors and four in subfamily II. All receptors contain a 'GAF' domain of unknown function, and a histidine kinase domain. Three of the five receptors also contain a receiver domain (ETR1, EIN4 and ETR2). The GAF, histidine kinase and receiver domains comprise the cytoplasmic 'transmitter', which is important for signal transmission.

binding are the conserved Cys^{65} and His^{69} residues in the second transmembrane domain of ETR1, and mutations in either one of these residues are sufficient to eliminate ethylene binding (Bleecker et al., 1998; Rodriguez et al., 1999). It is likely that these residues form a hydrophobic pocket in the ETR1 homodimer to bind two copper (I) ions (Schaller and Bleecker, 1995; Schaller et al., 1995; Bleecker et al., 1998; Rodriguez et al., 1999). Once copper is bound, and ethylene is able to associate, a conformational change occurs in the transmembrane binding domain, causing the receptor to inactivate, and therefore allowing downstream responses to proceed (Bleecker et al. 1998, Wang et al., 2006). It is believed that the other ethylene receptors utilize similar signaling mechanisms.

Delivery of copper to the receptors is dependent on RAN1 – a copper transporter with homology to the Menkes/Wilson P-type ATPase found in mammalian systems (Hirayama et al., 1999; Woeste and Kieber, 2000). Loss of RAN1 results in a severe constitutive ethylene response in *Arabidopsis* seedlings (Woeste and Kieber, 2000), implying that it causes a major disruption to the ethylene receptors, such that they become essentially non-functional. Addition of copper to the plant growth media partially rescues this constitutive phenotype.

It is likely that the carboxyl-terminal portion of the receptor is responsible for the signal output, although whether the receptors signal in a manner similar to two-component systems has yet to be demonstrated. In two-component systems, the binding of a ligand initiates autophosphorylation of the histidine kinases to a conserved histidine residue. This phosphate molecule is then transferred to an aspartic acid residue in a receiver domain, which may be associated with the receptor (such as

in hybrid histidine protein kinases), or in separate proteins known as response regulators (RRs) (Parkinson and Kofoid, 1992; Swanson et al., 1994). The hormone cytokinin is known to utilize two-component-like signaling, including the use of RRs to transfer the signal to downstream components (Hwang and Sheen, 2001; Inoue et al., 2001). However, phosphotransfer and the employment of RRs have not been shown to occur in the ethylene-signaling pathway. It is known that ETR1 exhibits histidine kinase activity *in vitro* (Gamble et al., 1998), but subsequent transfer of the phosphate to a receiver domain has yet to be demonstrated. Interestingly, mutations that disrupt the kinase activity of ETR1 do not seem to alter its ethylene signaling capabilities (Gamble et al., 2002; Wang et al., 2003), indicating that phosphotransfer to a receiver domain may not be essential for ethylene signaling. Indeed, activation of the next downstream component, CTR1, through a phosphorelay-type mechanism has not been demonstrated. In fact, CTR1 is capable of physically interacting with both ETR1 and ERS1 (Clark et al., 1998), indicating that the signal may be transmitted through an allosteric mechanism. We cannot rule out that the histidine kinase activity of ETR1 may be important for other functions, such as allowing for cross-talk with other signaling mechanisms, or maybe even for localization or protein stability.

The ethylene receptors have been described as having a 'redundant' function (Hua and Meyerowitz, 1998), although based on phenotypic variations, we can assume that different receptors are likely to carry out slightly different roles. A single loss-of-function mutant in any one receptor yields a phenotype virtually identical to the wild type, the one exception being an *etr1* null mutant, which exhibits enhanced sensitivity to ethylene (Hua and Meyerowitz, 1998). However, as multiple receptors

are eliminated, an increasingly constitutive response phenotype is observed, indicating that the receptors are negative regulators of ethylene responses (Hua and Meyerowitz, 1998). Interestingly, loss of both members of subfamily I has a more dramatic effect on responses than loss of all three of the subfamily II receptors (Hall and Bleecker, 2003; G.E. Schaller, personal communication). This indicates that the subfamily I receptors might play a larger and more important role in ethylene signaling. However, it is still widely believed that the members of subfamily II do in fact signal in a similar way to subfamily I, since there is some evidence to suggest that these receptors are also able to interact with CTR1 (Cancel and Larsen, 2002; Guo and Ecker, 2003).

The finding that the receptors are negative regulators of ethylene responses suggests that in the absence of ethylene, the receptors are actively signaling to inhibit ethylene responses. The association of ethylene inactivates the receptor, allowing downstream ethylene responses to proceed. As mentioned above, the receptors are largely redundant, and loss of one receptor has little effect on the phenotype. However, closer analysis does indicate that sensitivity to ethylene is altered. Loss of *ETR1* function results in ethylene hypersensitivity, such that seedlings respond to much lower ethylene concentrations and in fact are shorter than wild-type seedlings even when no ethylene is present (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002). It is likely that *Arabidopsis* and other plants regulate receptor levels as a way to manage the degree of sensitivity exhibited. Variations in environmental conditions have been shown to cause changes in receptor levels (Klee and Tieman, 2002; Zhao and Schaller, 2004), and tomato plants are known to regulate receptor expression in a

tissue and developmental-stage dependent manner (Payton et al., 1996; Lashbrook et al., 1998; Tieman and Klee, 1999).

When it comes to the subject of ligand-receptor binding, it is generally assumed that receptors localize to the plasma membrane, making it readily available to an external ligand. However, ethylene is lipid soluble, and therefore localization to the plasma membrane offers no advantage over other cell membranes. Indeed, recent localization experiments indicate that ETR1 is present in the endoplasmic reticulum (Chen et al., 2002), and it is anticipated that other receptors also share the same sub-cellular address. It may well be that localization to the ER is more efficient for signaling, or simply that the location of the receptors is inconsequential.

The ethylene receptors are a unique type of receptor, participating in an unusual, and somewhat ambiguous signaling pathway. Binding of ethylene inactivates the receptors, which is counter-intuitive to our usual concept of signaling pathways. In addition, the receptors have striking similarity to prokaryotic two-component regulators, and yet signal to a eukaryotic-like MAPKKK (CTR1), probably through direct physical interaction (Clark et al., 1998; Gao et al., 2003); this is another novel feature of the ethylene-signaling pathway

CTR1

Downstream of the ethylene receptors, the signal is transmitted to a raf-like mitogen activated kinase kinase kinase (MAPKKK). *CTR1* was so named since it was identified in a screen for **C**onstitutive **T**riple-**R**esponse mutants – i.e. mutants that exhibit the triple response regardless of whether ethylene is present. It is also a

negative regulator of the ethylene-signaling pathway, and is likely to be inactivated

upon ethylene binding. As described in the previous section, CTR1 has been shown to

physically interact with the ETR1 receptor (Clark et al., 1998), suggesting that ETR1

may regulate CTR1 through an allosteric mechanism. ERS1 and ETR2 are also able

to interact with CTR1, although seemingly to a lesser degree. Supporting this

hypothesis is the recent finding that CTR1 co-localizes with ETR1 to the ER

membrane (Gao et al., 2003). Since CTR1 does not contain any transmembrane

domains, this indicates that a physical interaction between CTR1 and the receptors is

likely necessary for transmission of the ethylene signal. A model is suggested where

in the absence of ethylene, CTR1 is associated with the ethylene receptors. When

ethylene binds, a conformational change leads to inactivation of the receptor,

meaning that it is no longer able to activate CTR1. CTR1 is therefore unable to

functionally repress downstream components, and ethylene responses are activated

(Kieber et al., 1993; Bleecker, 1999; Huang et al., 2003). This hypothesis might also

help us understand the phenotype seen in multiple receptor nulls. The levels of

membrane-associated CTR1 are similar between the wild type and single receptor

null mutants, but if you eliminate two or more receptors, increased quantities of

soluble CTR1 protein are observed. This correlates with the strength of the

constitutive response seen if multiple receptors are eliminated, such that elimination

of three receptors, which has a strong constitutive response, results in a large amount

of cytoplasmic CTR1. Although CTR1-receptor interaction has only been

demonstrated for three of the receptors, these results also support the likely

hypothesis that all receptors regulate CTR1 action through direct physical interaction.

It is believed that the majority of the CTR1 protein remains at the ER during normal signaling conditions, implying that CTR1 is always associated with the receptors. Exactly how the CTR1 kinase domain is regulated remains to be shown, although one possibility commonly observed in kinase proteins is that the N-terminal region regulates the C-terminus through a direct interaction (Chong and Guan, 2003).

CTR1 contains two distinct domains: the amino-terminal end, which is necessary for interaction with the receptors (Clark et al., 1998; Gao et al., 2003), and the carboxyl-terminal end, which has similarity to the raf family of protein kinases (Kieber et al., 1993). A missense mutation in a highly conserved residue of the N-terminal end disables the ability of CTR1 to physically associate with the receptors, and results in a non-functional CTR1 protein (Huang et al., 2003). A functional and sequence analysis of the C-terminal region indicates that CTR1 contains a Ser/Thr kinase, and a missense mutation in this kinase domain results in a loss of CTR1 function (Kieber et al., 1993). Combined, this indicates that both domains are essential for CTR1 function, and that CTR1 must be localized to the receptors at the ER membrane with a functional Ser/Thr kinase domain in order to inhibit downstream ethylene responses. This is interesting since it was assumed that the ethylene receptors, with their high degree of similarity to two-component regulators, would follow a signaling cascade reminiscent of a two-component system. The physical interaction between the receptors and CTR1, which is required for transmission of a downstream signal, implies that the ethylene receptors directly activate a MAPK cascade. Supporting this, there is no evidence to suggest that the ethylene receptors recruit *Arabidopsis* response regulators (ARRs), such as those

found in other two-component pathways like the cytokinin signaling pathway

(D'Agostino and Kieber, 1999; D'Agostino et al., 2000; Mason et al., 2005)

The necessity for a functional kinase domain implicates CTR1 as a regulator

of a downstream MAPK cascade, which has long been predicted due to the similarity

of CTR1 to the Raf protein kinases. *CTR1* has sequence similarity to the raf family of

mitogen activated protein kinase kinase kinases (MAPKKKs). These are Eukaryotic

proteins that are important for the activation of MAPK cascades, which are involved

in a variety of signaling cascades to regulate many intracellular processes. A typical

MAPK cascade involves the activation of a MAPKKK, which phosophorylates a

MAPKK, which then phosphorylates a MAPK, which in turn phosphorylates

downstream proteins to regulate responses. The role of *CTR1* in the ethylene-

signaling pathway is somewhat unusual, since it is uncommon for a MAPK cascade

to lie downstream of a two-component receptor. However, several proteins that may

participate in a kinase cascade downstream of *CTR1* have recently been identified

(Ouaked et al., 2003). This group identified two MAPKs from *Medicago – MMK3*

and *SIMK*, which were activated after a short treatment with the ethylene precursor

aminocyclopropane-1-carboxylic acid (ACC). In *Arabidopsis*, *MPK6* (which is a

close homolog to *SIMK*) was also activated upon treatment with ACC, although this

was later shown by a different group to be important as a regulator of the ethylene-

biosynthesis pathway (Ecker, 2004; Liu and Zhang, 2004). Ouaked et al. next

identified a MAPKK in *Medicago – SIMKK*, which was also activated by ACC

treatment, and which regulated ACC-induced activation of both *SIMK* and *MPK3*.

Overexpression of *SIMKK* resulted in a constitutive phenotype in dark-grown

Arabidopsis seedlings, indicating that it is a positive regulator of the ethylene-response pathway. If *SlMKK* does indeed act downstream of *CTR1*, this would imply that *CTR1*, acting as a MAPKKK, negatively regulates *SlMKK*: this is unusual, since MAPKKKs usually act as positive regulators in a MAPK cascade. More research and biochemical evidence is required before these components can definitively be put into the ethylene-signaling pathway, but this research does indicate that *CTR1* is highly likely to act in a MAPK cascade to regulate the ethylene-signaling pathway.

Although no other components acting directly downstream of the receptors have been identified, there is some evidence to suggest that *CTR1* may not be the only mechanism through which the ethylene receptors transmit their signal. *ctr1* loss-of-function mutants exhibit a strong constitutive response, but it has been shown that they are still able to respond to ethylene (Roman et al., 1995; Larsen and Chang, 2001). In addition, both the subfamily I null mutant and quadruple receptor null mutant exhibit constitutive responses that are even stronger than the *ctr1* loss-of-function mutant (Hall and Bleecker, 2003; G.E. Schaller, personal communication), indicating that an alternative pathway may also be affected.

EIN2

The next known component downstream in the ethylene-signaling pathway is *EIN2*, which encodes a membrane bound protein that acts to positively regulate downstream ethylene responses. It is likely that *EIN2* represents an essential 'bottleneck' in the pathway, since *ein2* loss-of-function mutants are the strongest of the ethylene mutants (Alonso et al., 1999) and represents the only non-receptor

insensitive mutant that can confer complete insensitivity to a subfamily I null mutant (Hall and Bleecker, 2003).

EIN2 is a large protein, containing 12 predicted membrane-spanning regions at the amino-terminus. This membrane-bound region has significant sequence similarity to the NRAMP family of metal ion transporters, which are known to transport various divalent cations, including Mn^{2+}, Zn^{2+} and Cu^{2+} (Cellier et al., 1995). However, there is no evidence that EIN2 acts to bind or transport any kind of metal ion, and expression of EIN2 in yeast strains deficient in Mn and Fe uptake yielded no difference in growth rates (Thomine et al., 2000). To date, no NRAMP protein is known to be regulated through a MAPK pathway, although since new precedents have already been set in the ethylene-signaling pathway, this should not be a reason to rule out metal binding in EIN2 proteins. It is hypothesized that the N-terminal NRAMP-like membrane bound region acts as an 'input' domain, possibly involving the binding of a metal ion (although this has not been demonstrated). EIN2 differs from NRAMP proteins in that it has a soluble carboxy-terminal region that extends into the cytoplasm. This region is believed to be important for signal output, since over-expression of this region alone is sufficient to cause constitutive expression of ethylene-response genes in an *ein2* null mutant (Alonso et al., 1999). Exactly how EIN2 transmits the signal to downstream components is unknown, and the C-terminal region does not contain any recognizable motifs or similarity to known proteins. It does, however, contain a coiled-coil domain, which is common in regions involved in protein-protein interactions. This suggests that EIN2 may transmit the

signal through direct protein interaction, although exactly which protein it might interact with is still unknown.

One possible analogy may be drawn between EIN2 and glucose sensors/transporters in budding yeast. Glucose sensors also have 12 N-terminal membrane-spanning regions, which probably bind to glucose and acts as an 'input' domain. This is followed by a soluble C-terminal tail that is important for signaling to downstream components. Glucose transporters, in contrast, do not have a cytoplasmic tail, but the N-terminal region has significant similarity to glucose sensors, such that glucose sensors were originally thought to be glucose transporters. It is possible therefore, to draw a parallel between EIN2 and the NRAMP transporters, such that EIN2 may require the association of a metal ion with it's N-terminal domain in order to transmit a signal via the C-terminal cytoplasmic domain. However, there is no evidence thus far to indicate that this is the case.

Transcription factors and nuclear events

Although it is unknown whether EIN2 directly interacts with a downstream target, the next defined component in the pathway is *EIN3*, which is another positive regulator of ethylene responses. EIN3, along with at least three EIN3-like (EIL) proteins are nuclear localized and contain features commonly associated with transcription factors (Chao et al., 1997). An *ein3* loss-of-function mutant confers weak ethylene insensitivity, which can be complemented by at least two of the EIL proteins (EIL1 and EIL2) (Chao et al., 1997), indicating that EIN3 and EILs play an important role in ethylene signaling. This is supported by the finding that

overexpression of both EIN3 and EIL1 in both wild type and an *ein2* loss-of-function

mutant resulted in a constitutive ethylene response (Chao et al., 1997). To date, five

EIN3-like proteins have been identified (Chao et al., 1997; Solano et al., 1998;

Alonso et al., 2003), and since both *ein3* and *eil1* loss-of-function mutants confer only

partial ethylene insensitivity, there is probably some level of redundancy between

these proteins. However, an *ein3 eil1* double mutant confers strong insensitivity, and

can fully suppress the *ctr1-1* constitutive response mutant, indicative of all ethylene

responses being blocked (Alonso et al., 2003). Therefore, only *EIN3* and *EIL1* can be

conclusively implicated in the ethylene-signaling pathway, and questions remain

regarding the exact role of other *EILs*. It is possible that other EIL proteins are

involved in regulating the pathway under specific conditions or at particular points

during development. It is expected that the ethylene-signaling pathway undergoes a

certain level of 'fine tuning', dependent on levels of ethylene, specific stresses, cross-

talk with other pathways, and developmental pressures; transcription factors such as

the EIL proteins may well be important in these processes.

EIN3 is regulated at a post-translational level, being stabilized in the presence

of ethylene, and rapidly degraded when ethylene is removed (Guo and Ecker, 2003;

Potuschak et al., 2003; Gagne et al., 2004). This method of regulation allows for rapid

responses once ethylene is detected. Under normal conditions, EIN3 is constitutively

made and ubiquitinated, which results in recruitment to the 26S proteasome and

consequent degradation. Targeting and ubiquitination of EIN3 is mediated by an SCF

complex containing EBF1/2; two F-box proteins that are able to interact directly with

EIN3 (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). In the

presence of ethylene, *EBF1* and *EBF2* mRNAs are targeted for degradation by *EIN5* (a 5'-3' exoribonuclease) and other as yet unknown factors (Olmedo et al., 2006). EIN3 is therefore no longer targeted for degradation, resulting in a rapid increase in protein levels and allowing ethylene responses to proceed. Both *EBF1* and *EBF2* are up-regulated in response to ethylene, implying that EIN3 levels are regulated through a negative-feedback mechanism. There are still unknown components and mechanisms involved in this complex regulation of EIN3, which is a representation of how much still needs to be learned about the ethylene-signaling pathway. It is unknown whether EIL1 and other transcription factors, in addition to other components in the pathway are also regulated post-translationally.

An analysis of genes regulated through the ethylene-signaling pathway revealed two types of *cis*-acting ethylene responsive elements (EREs). The first is known as the primary ethylene response element (PERE), and is important for the regulation of genes involved in senescence and ripening. The second element is actually a GCC box, and binding to this element primarily activates genes important for stress and pathogen responses. Further analysis revealed a transcriptional cascade, where EIN3 (and possibly EIL1 and EIL2) binds to a primary ethylene response element (PERE) to regulate the transcription of *ERF1*, which itself is an AP2-domain containing transcription factor, and a member of the *ERF* (ethylene response element binding factor) family (Solano et al., 1998). Overexpression of *ERF1* results in a weak ethylene-response phenotype (Solano et al., 1998), indicating that *EIN3* likely has other targets in the ethylene response pathway. It also implies that *ERF1* is not sufficient to initiate a full ethylene response, raising the possibility that other

22

transcription factors (possibly other ERF proteins) are also involved in regulating ethylene-response genes. ERF proteins (also known as EREBPs, or Ethylene Response Element Binding Proteins) have been shown to bind to GCC boxes such as those found in the second type of *cis*-acting ethylene response elements (Ohme-Takagi and Shinshi, 1995; Solano et al., 1998), and have been implicated as both repressors and activators of various ethylene-response genes (including, but not limited to those involved stress and pathogen responses) (Solano et al., 1998; Fujimoto et al., 2000). Some ERF proteins are themselves regulated through GCC boxes contained in their promoters (Solano et al., 1998), indicating that a potentially large and complex transcriptional cascade may be important for finely tuning ethylene responses in *Arabidopsis*.

Ethylene-signaling and other plants

The analysis of ethylene signaling in crop plants is of obvious importance. While a large degree of the research carried out in *Arabidopsis thaliana* is transferable to other plants, work carried out at a more species-specific level has provided a more detailed insight into the fine level of regulation exhibited on the ethylene-signaling pathway. The impact of ethylene on crop plants has huge economic consequences, and understanding it more fully will mean that we can exert a level of control over the pathway.

Although the ethylene-signaling pathway has mostly been studied using *Arabidopsis* as a model organism, homologs of the ethylene receptors have been identified in many plant species, including rice (Goff et al., 2002; Yu et al., 2002),

tobacco (Zhang et al., 2001; Xie et al., 2002) and tomato (Tieman and Klee, 1999;

Tieman et al., 2000). Interestingly, cyanobacteria *Synechocystis* and *Anabaena* also

encode proteins with structural similarity to the ethylene-binding, GAF and HK

domains, suggesting that eukaryotic ethylene receptors may have evolved from

plastid origins (Bleecker, 1999; Rodriguez et al., 1999; Mount and Chang, 2002).

Although these proteins are known to bind to ethylene (Rodriguez et al., 1999), it is

not known whether they act to regulate a downstream signaling pathway.

Tomato is perhaps the most studied organism after *Arabidopsis*, and contains

similar pathway components. Tomato contains 6 ethylene receptors: LeETR1,

LeETR2, LeETR3, LeETR4, LeETR5 and LeETR6. Receptors 1-3 constitute

subfamily I, and 4-6 constitute subfamily II (Klee and Tieman, 2002). Interestingly,

only LeETR3 lacks a receiver domain (Lanahan et al., 1994; Lashbrook et al., 1998).

An *etr1-1* equivalent mutation introduced into *LeETR4* and *LeETR5* conferred strong

insensitivity in *Arabidopsis* (Tieman and Klee, 1999), indicating that the receptors are

functionally similar. Studies indicate that ethylene receptors in tomato are regulated

in a developmental and tissue-specific manner (Zhou et al., 1996; Lashbrook et al.,

1998; Tieman and Klee, 1999), implying that a complex mechanism exists to regulate

the fine-tuning of ethylene responses in response to environmental and developmental

pressures. Homologs of *Arabidopsis CTR1* can also be found in tomato. Where

Arabidopsis only contains one *CTR1* gene, tomato has three, all of which can rescue

an *Arabidopsis ctr1* mutant, and which also appear to be regulated at a tissue-specific

manner (Leclercq et al., 2002; Adams-Phillips et al., 2004). Transcription factors

involved in regulating ethylene-response genes have also been identified in tomato:

three homologs of *EIN3* can functionally complement an *ein3* mutation in *Arabidopsis*. Interestingly, there are no homologs to *EIL2, EIL3, EIL4* and *EIL5* in tomato (Tieman et al., 2001), perhaps indicating that these four transcription factors are actually of limited importance in the *Arabidopsis* ethylene-signaling pathway.

The existence of these homologs indicates that the mechanism of ethylene signaling does not vary much between different plant species.

Ethylene and other signaling pathways

Cross-talk between ethylene and other signaling pathways is very complex and not well understood. Ethylene might act to regulate the levels of another signal; to modulate the activity of other signaling components; or to regulate the response transmitted to other signals (Stepanova and Alonso, 2005). Ethylene can also act equally and alongside other signals to elicit responses. For example, ethylene, salicylic acid (SA) and jasmonic acid (JA) can act coordinately to regulate a finely tuned defensive response to pathogens. In some cases, the signals act independently, and at other times together to tailor specific response (Clarke et al., 2000).

Auxin and ethylene have a relatively well-documented interactive relationship. An increase in auxin levels induces ethylene biosynthesis through up-regulation of the ACC synthase gene *ACS4* (Abel et al., 1995; Woeste et al., 1999), indicating that processes or stages that are regulated by auxin also require ethylene-signaling. Both ethylene and auxin play a part in processes such as root proliferation, leaf morphology and cell elongation (Swarup et al., 2002), although the analysis of ethylene and auxin mutants has helped distinguish the key players in more specific

physiological processes (Romano et al., 1993). One example of a developmental process requiring both ethylene and auxin is in the elongation of root hairs (Pitts et al., 1998; Rahman et al., 2002). Auxin plays an important role in the initiation of root hair formation, whereas both have independent rolls in regulating the physiological process of elongation (Rahman et al., 2002).

Abscisic acid (ABA) and ethylene signaling also exhibit a degree of cross-talk. The ABA signal-transduction pathway can act both upstream and downstream of ethylene-signaling, dependent on the physiological process. Ethylene-induced promotion of seed germination is the result of up-regulated levels of ABA and/or a decreased sensitivity of the seed to ABA, and in this case ABA signaling is downstream of ethylene responses (Ghassemian et al., 2000). In contrast, inhibition of root growth by ABA is the consequence of an activated ethylene-signaling pathway, and ABA signaling is believed to be upstream in this case.

In plants, glucose can act as a signaling molecule as well as an energy source; and has also been shown to affect ethylene signaling (Leon and Sheen, 2003). Mutants that are insensitive to ethylene exhibit hypersensitivity to glucose, but when treated with ethylene this response is alleviated (Leon and Sheen, 2003). Glucose also interacts with the ABA pathway (Gazzarrini and McCourt, 2001; Cheng et al., 2002) and so it is conceivable that these three pathways closely interact to mediate responses. Interestingly, it was recently found that EIN3 protein levels are regulated by glucose. Where an increase in the concentration of ethylene acts to stabilize EIN3 protein, increased glucose levels down-regulates EIN3 levels (Yanagisawa et al., 2003). The physiological consequence of this is not yet fully understood.

These are just a few examples of the very complex level of integrated

regulation exhibited by signaling pathways in plants. New interactions are continually

being discovered, which will help us understand more fully the extensive level of fine

tuning that exists to regulate every developmental, physiological, environmental and

stress response exhibited throughout the lifetime of a plant.

Conclusion

The ethylene-signaling pathway is complex, with several novel components

and mechanisms. Ethylene is important for many physiological responses throughout

the lifetime of the plant, and is able to act coordinately with other signaling pathways

through cross-talk and interactive mechanisms that are not well understood. While the

key players that comprise the linear components of the signaling pathway have been

identified, there are still many unknowns. The identification of new components of

the will help us to better understand the molecular mechanisms of signaling within

the ethylene-signaling pathway and to other signaling pathways within the plant. This

has important implications not only in *Arabidopsis*, but also crop plants, which are of

huge agricultural and economic importance worldwide.

Chapter 2: *REVERSION-TO-ETHYLENE-SENSITIVITY1*, a conserved gene that regulates ethylene receptor function in *Arabidopsis*

Introduction

The known components involved in ethylene signaling form a mostly linear signaling pathway model, beginning with ethylene binding at the receptors and leading to the activation of ethylene-response genes. The dissection of this pathway has been possible through the isolation of genetic mutants displaying altered responses to ethylene, i.e. screening for mutants that exhibit either ethylene insensitivity or a constitutive ethylene response. It is widely believed that such primary screens have been saturated, but secondary screens are still uncovering important new components in the pathway. *REVERSION-TO-ETHYLENE-SENSITIVITY1* (*RTE1*) was isolated in a suppressor screen utilizing the dominant ethylene insensitive mutant, *etr1-2*, which does not display a characteristic triple response phenotype when grown in the presence of ethylene. Mutagenized seeds were germinated on media containing ACC and screened for seedlings exhibiting the triple response. Two mutants were found to be extragenic suppressors, and complementation analysis showed them to be allelic. These mutants were named *rte1-1* and *rte1-2* (for *REVERSION-TO-ETHYLENE-SENSITIVITY1*). This initial work was carried out by Dr. Chi-Kuang Wen, a former Post-doc in Dr. Caren Chang's lab, who identified the gene in March of 2002 as the locus At2g26070 in *Arabidopsis*

using map-based cloning. The suppression of *etr1-2* insensitivity through mutations in the *RTE1* gene suggested a possible connection between *RTE1* and *ETR1*, and an implication that *RTE1* may act in the ethylene-signaling pathway. Genetic analysis of the *rte1* mutants and their effects on ETR1 and other ethylene receptors is important for placement of *RTE1* in the pathway and understanding its function. In order to address these important points, I sought to answer several key questions. Firstly, what do we know about the *rte1* mutant alleles? Two were obtained in the initial screens and a third was later obtained after the gene was cloned. Are these loss-of-function alleles, and what do we know about these mutations at a genetic level? Secondly, does the single *rte1* mutant exhibit an ethylene-response phenotype? The suppressor screen carried out initially suggested that this mutant should look like the wild type, but it needed to be closely analyzed to assess subtle differences, which could suggest otherwise. Thirdly, do mutations in the *RTE1* gene suppress other dominant ethylene insensitive mutations? Since *rte1-1* and *rte1-2* were isolated as suppressors of *etr1-2*, it was of interest to assess whether *rte1* mutants could suppress insensitive mutants of other ethylene receptor genes. This could indicate whether *RTE1* is specific to *ETR1*, or whether it has importance for several, or all of the ethylene receptors. Finally, is *RTE1* regulated in response to ethylene, and does overexpression of the *RTE1* gene result in an ethylene-response phenotype? If *rte1* single mutants have an ethylene-response phenotype, we might expect overexpression of *RTE1* to have an opposite effect. The answers to these questions would help us to understand the role of *RTE1* in the ethylene-signaling pathway.

Results

Analysis of the *RTE1* gene and the *rte1* mutant alleles

The *rte1* alleles isolated in the initial screens were both found to carry a single nucleotide mutation in the At2g26070 gene: *rte1-1* contains a G-to-A missense mutation that results in a Cys-Tyr substitution at the conserved Cys^{161} residue; *rte1-2* has a deletion of a single cytosine at the 3' end of the nucleotide sequence, causing a frameshift mutation that replaces the last 27 residues with 15 incorrect residues. A third allele (*rte1-3*) was obtained through TILLING (Till et al., 2003), which introduces a stop codon at residue 57, and is therefore likely a null allele (Fig. 2-1A). An RT-PCR analysis of *rte1-3* shows that *RTE1* transcript levels are decreased, probably due to nonsense-mediated decay (Fig. 2-1B) (Maquat, 2004), supporting *rte1-3* being a true loss-of-function allele.

In order to confirm that At2g26070 was the *RTE1* gene, we complemented the suppressed phenotype of the *etr1-2 rte1-2* double mutant with a genomic DNA fragment containing the At2g26070 gene. This fragment was 4.33kb in length, and encompassed a region 2.66kb upstream and 0.8kb downstream of the predicted start and stop codons. Transformed seedlings expressing this transgene exhibited ethylene insensitivity similar to that seen in *etr1-2* seedlings. (Fig. 2-1C). *rte1-2* is a recessive mutation, which was determined by crossing *etr1-2* to the *rte1-2 etr1-2* double. The resulting *rte1-2/+ etr1-2* mutant exhibited ethylene insensitivity, indicating that in a heterozygous state, *rte1-2* is unable to suppress *etr1-2*.

Crossing the *rte1-2 etr1-2* double mutant to wild type enabled us to eliminate the *etr1-2* mutation, and obtain an *rte1-2* single mutant for closer analysis. Once the

Figure 2-1. *RTE1* encodes a novel conserved gene.

(A) Alignment of the predicted protein sequences of *Arabidopsis* RTE1 "AtRTE1"

(NP_180177), *Arabidopsis* RTH (*RTE1-HOMOLOG*) "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]. (B) RT-PCR showing

the decrease in *RTE1* RNA levels in *rte1-3* compared to Col-0 wild type and the *rte1-2* mutant. RNA levels of β-tubulin are shown as a control. (C) Rescue of the *etr1-2*

rte1-2 mutant phenotype, using a wild-type 4.33-kb genomic DNA fragment

"gRTE1", which incorporates the entire *RTE1* coding region.

A

```
                                                                    (rte1-3)
                                                                        *
AtRTE1   -MSRGRGVPMMDLKRSYDVEDRVVSVSIPSIIEADEADLWPLPEIDTKKSKFPCCIVWTPLPVV   63
AtRTH    ----------MGETATDSEHRMMIGLSD--------------PMKIDPKRDRFPCCIVWTPLPFI   41
Rice1A   -MAPNK-ISSMDAGAAFD--DEDASSSN------SLQELWPVGEIDPKRARFPCCIVWTPLPIV   54
Rice1B   -MEVEAACG--DGVVSSSN------EMQELWPLGEVDQKGTRFPCCIVWTPLPVV          46
Rice2    -------------------METDRSQ---------PAPIDPRRARFPCCIVWTPLPLI         30
Tcruzi   ---------MTGRKGMGENRSVALPLPP---------------RIDPSEEHYPFCIVWTPIPVL   40
Human    MAEAEGSSLLLLPPPPPPPRMAEVEAPTAAETDMKQYQGSGGVAMDVERSRFPYCVVWTPIPVL   64
            .              . .:. .  . .         :*     ::* *:****:*.:

AtRTE1   SWLAPFIGHIGLCREDGVILDFAGSNFINVDDFAFGPPARYLQLDRTKC-CLPPNMGGHTCKYG   126
AtRTH    SWLVPFIGHVGICREDGVILDFAGPNFVCVDNFAFGAVSRYIQINKEMESSRSSSSGMFNGERR   105
Rice1A   SWLAPYIGHAGICREDGTVLDFAGSNLVSMDNFAYGSIARYLQLDRKKC-CFPVNLATHVCERS   117
Rice1B   SWLAPYIGHVGIAREDGTVMDFAGSNFVSVDDLAYGSAARYLQLDRRKC-CFPANLAAHVCARS   109
Rice2    SWLIPFIGHIGICREDGVILDFAGPNFVSVDNFAFGAVARYIQVNSDEC---------------   79
Tcruzi   SWIFPFIGHVGICDSAGRIHDFEGPYHIGVDKMLFGNPVKYWNISRMY----VPTFYRSQGENP   100
Human    TWFFPIIGHMGICTSTGVIRDFAGPYFVSEDNMAFGKPAKYWKLDPAQ---------------   112
          :*: * *** *:. . * :  ** *.  :  *.: :*   :* ::.     .   .

                                         (rte1-1)
                                            Y
AtRTE1   FKHTDFG--TARTWDNALSSSTRSF-EHKTYNIFTCNCHSFVANCLNRLCYGGSMEWNMVNVAI   187
AtRTH    YEQEEDSHEKEPTWDDALRKSTQEY-QHHSYNILTCNCHSFVANNLNRLSIK-SGGWNVVNLAT   167
Rice1A   YKHAEAG--TAISWDDALQLGMRSF-GHKFYNLFTCNCLNRLAYNGSVKWNVLNVAA         178
Rice1B   YEHSEAG--TAISWDDALQSGARRF-EHKCYNLFTCNSHSFVASCLNRLAYGGSVGWNVLNLAA   170
Rice2    YKLLEP--EGASTWDDALRKGVQEF-QHRGYSLFTCNCHSFVVNNLNRLFYSGHDKWNVVSLAA   140
Tcruzi   RTTEETCRREVEEYDAAVERVTKHFRKTQLYNFFTNNCHSYVACVLRDHPLTAAGSFSVFRVAW   164
Human    -VYASG----PNAWDTAVHDASEEY-KHRMHNLCCDNCHSHVALALNLMRYNNSTNWNMVTLCF   170
            .          :* *:   .: .   .: :  :.: . *.:*.*. *.   :.:. :.

                                      (rte1-2)
                                          ↓
AtRTE1   LLMIKGKWINGSSVVRSFLPCAVVTSLGVVLVGWPFLIGLSSFSLLLFAWFIIATYCFKNIIT-   250
AtRTH    LVLFKGRWVNKTAIVKSLLPPLIVYTIGILLGGWTFIASCSILVVLLTGWFIIGTYCFKKLIQL   231
Rice1A   LVWLRGQWVDKMSVVRSFFPFLTVTCVGILMAGWPFLIGMAAFSSLLIGWFVFAVYCMKDLVC-   241
Rice1B   LVWLRGRWLGKMAVVRSLLPPAAVACVGVLMAGWSFLISMAAFSSLLLGWFVLGVYCFKGLVC-   233
Rice2    VMFLRGRWVSTASVVKTFFFPPALVITIGTLLGGATFLIGLLAFAAVMTGWFLVGTYCIKSLVEL   204
Tcruzi   GLLIHGRYVSAGRFFRAHLPFFLLMVAVVLLCVMLT--------------------------   200
Human    FCLLYGKYVSVGAFVKTWLPFILLLGIILTVSLVFNLR--------------------------   208
          : *:::.   ..:: :*    :        :       .         .      . . .
```

B

C
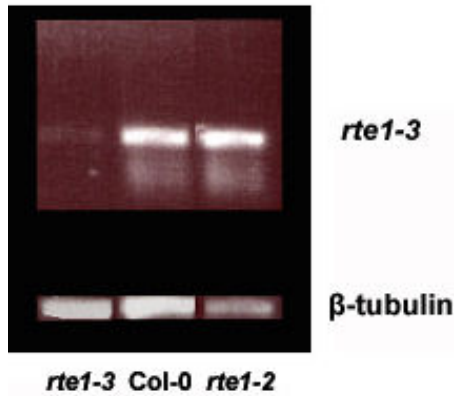
+100µM ACC

rte1-3 allele was obtained from the TILLING facility, we were also able to work with this (probable null) mutant. All of the *rte1* mutants exhibit similar phenotypes, and are therefore predicted to be loss-of-function mutants.

etr1-2 ethylene insensitivity is dependent on *RTE1*

All mutant *rte1* alleles revert the insensitivity exhibited by *etr1-2* to a phenotype comparable to that of the wild-type. This includes the *rte1-1 etr1-2* and *rte1-2 etr1-2* mutants isolated in the mutant screen, along with the *rte1-3 etr1-2* double mutant, which was constructed once the *rte1-3* allele was obtained. This suppression is apparent in seedling triple responses (Fig. 2-2A) and in an ethylene dose-response analysis (Fig. 2-2B). However, it is noteworthy that the hypocotyl and root appear to be slightly shorter than the wild type under all concentrations of ethylene, reminiscent of an *etr1* null mutant (Hua and Meyerowitz, 1998). In adults, *etr1-2* insensitive mutants are resistant to the ethylene-induced senescence that is seen in wild-type plants exposed to ethylene for several days. The suppressed mutant lines, however, do exhibit signs of senescence upon prolonged exposure to ethylene (Fig. 2-2C), indicating that *RTE1* is required for the ethylene insensitivity exhibited by *etr1-2* mutants in both seedling and adult stages.

The *rte1* single mutant is similar to the *etr1-7* null mutant

As mentioned above, the suppressed mutant lines all display a shorter hypocotyl and root when compared to the wild type (Fig. 2-2B). When these seedlings were germinated in the light, they also exhibited roots that were slightly

Figure 2-2. Mutant alleles of *rte1* suppress *etr1-2* ethylene insensitivity.

(A) Analysis of dark-grown seedlings grown in the presence and absence of the ethylene precursor ACC (100μM). Representative seedlings of three suppressor lines (*etr1-2 rte1-1, etr1-2 rte1-2, etr1-2 rte1-3*) and two single mutants (*rte1-2* and *rte1-3*) are compared alongside *etr1-7*. (B) Ethylene dose-response analysis of hypocotyl lengths in 4-day old *Arabidopsis* seedlings. The mean +/- SE is shown for 10 seedlings measured for each dose. Two mutant lines (*etr1-2 rte1-1* and *etr1-2 rte1-2*) suppress the insensitivity exhibited by *etr1-2*. (C) Ethylene-induced leaf senescence in 5-week old plants treated with or without 100ppm ethylene for four days. The degree of senescence is shown in wild type, *etr1-2* and three suppressor lines (*etr1-2 rte1-2, etr1-2 rte1-1, etr1-2 rte1-3*).

A

Dark + ACC

Dark − ACC

WT *etr1-2* *etr1-2* *etr1-2* *etr1-2* *rte1-2* *rte1-3* *etr1-7*
　　　　　rte1-1 *rte1-2* *rte1-3*

B

Hypocotyl Length (mm)

14
12
10
8
6
4
2
0

- WT
○ *etr1-2*
▲ *etr1-2 rte1-1*
△ *etr1-2 rte1-2*

0　0.01　0.1　1　10

[Ethylene] (ppm)

C

Air　Ethylene

WT

etr1-2

etr1-2 rte1-1

etr1-2 rte1-2

etr1-2 rte1-3
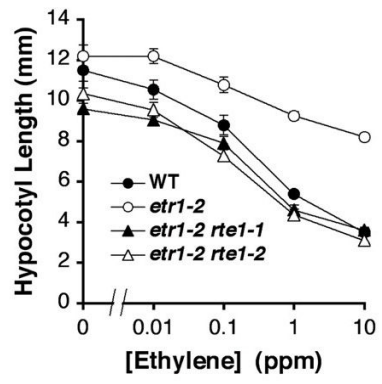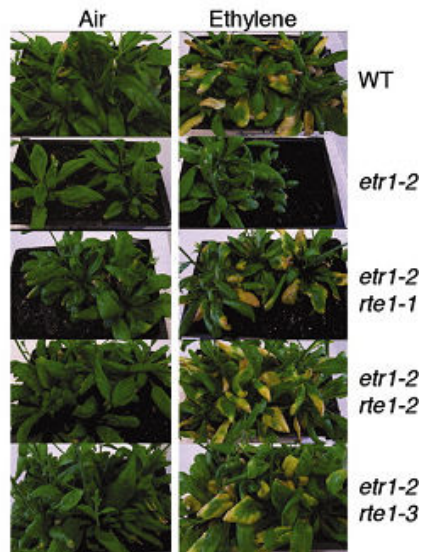
shorter than either the wild type or *etr1-2*, along with smaller cotyledons (Fig. 2-3A).

In an ethylene dose-response analysis, both *rte1-2* and *rte1-3* single mutants strongly

resemble the *etr1-7* null mutant, all exhibiting an enhanced response to ethylene and

therefore are shorter than the wild-type at all concentrations of ethylene (Fig. 2-3B).

In addition, a low concentration of the ethylene precursor ACC is able to elicit the

triple response in *rte1-2* and *etr1-7*, but not in the wild type (Fig. 2-3C). Like *etr1-7,

rte1-2* and *rte1-3* also have shorter hypocotyls than wild type in the absence of

ethylene. In *etr1-7* mutants, this is known to be largely due to an enhanced response

to endogenous ethylene (Cancel and Larsen, 2002), since when it is grown in the

presence of the ethylene biosynthesis inhibitor AVG, shortening is partially alleviated

(Cancel and Larsen, 2002). When *rte1-2* and *etr1-7* were grown side-by-side on

AVG, hypocotyl shortening was alleviated to similar degrees (Fig. 2-3D), indicating

that *rte1* mutants may be sensitive to endogenously produced ethylene. There were

no other obvious phenotypes detected in *rte1* mutant plants. We were also able to

reverse hypocotyl shortening by treatment with silver nitrate. It is believed that silver

is able to replace copper at its binding site in the ethylene receptors, causing a

conformational change that results in ethylene insensitivity (Hirayama and Alonso,

2000). When *rte1-2* and *etr1-7* seedlings were grown on silver nitrate in the presence

of a high concentration of ACC, both exhibited approximately equal degrees of

insensitivity. When just silver nitrate was present, both mutants were phenotypically

identical in their degree of hypocotyl lengthening (Fig. 2-3E).

     These results demonstrate the striking similarity between *rte1* loss-of-function

mutants and the *etr1* null mutant, *etr1-7*. As discussed earlier, it is apparent that *RTE1*

Figure 2-3. *rte1-2* has enhanced ethylene sensitivity resembling the *etr1-7* null mutant.

(A) Comparison of four-day old light grown *Arabidopsis* seedlings grown in the absence of ethylene. Representative seedlings are shown for three suppressor lines (*etr1-2 rte1-1, etr1-2 rte1-2, etr1-2 rte1-3*) and two single mutants (*rte1-2* and *rte1-3*), all showing similarity to *etr1-7*. (B) Dose response analysis of hypocotyl length in four-day old seedlings shows the similarity of *rte1-2, rte1-3* and *etr1-7*, all of which exhibit enhanced ethylene sensitivity compared to wild type. The mean +/-SE is shown for 17-30 seedlings measured for each dose. (C) Four-day old *rte1-2* and *etr1-7 Arabidopsis* seedlings exhibit the triple response when grown on 0.5μM ACC, whereas wild type does not. (D) Treatment with the ethylene biosynthesis inhibitor 1-aminoethoxyvinylglycine (AVG) (10μM) partially alleviates hypocotyl shortening in *rte1-2* and *etr1-7* ($P < 0.0001$), in the absence of ethylene treatment. The mean +/-SE is shown for 10-15 seedlings per genotype. (E) Treatment with the ethylene response inhibitor $AgNO_3$ (100μM) alleviates the enhanced ethylene sensitivity of *rte1-2* and *etr1-7*. Similarly, the wild type does not respond to ACC in the presence of $AgNO_3$. The *ctr1-1* control does not show alleviation, since it acts downstream of the receptors in the pathway (the receptors being the likely target of $AgNO_3$). For each treatment, the mean +/- SE is shown for 10-12 seedlings per genotype.

A

WT etr1-2 etr1-2 etr1-2 etr1-2 rte1-2 rte1-3 etr1-7
            rte1-1 rte1-2 rte1-3

Light

B

Hypocotyl Length (mm)

△ WT
● rte1-2
□ rte1-3
▲ etr1-7

[Ethylene] (ppm)

C

0.5 μM ACC

WT rte1-2 etr1-7

D

−AVG   +AVG

Hypocotyl Length (mm)

WT rte1-2 etr1-7

E

− − + + ACC
− + − + AgNo₃

Hypocotyl Length (mm)

WT rte1-2 etr1-7 ctr1-1
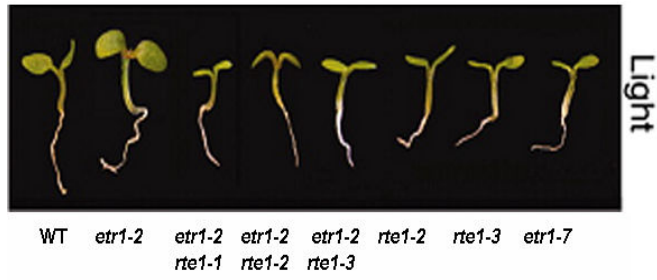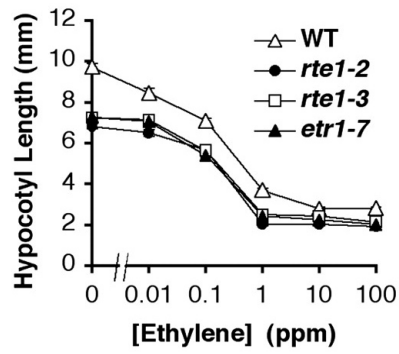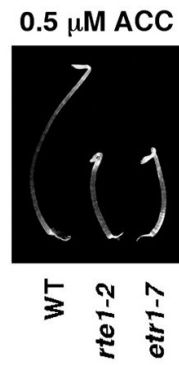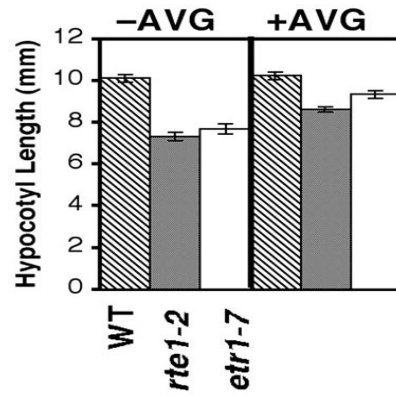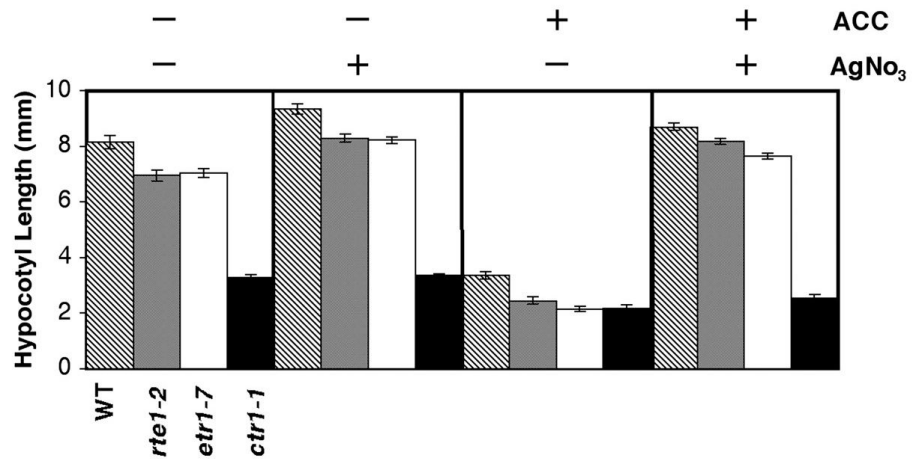
is required for the ethylene insensitivity exhibited by the *etr1-2* dominant insensitive

mutant, but it also appears that *RTE1* is required (or largely required) for wild-type

ETR1 receptors to be fully functional. To test this hypothesis further, we constructed

two double mutants: an *etr1-7 rte1-2* double mutant, to see whether *RTE1* and *ETR1*

act in the same pathway, and an *ers1-3 rte1-2* double mutant, to assess whether *RTE1*

also likely acts through this second member of the subfamily I receptors (the other

member being ETR1). The *etr1-7 rte1-2* double mutant in an ethylene dose response

analysis was phenotypically similar to both the *etr1-7* and *rte1-2* single mutants (Fig.

2-4A), consistent with *ETR1* and *RTE1* acting in the same pathway, both acting as

negative regulators of the ethylene-signaling pathway. In contrast, the *ers1-3 rte1-3*

dose response curve was significantly different from the *ers1-3* single mutant (Fig. 2-

4B), indicating that *ETR1* is likely the primary target for *RTE1*. However, the *ers1-3*

rte1-3 double mutant did not fully phenocopy the *ers1-3 etr1-7* double mutant, which

has a severe constitutive response, suggesting that loss of *RTE1* may not fully

eliminate the functionality of *ETR1*. However, results thus far are consistent with

RTE1 being largely required for *ETR1* function.

rte1 is unable to suppress other dominant ethylene receptor mutants

Since *rte1-2* was isolated as a suppressor of *etr1-2*, it was of interest to assess

whether *rte1* loss-of-function mutants would be able to suppress other dominant

insensitive alleles. Since there are five ethylene receptors, we wanted to test

insensitive mutants from each one. Firstly, we tested another *ETR1* gain-of-function

allele, *etr1-1*. Interestingly, the *etr1-1 rte1-2* double mutant retained dominant
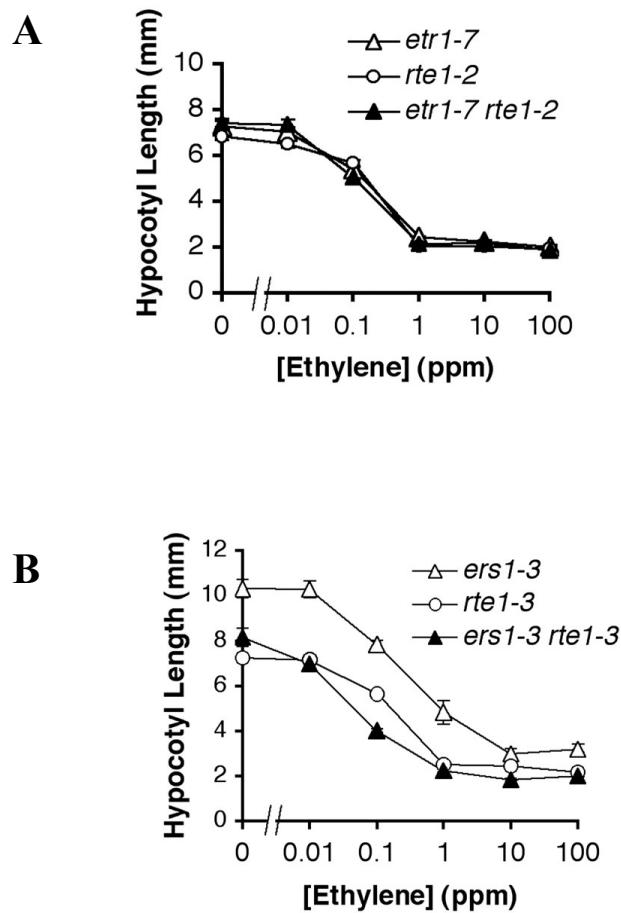
Figure 2-4. Dose-response analyses suggest that *RTE1* acts in the same pathway as *ETR1*.

(A) The *etr1-7 rte1-2* double mutant does not display an enhanced phenotype when compared to *etr1-7* and *rte1-2* single mutants, suggesting that *RTE1* acts in the same pathway as *ETR1*. (B) The *ers1-3 rte1-2* double mutant has an enhanced ethylene response that is distinct from the *ers1-3* single mutant, consistent with *RTE1* being largely independent of *ERS1*. For both graphs, the mean +/- SE is shown for 11-30 seedlings per genotype for each dose.

ethylene insensitivity, and was indistinguishable from the *etr1-1* single mutant in both hypocotyl and root length (Fig. 2-5A and Table 2-1). This indicated that *rte1-2* is unable to suppress *etr1-1*. The fact that *rte1-2* can suppress *etr1-2* but not *etr1-1* is intriguing, and indicates that the difference between these two alleles is important for the functionality of *RTE1*. This question is addressed further in Chapter 5. *rte1-2* was also unable to suppress the dominant insensitivity exhibited by the *etr2-1, ers2-1,* and *ein4-1* mutants (Table 2-1). We also tested whether the *rte1-3* null allele could suppress *ers1-10*, which is likely the weakest ethylene receptor gain-of-function mutant (Alonso et al., 2003). This is of interest not only because *ers1-10* is a weak allele, but also since *ERS1* makes up the second member of the subfamily I group of ethylene receptors, along with *ETR1*. Notably, *rte1-3* did not suppress the *ers1-10* mutant (Fig. 2-5b and Table 2-1). It therefore appears that suppression by *rte1* is specific to *ETR1*, and perhaps even towards particular receptor alleles. The fact that *rte1* can suppress *etr1-2* but not *etr1-1* raised the possibility that *rte1* may be allele-specific. A suppression analysis of additional *ETR1* insensitive alleles is described in Chapter 5.

Additionally, *rte1-2* did not enhance the constitutive phenotype observed in the *ctr1-1* constitutive-response mutant, nor was it able to suppress the insensitivity conferred by the recessive ethylene-insensitive mutant *ein2-1* (Table 2-1). This indicated that *RTE1* acts in the same pathway as *CTR1*, and that it is upstream of *EIN2*.
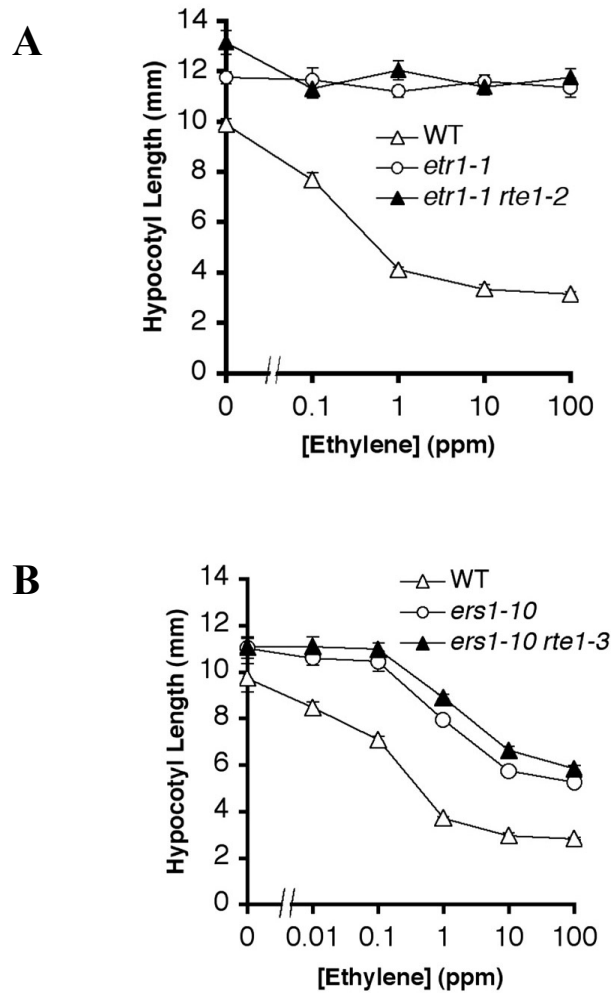
Figure 2-5. *rte1-2* **is unable to suppress other insensitive mutants.**

(A) *rte1-2* does not suppress the insensitivity of the strong ethylene gain-of-function

insensitive mutant *etr1-1*. (B) *rte1-3* does not suppress the insensitivity of *ers1-10*,

which is a weak gain-of-function insensitive mutation in the *ERS1* receptor gene. For

both graphs, the mean +/- SE is shown for 11-30 seedlings per genotype for each

dose.

Table 2-1. Hypocotyl and root lengths (mm) in etiolated seedlings germinated on media supplemented with 0 or 100μM ACC

Genotype	Hypocotyl Length (mm)		Root Length (mm)	
	–ACC	+ACC	–ACC	+ACC
WT (Col-0)	9.5 +/- 0.2	3.2 +/- 0.1	3.0 +/- 0.17	0.6 +/- 0.05
WT (Col-0)*		3.5 +/- 0.1		0.6 +/- 0.03
rte1-2	7.7 +/ -0.2	2.2 +/- 0.04	1.6 +/- 0.08	0.4 +/- 0.02
rte1-3	7.3 +/-0.2	2.7 +/- 0.1	1.7 +/- 0.09	0.3 +/- 0.01
rte1-3*		2.2 +/- 0.1		0.4 +/- 0.04
etr1-1	11.1 +/- 0.5	11.3 +/- 0.3	3.6 +/- 0.22	3.2 +/- 0.16
etr1-1 rte1-2	11.6 +/- 0.3	3.1 +/- 0.2	3.2 +/- 0.16	3.1 +/- 0.22
ein4-1	10.2 +/- 0.2	9.6 +/- 0.2	3.1 +/- 0.07	3.3 +/- 0.14
ein4-1 rte1-2	10.6 +/- 0.4	9.2 +/- 0.2	3.4 +/- 0.31	2.6 +/- 0.09
etr2-1	10.3 +/- 0.3	10.7 +/- 0.2	3.3 +/- 0.19	2.9 +/- 0.09
etr2-1 rte1-2	9.9 +/- 0.3	10.5 +/- 0.3	3.2 +/- 0.24	3.3 +/- 0.12
ers2-2	10.7 +/- 0.3	10.0 +/- 0.2	3.1 +/- 0.10	2.3 +/- 0.14
ers2-2 rte1-2	10.2 +/- 0.2	10.1 +/- 0.2	3.1 +/- 0.14	2.7 +/- 0.09
ers1-10*	11.0 +/- 0.03	4.9 +/- 0.2	2.6 +/- 0.18	1.9 +/- 0.10
ers1-10 rte1-3*	11.1 +/- 0.5	5.4 +/- 0.2	3.1 +/- 0.13	2.8 +/- 0.11
ctr1-1	3.76 +/- 0.09	2.61 +/- 0.08	0.57 +/- 0.04	0.34 +/- 0.03
ctr1-1 rte1-2	3.64 +/- 0.19	2.69 +/- 0.07	0.54 +/- 0.03	0.36 +/- 0.06
ein2-1	10.42 +/- 0.43	10.35 +/- 36	4.97 +- 0.15	4.61 +/- 0.15
ein2-1 rte1-2	10.21 +/- 0.39	10.48 +/- 0.39	4.61 +/- 0.15	4.74 +/- 0.14

* 10μM ACC was used. Mean +/-SE shown for 15 seedlings

RTE1 expression and induction

Arabidopsis gene array data indicates that *RTE1* is expressed at detectable

levels in most organs and at most stages of development, but is most highly expressed

in late stage siliques and in seeds (www.cbs.umd.edu/arabidopsis and

www.genevestigator.ethz.ch/at). *RTE1* levels also appear to be induced upon

treatment with endogenous ethylene: microarray data indicates that *RTE1* transcript

levels increase 4-fold on exposure to ethylene (Alonso et al., 2003), and we were able

to observe a similar increase in adult leaves after ethylene treatment using semi-

quantitative RT-PCR analysis (Fig. 2-6).

RTE1 overexpression causes reduced ethylene sensitivity that is largely

dependent on ETR1

Using the *35S* promoter from the cauliflower mosaic virus we were able to

overexpress *RTE1* in a variety of mutant genotypes. To confirm that the *RTE1*

overexpression construct was functional, we overexpressed *RTE1* in *rte1-2* and *rte1-3*

backgrounds. On MS alone, transgenic overexpression lines were a similar length to

wild type, indicating that the *35S:RTE1* construct does indeed complement the null

rte1 phenotype. On 10 and 50μM ACC, all lines tested exhibited weak insensitivity,

being slighter longer than wild-type seedlings (Table 2-2).

Overexpression of *RTE1* in the wild type also conferred weak ethylene

insensitivity, which is consistent with *RTE1* being a negative regulator of ethylene

responses (Fig. 2-7A,C). When *RTE1* was overexpressed in the *etr1-7* null mutant,

insensitivity was much less pronounced (Fig. 2-7B,C). In contrast, overexpression in
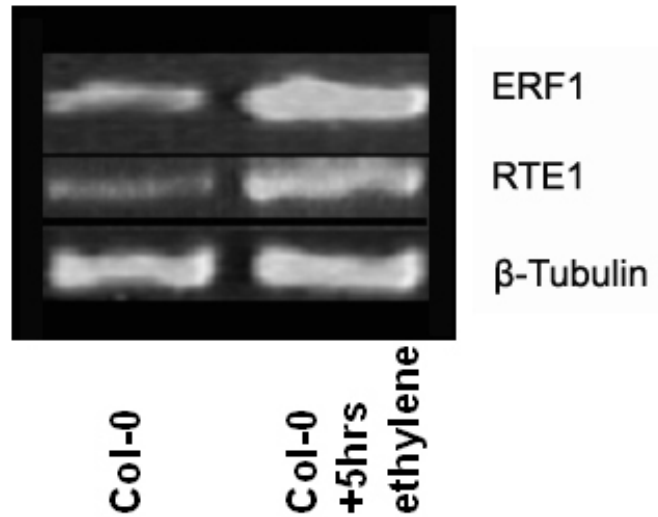
Figure 2-6. *RTE1* is up-regulated by ethylene.

RT-PCR of Col-0 wild-type plants exposed to ethylene for five hours shows increased

levels of *RTE1* transcript compared to plants grown in air. *ERF1* (*ETHYLENE-*

RESPONSE-FACTOR1) is a positive control known to be up-regulated by ethylene,

and also shows elevated transcript. The β-tubulin control shows approximately equal

amounts of RNA.

Table 2-2. *RTE1* Overexpression causes insensitivity in *rte1* loss-of-function mutants.

Genotype	Hypocotyl Length (mm) on MS	Hypocotyl Length (mm) on 10µM ACC
Col-0	11.5 +/- 0.23	4.87 +/- 0.17
rte1-3	7.95 +/- 0.18	3.14 +/- 0.06
rte1-2	7.67 +/- 0.29	3.12 +/- 0.09
35S:RTE1 in *rte1-3*	11.26 +/- 0.33	7.17 +/- 0.22
35S:RTE1 in *rte1-2*	10.38 +/- 0.24	6.39 +/- 0.27

Figure 2-7. Overexpression of *RTE1* confers weak insensitivity to ethylene.

(A) Measurements of hypocotyl length for wild-type seedlings either untransformed (-) or transformed (+) with *35S:RTE1,* with and without ethylene treatment. Two independent transgenic lines are shown. For each treatment, the mean +/- SE is shown for 14-20 seedlings per line. (B) Measurements of hypocotyl length for *etr1-7* seedlings either untransformed (-) or transformed (+) with *35S:RTE1,* with and without ethylene treatment. For each treatment, the mean +/- SE is shown for 14-19 seedlings per line. Differences for each transformed line compared to untransformed *etr1-7* are significant as determined by a T-test ($P < 0.0001$). (C) Representative four-day old dark-grown seedlings of wild-type, *etr1-7* and *ers1-2* either untransformed (-) or stably transformed (+) with *35S:RTE1*, germinated in the presence or absence of ACC.
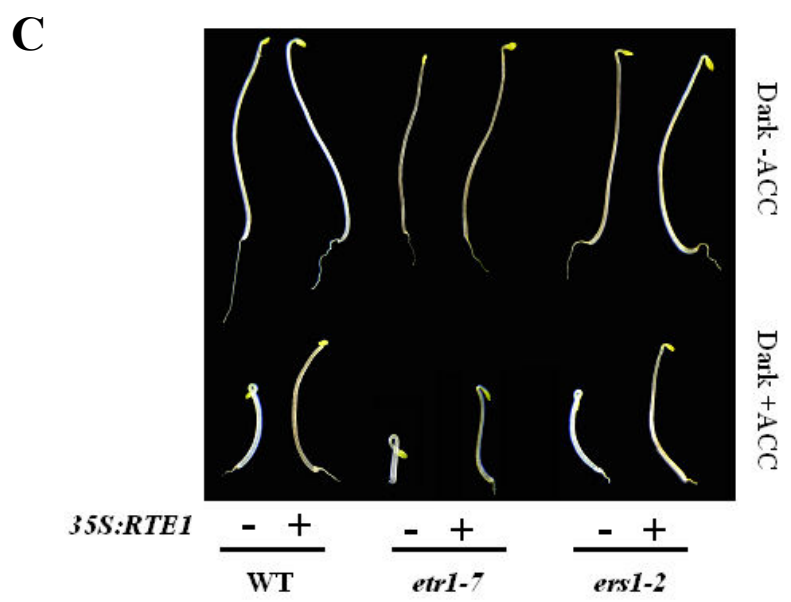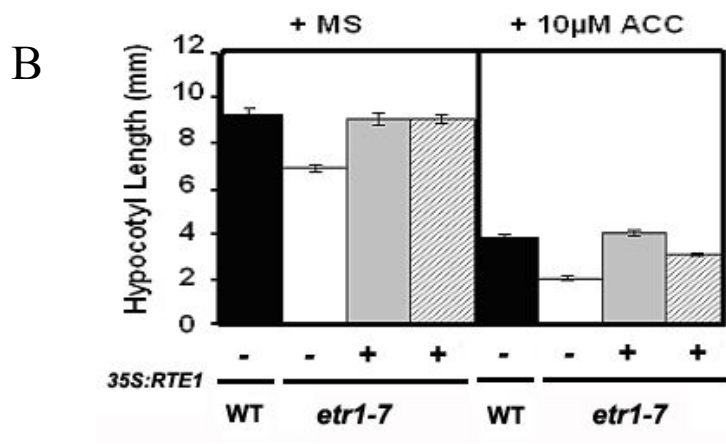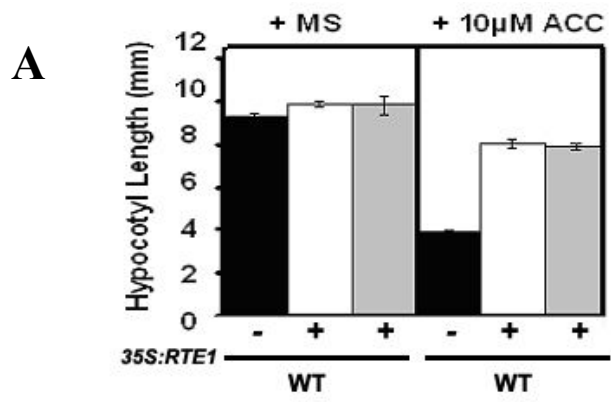
the *ers1-2* null mutant resulted in a level of insensitivity comparable to that seen in wild-type seedlings (Fig. 2-7C). This suggests that the ETR1 receptor is largely required for the insensitive phenotype to be observed, and further supports the hypothesis that *RTE1* is a regulator of *ETR1*.

Since the ethylene receptors exhibit redundancy and are negative regulators of the pathway, loss of multiple receptors results in a constitutive-response phenotype. It was of interest to see whether seedlings would still exhibit ethylene insensitivity when two or more receptors are absent, and whether the presence of *ETR1* is important in these cases. We overexpressed *RTE1* in a variety of multiple null mutant genotypes and compared responses when *ETR1* was either present or absent. The five multiple null mutants tested were as follows: *etr1 etr2*, *etr1 ein4*, *etr1 etr2 ein4*, *etr2 ein4*, *etr2 ers2 ein4* (Figs. 2-8A-E). Ethylene insensitivity was observed in all cases, but was more pronounced in seedlings that retained ETR1 receptor activity.

Double mutant analysis suggests that *RTE1* acts at or upstream of *ETR1*, and to support this hypothesis we overexpressed *RTE1* in various mutants of downstream pathway components. The constitutive response mutant *ctr1-1* displays a constitutive triple response even in the absence of ethylene. However, *ctr1-1* still exhibits weak responsiveness to ethylene, presumably due to the presence of an 'alternative' ethylene pathway which has not yet been described (Larsen and Chang, 2001). Overexpression of *RTE1* in a *ctr1-1* background resulted in a reduced sensitivity to ethylene, such that we do not see increased shortening of the hypocotyl to the same degree as in *ctr1-1* alone (Fig. 2-9A). We also overexpressed *RTE1* in *ein2-1* and

Figure 2-8. Loss of *ETR1* decreases the insensitivity conferred by *RTE1*

overexpression.

For each graph, measurements of hypocotyl length for control and two independent

transgenic lines transformed with *35S:RTE1* in the indicated background are shown,

with and without ethylene treatment. For each treatment, the mean +/- SE is shown

for 13-21 seedlings. (A), (D) Ethylene insensitivity conferred by *35S:RTE1* is

pronounced in an *etr2 ers2 ein4* triple null mutant and an *etr2 ein4* double mutant,

compared to the control null mutants. (B), (C), (E) Weaker ethylene insensitivity is

conferred by *35S:RTE1* in *etr1 ein2, etr1 ein4* and *etr1 etr2 ein4* multiple receptor

null mutants.
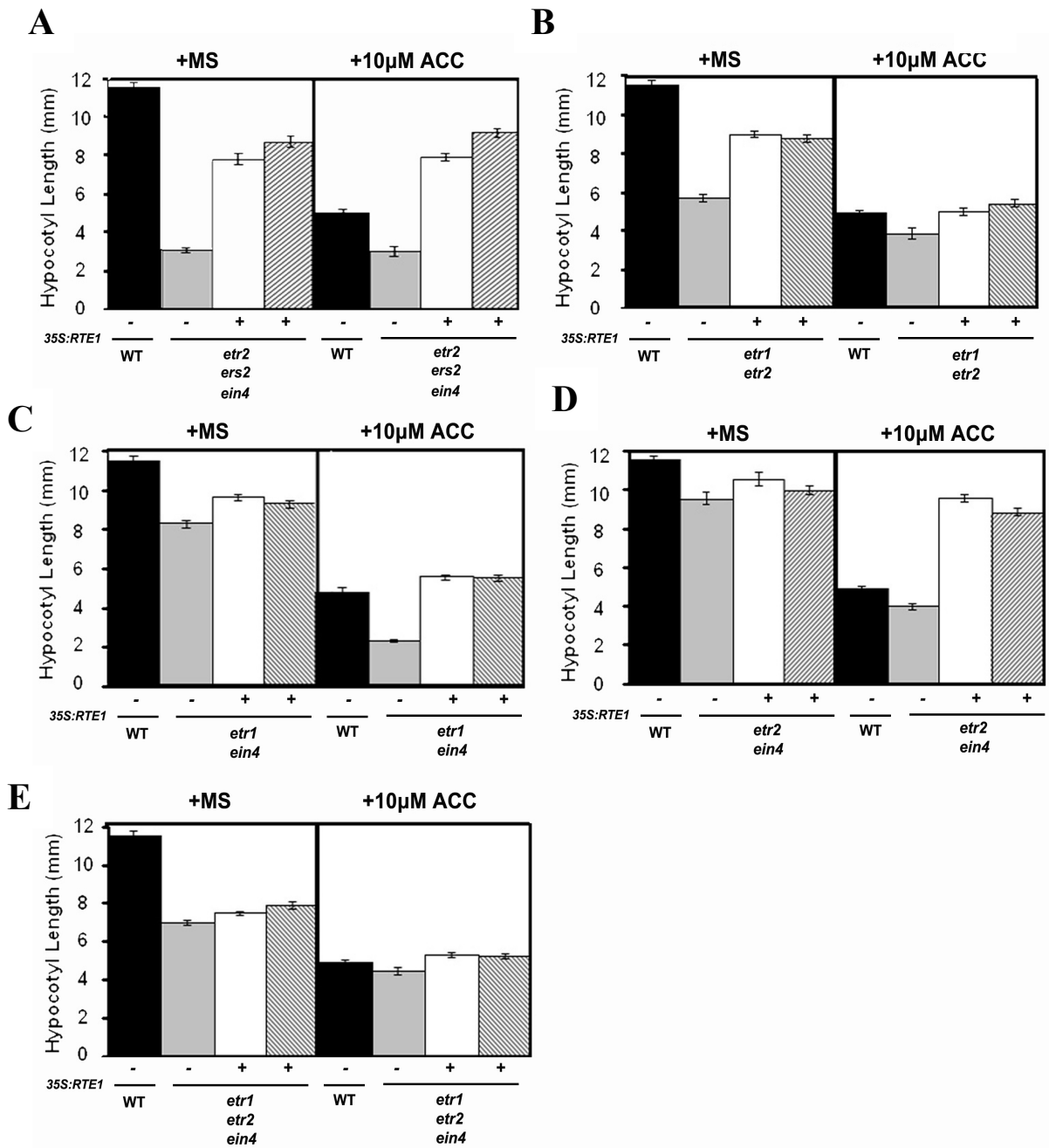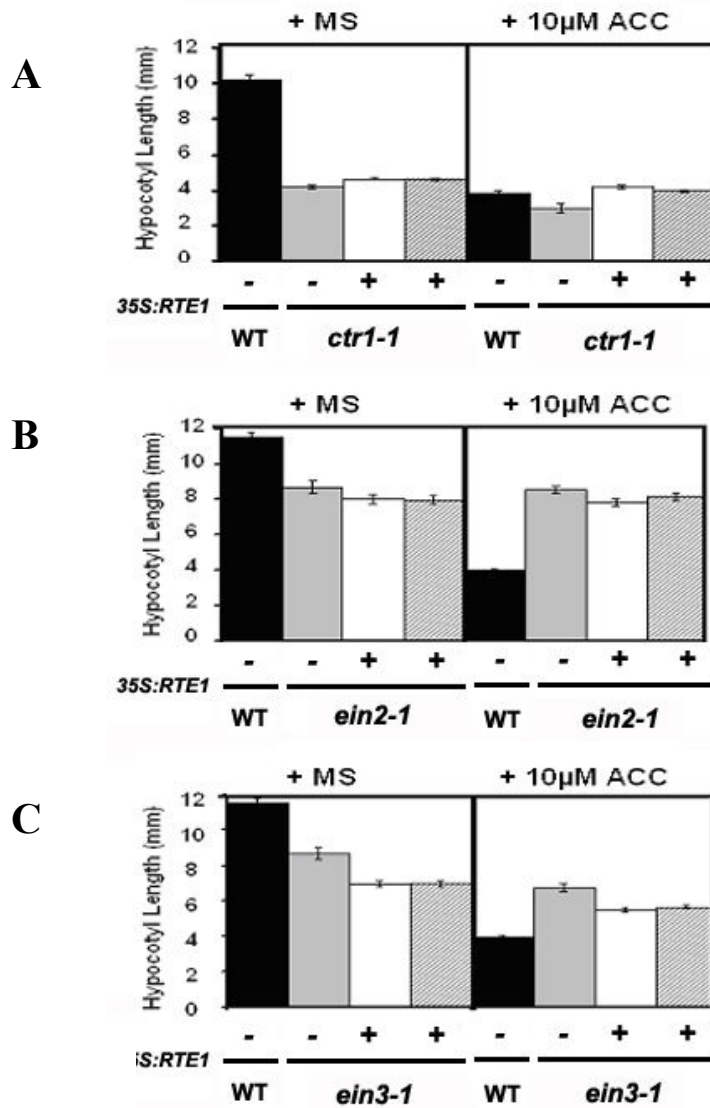
Figure 2-9. *RTE1* acts upstream of *EIN2* and *EIN3* in the ethylene-signaling pathway.

For each graph, measurements of hypocotyl length for control and two independent transgenic lines transformed with *35S:RTE1* in the indicated background are shown, with and without ethylene treatment. For each treatment, the mean +/- SE is shown for 10-20 seedlings. (A) *35S:RTE1* does not confer ethylene insensitivity in a *ctr1-1* background. (B), (C) *35S:RTE1* does not enhance the insensitivity exhibited by both *ein2-1* and *ein3-1* single mutants.

ein3-1 backgrounds, both of which are ethylene-insensitive mutants in downstream components (Chao et al., 1997; Alonso et al., 1999). In both cases, insensitivity was retained but not enhanced. (Figs. 2-9B-C). Interestingly, when *RTE1* was overexpressed in *etr1-2,* the insensitivity exhibited under normal circumstances was enhanced (Fig. 2-10A). This was also observed in the weak insensitive mutant *ers1-10,* but not in the strong insensitive mutant *etr1-1* (Fig. 2-10).

Discussion

  RTE1 is a novel and previously undescribed gene that acts to negatively regulate ethylene responses in *Arabidopsis*. Genetic analysis indicates that *RTE1* acts as a regulator of *ETR1* ethylene receptor function. *RTE1* was isolated in a screen for suppressors of the *etr1-2* ethylene insensitive mutant, and is required for the insensitive phenotype observed in this mutant. In addition, *rte1* loss-of-function mutants phenotypically resemble the *etr1* loss-of-function mutant, indicating that RTE1 may also be required for wild-type ETR1 functionality. The *rte1-2 etr1-7* double mutant phenocopies the *etr1* single mutant, indicating that *RTE1* acts in the same pathway as *ETR1*. In contrast, the *rte1-3 ers1-3* double mutant does not look like the *ers1-3* single null mutant, nor does it appear like the *etr1-7 ers1-3* double mutant, which has a severe constitutive phenotype. We can draw two conclusions from this: the first is that *RTE1* probably does not act in the same pathway as *ERS1*; and secondly, loss of *RTE1* probably does not result in a complete loss of *ETR1* function. In addition, overexpression of *RTE1* in *etr1* loss-of-function mutants results
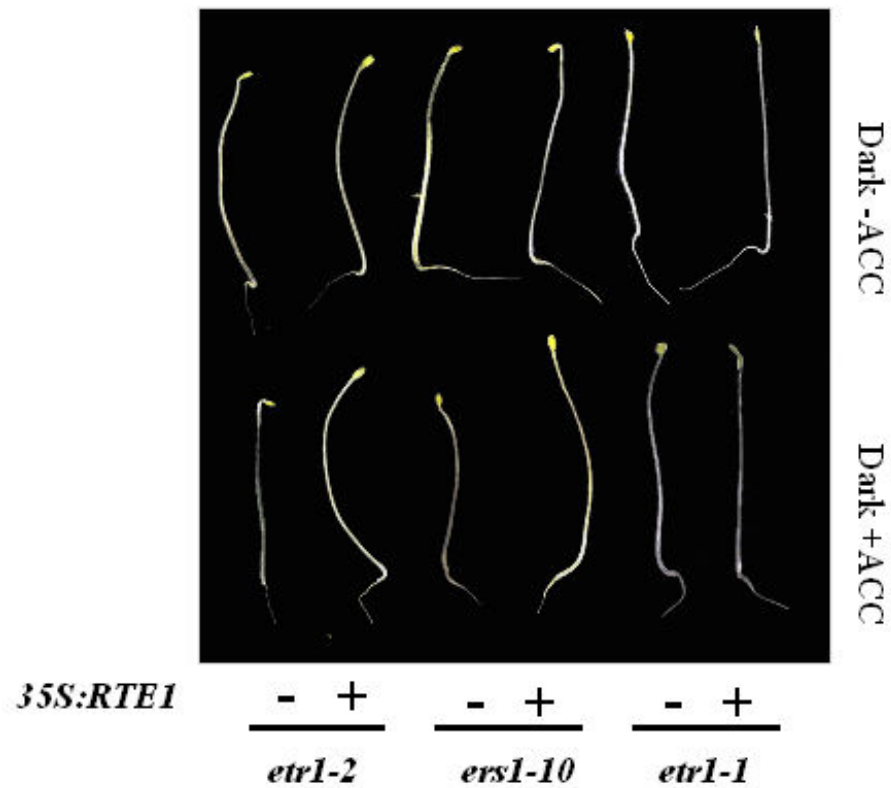
Figure 2-10. Overexpression of *RTE1* enhances the ethylene insensitivity in weak insensitive mutants.

Representative four-day old dark-grown seedlings of etr1-2, *ers1-10 and etr1-1* either untransformed (-) or stably transformed (+) with *35S:RTE1*, germinated in the presence or absence of 10μM ACC.

in partial ethylene insensitivity (although not to the same degree as overexpression in wild-type seedlings), which may imply that *RTE1* is able to act partially through the other receptors, or through other components in the ethylene-signaling pathway. Hypocotyl lengthening due to *RTE1* overexpression may also be due to non-ethylene related targets.

Slightly reduced sensitivity to ethylene was observed when RTE1 was overexpressed in *ctr1-1* mutants. Since *RTE1* is believed to act upstream of *CTR1*, and we have seen that the *rte1-2 ctr1-1* double mutant phenocopies *ctr1-1* single mutants, this may imply that the insensitivity observed when *RTE1* is overexpressed in *etr1* null mutants is due to ectopic responses. Alternatively, this could also be due to an increase in receptor activity, since ETR1 likely retains some slight functionality in *ctr1* mutants (this is supported by the finding that an *etr1-1 ctr1-1* double mutant has a longer hypocotyl than *ctr1-1* alone (Chang, unpublished)).

At this point we cannot rule out the possibility that loss of *RTE1* impacts several or all of the receptors to some degree, and the phenotype seen in *rte1* loss-of-function mutants is due to partial loss of function in each of the receptors. This would be consistent with the finding that the *rte1-3 ers1-3* double mutant is not as severe as the *etr1-7 ers1-3* double mutant. In fact, the *rte1-3 ers1-3* mutant is a viable plant that grows to adulthood and is fertile, whereas the *etr1-7 ers1-3* double mutant is lethal at an early rosette stage. If this is the case, *ETR1* may appear to be affected more by a loss of *RTE1* due to the abundance of this receptor, which under normal conditions is more prevalent than the others (O'Malley et al., 2005).

Loss of *RTE1* did not suppress dominant insensitive mutants in each of the other receptors, indicating that *RTE1* is probably specific to *ETR1*, although this could be due to the alleles tested, such that a different insensitive allele of the same receptor may have been suppressed. Alleles tested in three of the receptors – *ETR2, ERS2* and *EIN4* were all strong dominant insensitive alleles, and since *rte1-2* was also unable to suppress the *etr1-1* strong insensitive mutant we cannot rule out that *RTE1* may play a role in regulating these other receptors. However, one of the alleles tested for the *ERS1* receptor was *ers1-10,* which is a weak insensitive mutant with some similarities to the *etr1-2* allele (Alonso et al., 2003). This mutant was also not suppressed by loss of *RTE1*, supporting the hypothesis that *RTE1* specifically regulates *ETR1*, or alternatively, raising the possibility that *rte1* mutants suppress *etr1-2* in an allele-specific manner. This is a particularly valid question since *rte1-2* is able to suppress *etr1-2* but not the *etr1-1* dominant insensitive allele. *etr1-2* contains an Ala^{102} – Thr substitution in the third transmembrane domain of the ETR1 ethylene-binding region, and differs from *etr1-1* and other dominant insensitive mutants in that the mutated protein can still bind wild-type levels of ethylene (Bleecker et al., 1998; Wang et al., 2006). In contrast, *etr1-1* contains a Cys^{65}- Tyr mutation, also in the ethylene-binding region, which prevents copper binding to the receptor and therefore eliminates any ethylene binding. As a result, *etr1-1* is a stronger dominant insensitive mutant that *etr1-2*, being permanently 'locked' in a signaling conformation. These differences may indicate that *rte1* mutations can either suppress *etr1* alleles that retain the ability to bind ethylene or alleles that are relatively weak. Whether these differences are significant is addressed further in Chapter 5.

As expected, overexpression of *RTE1* resulted in weak ethylene insensitivity in *Arabidopsis* seedlings. This is consistent with the hypersensitivity observed in *RTE1* loss-of-function mutants. This insensitivity is much more pronounced in seedlings that express the wild-type *ETR1* gene, although *etr1* null transgenic lines are still slightly longer than untransformed controls when grown on ACC. The weak insensitivity seen in lines where *ETR1* is absent could be due to a slight increase in activity of other ethylene receptors, or due to ectopic overexpression of *RTE1* that may affect other pathways or processes. In either case, these results support the hypothesis that *RTE1* is at least primarily a regulator of *ETR1*, and likely has little effect on the other ethylene receptors. The fact that enhanced ethylene insensitivity was not observed in *ein2-1* and *ein3-1* mutants indicates that *RTE1* most likely does act at the receptor level. Overexpression of *RTE1* in *etr1-2* results in a stronger insensitivity to ethylene, but not in *etr1-1*, which is perhaps the strongest of the ethylene receptor mutants. This may indicate that RTE1 is still able to positively regulate ETR1-2, but not the ETR1-1 receptor, perhaps due to a structural or mechanistic difference between the two. (It is also possible that the insensitivity exhibited by *etr1-1* is at a maximum, such that any additional lengthening of the hypocotyl cannot occur). However, the differences between *etr1-1* and *etr1-2* may be important, and could potentially offer some insight into the functionality of *RTE1*. This is addressed further in Chapter 5.

Work presented in this Chapter supports the hypothesis that *RTE1* is a positive regulator of *ETR1*, although how RTE1 functions at the molecular level is unknown.

Since *RTE1* has a novel sequence, it is important to look closely at its sequence and carry out further analyses and screens to help answer these questions.

Materials and Methods

Plant Strains and Growth Conditions

Unless otherwise stated, the *Arabidopsis thaliana* Columbia ecotype was used. The *ers1-3* T-DNA mutant (provided by Dr. G. Eric Schaller (Dartmouth College, Hanover, NH) is the ecotype Wassilewskija. For all seedling analyses, seeds were sown on Murashige and Skoog (MS) medium containing 0.9% agar. Following a three-day stratification at 4^oC, seeds were incubated at 20^oC for 4 days under either complete dark (for etiolated response analysis) or 24-hour light (for light grown seedling analysis). Plants were grown in soil at 16-hour light and 8-hour dark in controlled environment chambers under fluorescent light.

For the triple response assay, seedlings were germinated in the presence of ethylene gas as described, or on MS medium containing ACC (Sigma Aldrich) at the stated concentrations. To inhibit ethylene responses, 100μM Silver Nitrate ($AgNO_3$) was added to the medium. To inhibit ethylene biosynthesis, 10μM AVG was added to the medium. To measure the seedlings, photographs were taken and measurements made using IMAGEJ software (http://rsb.info.nih.gov/jj/).

Ethylene treatment of adult plants was carried out in airtight clear acrylic chamber. Ethylene gas (specialty gases of America, Toledo, OH) was injected into the chamber to a final concentration of 100ppm. A control chamber was injected with air. Plants were placed at 20^oC under 24-hour light.

Mutagenesis, suppressor screen and genetic analysis

Arabidopsis etr1-2 mutant seeds were mutagenized using ethylmethylsulfonate (EMS) as described (Resnick et al., 2006), or by fast neutron irradiation (60 Gy) (International Atomic Energy Agency in Vienna, Austria). Seedlings were screened for loss of insensitivity on medium containing 100μM ACC. *rte1-1 etr1-2* and *rte1-2 etr1-2* were crossed to both wild-type and *etr1-2*, and all progeny were insensitive, indicating that both *rte1-1* and *rte1-2* are extragenic and recessive. When *rte1-1 etr1-2* was crossed to *rte1-2 etr1-2*, all F_1 progeny displayed the suppressed phenotype, indicating that they are allelic. Cloning of the *RTE1* gene was carried out by Dr. Chi-Kuang Wen.

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 crossing to wild type Col-0.

To obtain the *rte1-2* single mutant, the *etr1-2 rte1-2* double mutant was crossed with wild type. After allowing the F_1 generation to self, *rte1-2* homozygotes carrying only the wild-type *ETR1* allele were identified in the F_2 generation.

To create double mutants with *rte1-2*, the *rte1-2* single mutant (or in some cases *etr1-2 rte1-2*) was crossed separately with *etr1-1*, *ein4-1*, *ers2-2*, *etr1-7*, *etr2-1*, *ctr1-1* and *ein2-1* single mutants. (The *ein4-1* mutation encodes Ile-84 to Phe (Hua et al., 1998), *ers2-2* is an *ers2* transgene encoding Ile-94 to Phe (Hua et al., 1998), and *etr2-1* encodes Pro-66 to Leu (Sakai et al., 1998)) After selfing of the F_1, double mutants were identified in the F_2 or F_3 generations by genotyping.

To create double mutants with *rte1-3*, the *rte1-3* single mutant was crossed separately with *etr1-2*, *ers1-3*, and *ers1-10* single mutants. (*ers1-10* encodes an Arg-320 to Cys mutation (Alonso et al., 2003), and *ers1-3* contains a T-DNA insertion in the coding region of the ERS1 gene (G.E. Schaller, personal communication). After selfing of the F_1, the double homozygotes were identified in the F_2 or F_3 generations by genotyping.

rte1-3 RNA levels were analyzed using a one-step RT-PCR reaction with Superscript II Reverse Transcriptase (Invitrogen), as per the manufacturers instructions. Primers (5'-GGACCAAGTGTTGCTTACCACC-3') and (5'-GTAGCAATTATGAACCAGGC-3') were used to amplify an intron-spanning region. As controls, primers (5'-CGTGGATCACAGCAATACAGAGCC-3') and (5'-CCTCCTGCACTTCCACTTCGTCTTC-3') were used to amplify a region of B-tubulin; and primers (5'-ATTCTATCGGATCTTCTCCAGATTC-3') and (5'-CCTAATCTTTCACCAAGTCCCACT-3') were used to amplify a region of *ERF1*.

Genotyping markers

Genotyping was carried out using cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993) or derived cleaved amplified polymorphic sequence (dCAPS) markers (Neff et al., 1998) unless stated otherwise. Total genomic DNA was isolated as described (Lukowitz et al., 2000).

The *rte1-2* allele was detected by CAPS, using primers (5'-CCTGCTCGCTATCTCC-3' and 5'-GATCGAAAGTTGAGG-3') to amplify a DNA fragment that is cleaved by the restriction enzyme *Mnl*I if the fragment is from the wild-type *RTE1* allele, but not from *rte1-2*.

The *rte1-3* allele was detected by CAPS, using primers (5-GGAGTTCCTATGATGGACCTGAAA-3' and 5-GTAAGTAGCAATTATGAACCA-3) to amplify a DNA fragment that is cleaved by the restriction enzyme *Alo*I if the fragment is from *rte1-3*, but not from the wild-type *RTE1* allele.

The *etr1-2* allele was detected by CAPS, using primers (5'-CCGATTTCTTCATTGCGATT-3' and 5'-ACCGTATACTCCACGGGATG-3') to amplify a DNA fragment that is cleaved by the restriction enzyme *Hpy*CH4IV if the fragment is from *etr1-2*, but not from the wild-type *ETR1* allele.

The *etr1-7* dCAPS primers (5-GCGATTGCGTATTTTTCGAT-3' and 5'-GTGCATAAGTTAATAAGATGAGTTGATGCA-3') introduced an *Nsi*I site in the *etr1-1* site present in *etr1-7* mutants, but not wild-type *ETR1*.

For *ers1-3*, the presence or absence of the T-DNA insertion was detected using the primer (5'-TCGAGCATGTACTGCCATCTCAGCCTCTT-3') paired with the JL202 primer for the T-DNA insertion, or the primer (5-GTGCCGTCTCGGGATAACAAACTTTCTAT-3') for wild-type *ERS1*.

The *etr1-1* dCAPS primers (5'-CGATTCCTCTTGAGTTGATTTAC-3' and 5'-TGCACCAACTCATCTTATTAACTTATGGACTTTCACTACG-3') introduced an *Apa*LI restriction site in the wild-type *ETR1* fragment, but not *etr1-1*.

The *ers1-10* dCAPS primers (5-GTGGCCACATGTGCCAATTTTGGAAGAATCCATGCGAGCT-3' and 5'-TGATGGCATGCATCGGTGTCCTCATC-3') introduced a *Sac*I site in the wild-type *ERS1* fragment but not *ers1-10*.

The *ein4-1* dCAPS primers (5'-GAGTCATTCCACATAGGACAT-3' and 5'-GTGATCTCTTAATAGCCATTG-3') introduced an *Mse*I restriction site in the wild-type *EIN4* fragment, but not *ein4-1*.

The *etr2-1* dCAPS primers (5'-AACTGCGAAGACGAAGGAAA-3' and 5'-GGAACAACTCACGAAGTAAAGTAACTCACTA-3') introduced a *Dde*I site in the mutant *etr2-1* fragment but not the wild-type *ETR2* fragment.

The *ein2-1* dCAPS primers (5-CGCCATCTTTGTTTCAACAATCAGATCC-3' and 5'-CCAGAGGAAAGAGAGTTGGATGTAAAGTACTCTACCGCT-3') introduced a *Bsr*B1 site in the wild-type *EIN2* fragment, but not *ein2-1*.

The *ers2-2* transgene was followed by using kanamycin selection.

Transgenic Constructs and Plant Transformation.

To rescue the *rte1-2* mutation, the *Not*I genomic DNA fragment of the bacterial artificial chromosome T19L18 was purified and digested with *Bam*HI and the resulting fragments were cloned into cosmid vector pCLD04541 (Jones et al., 1998). A 4.3-kb genomic DNA fragment of *RTE1* was released from pCLD04541 by using a *Hind*III partial digest and *Bam*H1 and ligated into plasmid pBJ36. This fragment was then released with *Not*I and ligated into the *Not*I site of binary vector pMLBart (Gleave, 1992), a derivative of pART27 containing the *bar* gene. The *etr1-2 rte1-2* mutant was transformed by the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* strain GV3101. Transformants were selected with the herbicide BASTA, and three-fourths of the progeny of each transformant showed ethylene insensitivity in the seedling triple-response assay, confirming the rescue of *rte1-2*.

For *RTE1* overexpression, the *RTE1* coding sequence was PCR-amplified from an *Arabidopsis* seedling cDNA library (*Arabidopsis* Biological Resource Center catalogue no. CD4-22), and cloned between the cauliflower mosaic virus (CaMV) 35S promoter and 3' OCS terminator sequence of pART7. The composite fragment was released with *Not*I and ligated into the *Not*I site of pMLBart. Transformation was carried out as above. Between 5-20 independent lines were analyzed for each genotype, with 12-25 seedling measurements taken for graphical analysis.

Chapter 3: *RTE1* encodes a novel conserved protein of unknown function

Introduction

RTE1 encodes a previously undescribed, conserved protein, 250 amino acids in length with a predicted molecular mass of 28kDa (Fig. 2-1A). Sequence analysis and hydrophobicity plots predict that RTE1 is a likely transmembrane protein with two to three predicted membrane domains (Fig. 3-1).

RTE1 is highly conserved among eukaryotes (see below), but contains no known motifs that might reveal its function, although a detailed sequence analysis may provide some clues. Due to its high degree of conservation, we predict that it carries out an essential conserved function. If we knew how *RTE1* functions in *Arabidopsis*, we could make a fair assumption as to what it does in other organisms.

In sequence analysis, the features that should be looked for are highly conserved residues, and interesting potential 'patterns' or regions that are rich in particular amino acids. Modeling programs can be used to identify regions with similarity to known motifs, followed by an assessment as to the statistical significance of the similarity. Since one *rte1* loss-of-function mutant is known to be the result of the mutation of a conserved Cys residue, it is reasonable to assume that this residue is important for the functionality of RTE1. Proteins that contain essential and conserved Cys residues are varied, but comparing known Cys-dependent proteins with RTE1 might provide some insight.

A

Red line: Transmembrane helix preference. (THM index). Blue line: Beta preference. (BET index) Gray line: Modified hydrophobic moment index. (INDA index). Violet boxes (below abscisa): Predicted transmembrane helix position. (DIG index)
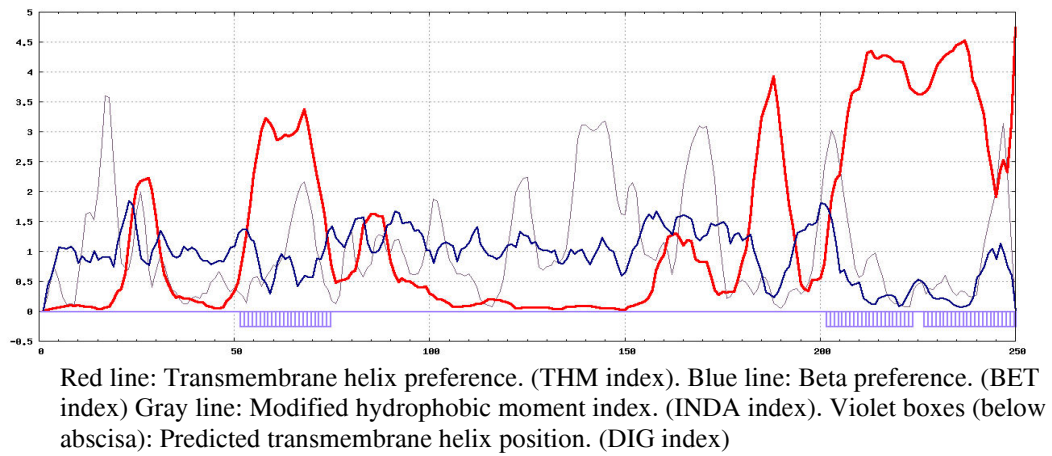
B
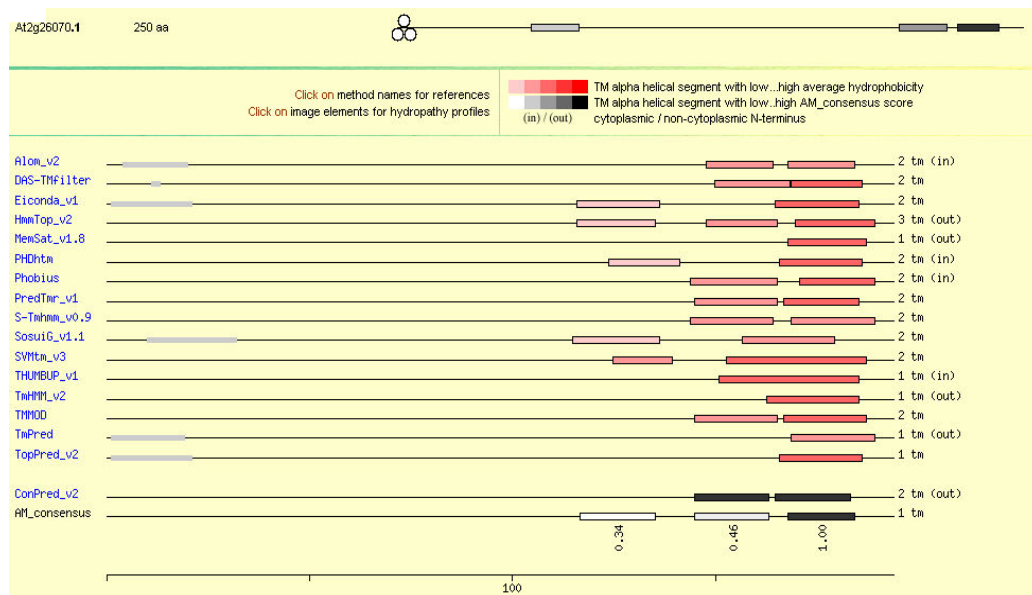
Figure 3-1. Predicted transmembrane domains of RTE1.

(A) A hydrophobicity plot indicating three putative transmembrane regions in the RTE1 sequence, corresponding to hydrophobicity peaks shown in red. Prediction as per SPLIT 4.0 server (http://split.pmfst.hr/split/4/). (B) An analysis carried out using the Aramemnon database indicating predicted transmembrane regions from multiple databases.

Many online databases exist with experimental data from microarrays or protein interaction experiments. We can collect data on *RTE1* and *RTE1* homologs that may provide useful information. For example, regulation of *RTE1* expression under certain circumstances or conditions might be significant, and RTE1 may interact with particular proteins that may be of interest.

The localization of RTE1 may also provide some insight. RTE1 is predicted to be a membrane protein, and so is probably present in either the plasma membrane or one of the intracellular organelle membranes. Since ETR1 has been localized to the ER (Chen et al., 2002), and due to the close relationship that has been demonstrated to exist between *ETR1* and *RTE1*, it seems likely that RTE1 is present in either the ER or the Golgi membrane. Online databases are available to predict likely protein localization sites, which can be confirmed through molecular localization techniques such as GFP analysis. The *rte1-2* mutation is a frameshift mutation that is indicated by membrane prediction programs to result in the loss of the third predicted membrane region. Since *rte1-2* is a loss-of-function mutant, the membrane regions may be important for *RTE1* function. Loss of this site may result in mis-localization of the entire protein, or of just the C-terminal portion, which would be positioned on the other (wrong) side of the membrane. If RTE1 is required to interact with another protein(s) in order to function, this mis-localization could easily result in loss of functionality.

A detailed sequence analysis of motifs or residues of interest in RTE1 and its homologs, along with data collected from online databases may help us to obtain a clearer idea as to what *RTE1* may be doing at the molecular level.

Results

***RTE1* is highly conserved among eukaryotes**

A BLAST search revealed that *RTE1* homologs exist in a wide variety of eukaryotic organisms, including *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Danio rerio*, *Plasmodium*, and *Trypanosoma* (Fig. 3-2A, B). *RTE1* is absent from prokaryotes, and also from fungi, which may have lost the *RTE1* gene. *RTE1* appears to be present in single copy in each species, except plants, where there are two copies in *Arabidopsis*, and three in rice and tomato. The second copy in *Arabidopsis* we have named *RTE1-HOMOLOG* (*RTH*), which encodes a protein 231 amino acids in length, with 51% identity to RTE1 over 209 amino acids. One interesting feature present in humans and some other animals (but not plants) is the presence of a poly-proline tail at the N-terminus. This may indicate a possible binding site for WW or SH3 domains, which interact with proline-rich regions to create important protein-protein interaction sites (Viguera et al., 1994). Through such interactions, protein complexes can form that are important for a variety of cellular processes, including cell signaling cascades, cell cycle control, ubiquitination, and regulation of transcription. The presence of this proline-rich region may or may not be significant, and since WW and SH3 domains are common protein-interaction motifs, it does not offer any real insight into which proteins (if any) these RTE proteins might interact with. It is a significant possibility, however, that protein-protein interaction via a WW or SH3 domain may be important for the function of RTE in some animals.

Figure 3-2. RTE1 alignment and phylogenetic tree.

(A) Alignment of RTE1 and predicted RTE1 homolog sequences using CLUSTALW. The following sequences are shown: *Arabidopsis thaliana* RTE1 "AtRTE1" (NP_180177), *Arabidopsis* RTH "AtRTH" (NP_190673), three *Oryza sativa* homologs "Rice1A" (NP_916598), "Rice1B" (AAV59409), and "Rice2" (AAO37528), *Caenorhabditis elegans* "Celegans" (AAF39886.1), *Danio rerio* "Drerio" (NP_001013334.1), *Xenopus laevis* "Xlaevis" (AAH87509.1), *Homo sapiens* homolog "Hsapiens" (NP_115501 extended at the N terminus based on the sequence of EST B1667401), *Mus musculus* "Mmusculus" (AAH37609.1), *Drosophila melanogaster* "Dmelanogaster" (NP_723362.1), *Anopheles gambiae* "Agambiae" (EAA00221.2), *Plasmodium falciparum 3D7* "Pfalciparum3D7" (NP_703394.1), *Giardia lamblia* " Glamblia" (XP_767284.1) *Leishmania major* "Lmajor" (CAJ08815.1), *Trypanosoma brucei* "Tbrucei" (EAN77990.1). and *Trypanosoma cruzi* "Tcruzi" (XP_804751.1) (B) An unrooted neighbor joining tree (2) for the RTE1 and RTE1 homolog protein sequences shown in (A).

A

```
AtRTE1         -MSRGRGVPMMDLKRSYDVEDRVVSVSIPSIIEADEADLWPLPEIDTKKSKFECCIVWTP
AtRTH          ----------MGETATDSEHRMMIGLSD-------------PMKIDPKRDRFECCIVWTP
Rice1.1        -MAPNK-ISSMDAGAAFD--DEDASSSN------SLQELWPVGEIDPKRARFECCIVWTP
Rice1.2        ----------MEVEAACG--DGVVSSSN------EMQELWPLGEVDQKGTRFECCIVWTP
Rice2.1        --------------------METDRSQ-------------PAPIDPRRARFECCIVWTP
Celegans       ----------------------------MPLTDRDICPENNRYEYCIVWTP
Drerio         -----------------------------MKHYHG-GFEKIDREMSRYEHCIVWTP
Xlaevis        ------------------------------MKLDPERSRYEHCIVWTP
Hsapiens       MAEAEGSSLLLLPPPPPPPRMAEVEAPTAAETDMKQYQGSGGVAMDVERSRFEYCVVWTP
Mmusculus      MAEAEGSSPLLLQPPPPPPRMAEVETPTGAETDMKQYHGSGGVVMDVERSRFEYCVVWTP
Dmelanogaster  -----------------------MASSGGGAGN-GVPDRLPPINVKDQRFEYCIVWTP
Agambiae       --------------------MSLHSDTSEDLSRNSAEMELLPPINFADDKYEYCIVWTP
Pfalciparum3D7 ------------------------------MPNDLLNNMTINKKDNKYEYCVVETY
Glamblia       ----------------------------------------------MAPLRSVVWAP
Lmajor         ----------------------MESRTRFKADT--PVQLPPAIDPVRERYEFCIVWTD
Tbrucei        -----------------------MTRRNAMSDNTTSTSSVTP-----ERYEFCIVWTD
Tcruzi         -----------------------MTGRKGMGENRSVALPLPPRIDPSEEHYEFCIVWTP
Consensus/80%  .............hs...p+aPhClVWTP

AtRTE1         LPVVSWLAPFIGHTGLCREDCVILDFACSNFIN--VDDFAFGPPARYLQLDRTK-CCLPP
AtRTH          LPFISWLVPFIGHVGICREDCVILDFACPNFVC--VDNFAFGAVSRYIQINKEMESSRSS
Rice1.1        LPIVSWLAPYIGHAGICREDCTVLDFACSNLVS--MDNFAYGSIARYLQLDRKK-CCFPV
Rice1.2        LPVVSWLAPYIGHVGIAREDCTVMDFACSNFVS--VDDLAYGSIARYLQLDRRK-CCFPA
Rice2.1        LPLISWLPFIGHIGICREDCVILDFACPNFVS--VDNFAFGAVARYIQVNSDE----
Celegans       IPCLTWFPFIGHMGIANSRCIILDFACSYYVA--EDDMGFGWPTRLWQLGPEK------
Drerio         IPVLTWFLPFIGHMGICSSACVILDFACPYFVS--EDNMAFGKPTKLWKLDKNK------
Xlaevis        IPVLTWLPFIGHMGICTSSCVILDFACPYYVS--EDCMAFGKPVKLWQLDPSL------
Hsapiens       IPVLTWFBPIIGHMGICTSTCVILDFACPYFVS--EDNMAFGKPAKLWKLDPAQ------
Mmusculus      IPVLTWFPIICHMGICTSACVILDFACPYFVS--EDNMAFGKPAKFWKLDPGQ------
Dmelanogaster  IPVLTWLMPMICHMGICTSSCVILDFACPYFVS--EDNMAFGRPTRYIRLHPKH------
Agambiae       IPVLTWFPIGHMGIAMSNGVILRDFACPYFVS--EDNMGFGRPTRYLRLHPAN------
Pfalciparum3D7 LPCASTFIPTVGHVGICTSTCVILDFGSGSYSVS--VDNMFFGDPMKYWQLDKNK------
Glamblia       IPCLSSSLPFMLGHFGITDSVCVILDFGCGDFYINRSETHTIFGPPTLYTQLSEEC------
Lmajor         IPVLSWIIPFVGHTAVCDSQCRIYDFDQCAYRIG--QDRMLFGNPVKYWVSRDYIP---
Tbrucei        IPLVSWLFPFVGHVGICDSTCRILDFECSYCIG--VDHMLFGNPVKLWDISAMYVPS---
Tcruzi         IPVLSWIFPFICHVGICDSACRILDFEEPYHIG--VDKMLFGNPVKLWNISRMYVP---
Consensus/80%  lPhlsWhhPhlGHhGIsppsGhIbDFTGs.hlt...DphhFG.ss+Yhpls..b......

AtRTE1         NMGGHTCKYGFKHTDFG--TARTWDNALSSSTRSFEH-KTYNIFTCNCHSFVANCLNRLC
AtRTH          SSGMFNGERRYEQEEDSHEKEPTWDDALRKSTQEYQH-HSYNILTCNCHSEVANNENRLS
Rice1.1        NLATHVCERSYKHAEAG--TAISWDDALQLGMRSFGH-KFYNLFTCNCYSFVANCLNRLA
Rice1.2        NLAAHVCARSYEHSEAG--TAISWDDALQSGARRFEH-KCYNLFTCNSHSFVASCLNRLA
Rice2.1        ---------CYKLLEP--EGASTWDDALRKGVQEFQH-RGYSLFTCNCHSFVVNNLNRLF
Celegans       ---------------VEG-GAEVFDRAVQDASDTYKT-RTHNLICDNCHSHVALNKMR
Drerio         --------------VYGGGANAWDVAVHEASEEYKN-RMHNLCCDNCHSHVMALNLMR
Xlaevis        --------------ILASGSNPWDTAVHEASEEYKH-HMHNLCCDNCHSHVALALNLMK
Hsapiens       --------------VYASGPNAWDTAVHDASEEYKH-RMHNLCCDNCHSHVALALNLMR
Mmusculus      --------------VYASGPNAWDTAVHDASEEYKH-RMHNLCCDNCHSHVALALNLMR
Dmelanogaster  --------------MVG-GSYAWDEAVSKASVLYGT-RIHNIFCDNCHSHVGMALALMR
Agambiae       --------------AFG-GTQNWDESVIKASATYGA-RMHNLFCDNCHSHVGMALALMR
Pfalciparum3D7 --------------LPLSISDKSYDDAIYKTDEIFKK-RREQLSPSRSDGPK--------
Glamblia       --------------WSAISDEEWDSALSITMANYQK-KRYNFFINNCHEFVAAVLKTLS
Lmajor         SFYNADQQNSAEREEAVKREVAAYDAALMSTISHFRQTEVYNFFTNNCHSFVAASMNEQQ
Tbrucei        SRFPPGGLLSGDVEERRRRETEEYDRALSGVTTRFRRKTQTYNFFTNNCHSYVASVFEEMT
Tcruzi         TFYRSQGENPRTTEETCRREVEEYDAAVERVTKHFRKTQLYNFFTNNCHSYVACVLRDHP
Consensus/80%  ...................s.saDpAlppssppapp.+haNhhssNCHSaVA.sLPbh.

AtRTE1         YGGSMEWNMVNVAILLMIKGK--WINGSSVVRSFLECAVVTSLGVVLVGWPFLIGLSSFS
AtRTH          IK-SGGWNVVNLATLVLFKGR--WVNKTAIVKSLLEPLIVYTIGILLGGWTFIASCSILV
Rice1.1        YNGSVKWNVLNVAALVWLRGQ--WVDKMSVVRSFFPFLTVTCVGILMAGWPFLIGMAAFS
Rice1.2        YGGSVGWNVLNLAALVWLRGR--WLGKMAVVRSLLPFAAVACVGVLMAGWSFLISMAAFS
Rice2.1        YSGHDKWNVVSLAAVMFLRGR--WVSTASVVKTFFEFALVITIGTLLGGATFLIGLLAFA
Celegans       YDEREDWNMINLAWYSLTKGS--FVRNTDMLAQYLEFVIIVFIFVALWAFL---------
Drerio         YNNSSSWNMANLCLRFLIHSK--HVSFVGFLKTWLEFLMICGVIVTIALAVNLR------
Xlaevis        YNN-TSWNMVKLCILCLVYSR--YISFGAFLKTWVEFILLAGAVMTVVLTLHLR------
Hsapiens       YNNSTNWNMVTLCFFCLLYGK--YVSVGAFVKTWLEFILLLGIILTVSLVFNLR------
Mmusculus      YNNSTNWNMVTLCCFCLIYGK--YVSVGAFVKTWLEFVLLLGIILTVSLVFNLR------
Dmelanogaster  YYDSTAWNMIILSMWLFVCGR--YVGIGGFIKTWLEFAILLSIFTILGIYF---------
Agambiae       YKEYTNWNMVVLAFWMFFRGK--YVGLRGFIKTWLEFLIILTICALVAMYSKWTI-----
Pfalciparum3D7 ------------------------------------------------------------
Glamblia       SG-KKNYTIFSLIRAFRLGRT-----VKKMPGHNAEPASHSDVLGCEDA-----------
Lmajor         LK-KQHMGMVSIAIGMMTRGR--YISVSRFMQAHLESILLIVIILILVALL--------
Tbrucei        NGPRRPWNMFWIAWGLAIHGRSLLKSPSSLPRYRRELCDRRCTLGLTRSK---------
Tcruzi         LTAAGSFSVFRVAWGLLIHGR--YVSAGRFFRAHLPFFLLMAVVLLCVMLT--------
Consensus/80%  h.....ashh.lshhhhhbtp..als...hhp.hhP.hhlhshhhhhshh.........

AtRTE1         LLLFAWFIIATYCFKNIIT-
AtRTH          VLLTGWFIIGTYCFKKLIQL
Rice1.1        SLLIGWFVFAVYCMKDLVC-
Rice1.2        SLLLGWFVLGVYCFKGLVC-
Rice2.1        AVMTGWFLVGTYCIKSLVEL
Celegans       --------------------
Drerio         --------------------
Xlaevis        --------------------
Hsapiens       --------------------
Mmusculus      --------------------
Dmelanogaster  --------------------
Agambiae       --------------------
Pfalciparum3D7 --------------------
Glamblia       --------------------
Lmajor         --------------------
Tbrucei        --------------------
Tcruzi         --------------------
Consensus/80%  ....................
```

B

Glamblia

Lmajor

Pfalciparum3D7

Tbrucei

Agambiae

Tcruzi

Dmelanogaster

Mmusculus

AtRTE1

Hsapiens

Xlaevis

Rice1.1

Drerio

Rice1.2

Celegans

Rice2.1   AtRTH
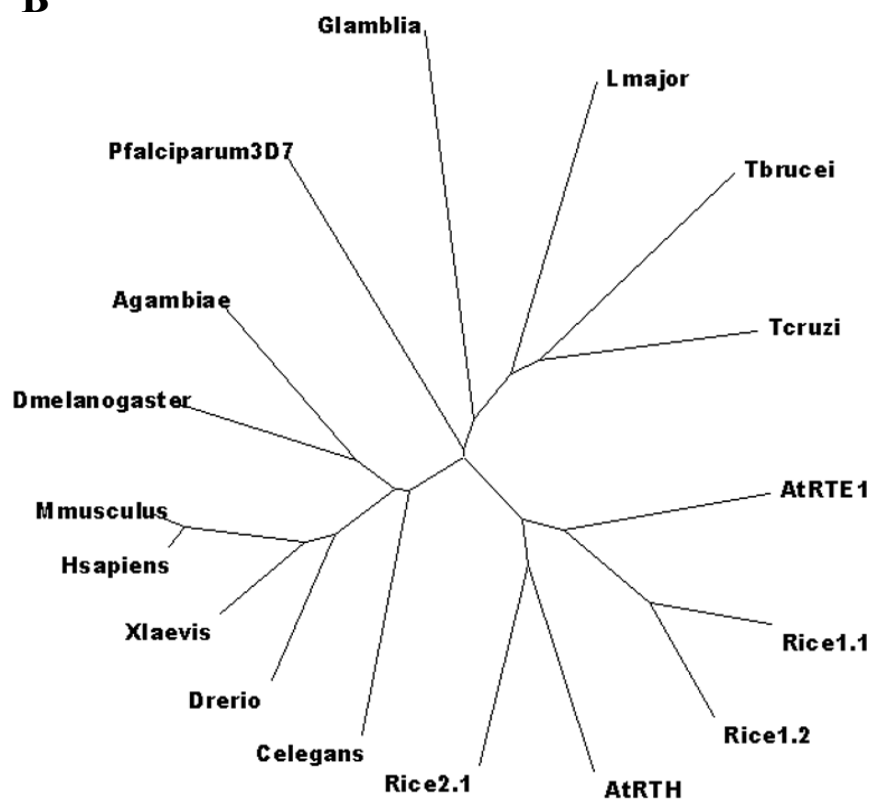
0.1

Interestingly, a yeast-2-hybrid analysis indicated that human RTE (hRTE) is likely to interact with BAT5 (Lehner et al., 2004), a protein expressed from a region of the genome that is associated with autoimmune disease (human major histocompatability class III region). Sequence analysis revealed that BAT5 contains an ά/β hydrolase domain, indicating that it may be a protease involved in cleavage of various C-C, C-O and C-N bonds (Spies et al., 1989; Fischer and Mayr, 2001). In addition, BAT5 interacts with proteins implicated in RNA processing, indicating that it may also play a role in mRNA regulation. BAT5 contains three transmembrane domains, although its subcellular localization is unknown. Interestingly, all ά/β hydrolases contain a catalytic triad, which are usually comprised of conserved His and Asp residues, with a third residue dependent on the protein type –usually a serine, cysteine or threonine residue. RTE1 also contains several conserved Cys, His and Asp residues, which could possibly make up a catalytic triad, and may be worth further examination.

Although these results are potentially interesting and exciting, we should use careful judgment when assessing the real value of such findings, since yeast-2-hybrid assays conducted using membrane proteins commonly give false-positive results (Serebriiskii et al., 2000). However, these data indicates that *hRTE* may be involved in the regulation of *BAT5*, and/or possibly in mRNA processing. It may also suggest that hRTE is post-translationally regulated by BAT5 or other proteases.

A 112-amino acid segment of hRTE defined as 'C1orf160', has been localized to the dorsal root ganglion (Strausberg et al., 2002). These are sensory nerve cells that pass information to nerve cells in the spinal cord in order to be relayed to the brain.

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A microarray analysis of *hRTE* indicates it is upregulated in the brain, cortex, thalamus, thymus and spinal cord. (http://meduza.thep.lu.se/index.html?org=Human&db=hg17&hgsid=368847). This high degree of expression in the central nervous system indicates a possible importance for hRTE in these specific tissues. Interestingly, GFP localization analysis carried out on *C. elegans* also localizes RTE to neurons (Liesch, Muresan, Haag and Chang, unpublished), which supports the above hypothesis. Of course, one obvious paradox here is that *Arabidopsis* has no nervous system! However, *RTE1* may carry out a conserved, essential, regulatory function that has evolved to be specific to individual proteins or protein types/families. In animals, this protein type may be most prevalent or important in neural tissues. This may also have implications for human disease. *hRTE* maps to the gene map locus 1p36.11 on the human genome, and many diseases are known to map to this locus; including several that can cause neural degeneration such as Parkinson's Disease (Samii et al., 2004). Although there are many genes that localize to this region, it is possible that one of these disease genes could indeed be *hRTE,* including *PARK7* or *PARK9* – two disease-causing genes known to localize to the 1p36.1 region of the human genome (Mori et al., 2003).

Some sequence analysis programs indicate that hRTE is a hypothetical Cytochrome c family heme-binding protein. This also applies to other animal RTE proteins, due to the presence of a highly conserved CXXCH motif. In plants this motif is a conserved CXCH motif, which is not implicated in the binding of heme molecules. However, we know that the second Cys residue is likely an essential one,

since the *rte1-1* loss-of-function mutation is the result of a Cys-Tyr mutation at this location. At first glance, this seems an exciting prospect in deciphering the function of *RTE1*. However, there are a high number of false positive hits with regards to CXXCH-containing proteins being labeled as Cytochrome C Oxidases; and other 'signature' motifs and structural components are required for a functional heme-binding protein (Jobson et al., 2004). In addition, Cytochrome C Oxidases are very large, multi-subunit proteins localized to the mitochondrial membrane. If RTE proteins are shown to be present in the mitochondria and/or to be part of a large complex, the possibility of it functioning as a Cytochrome C Oxidase could be addressed further.

There are many protein types that utilize Cys residue-containing motifs such as CXXC and CXC. For example, Chemokines, Metallothioneins (MTs) and Lipid Transfer Proteins (LTPs) in plants all contain CXC motifs (Giritch et al., 1998; Douliez et al., 2001; Belperio et al., 2006). Several metal-binding proteins are known to contain CXXC motifs: for example, the copper chaperone CopA contains a CXXC motif that is essential for copper binding (Multhaup et al., 2001). Superoxide dismutase (SOD) also contains a CXXC copper-binding motif (Rae et al., 2001).

However, CXXC motifs are also important in other protein types. Thiol:disulfide oxidoreductases require the CXXC motif for the catalysis of redox reactions in electron transfer chains (Chivers et al., 1997). These proteins are important for the oxidation of protein thiols and isomerization of disulfide bonds. Another protein that contains a redox-active CXXC motif are protein-disulfide isomerases, although most proteins of this type contain multiple CXXC motifs within

their peptide sequence. Although these motif comparisons may provide some insight, they cannot point towards a definitive function for *RTE1*, since many other criteria would have to be met in order to fit into one of these protein categories. However, since it is likely that at least one of the Cys residues within this motif is essential, it is likely an important region within the protein.

RTE1 is the *GR* gene in tomato

Research from Dr James Giovannoni's lab at The Boyce Thompson Institute for Plant Research has shown that the tomato *Green Ripe (GR)* gene cloned by them is homologous to *RTE1* (Barry and Giovannoni, 2006). The *Gr* mutant exhibited reduced ethylene sensitivity in fruit, flowers and roots (although not in hypocotyls). The mutant phenotype was found to be due to ectopic overexpression of the *GR* gene, caused by the deletion of a segment of the 5'UTR and upstream regulatory regions.

This result is consistent with the insensitivity observed when *RTE1* is overexpressed in *Arabidopsis* seedlings. Since not all tissues were affected by the ectopic overexpression, Barry hypothesizes that *GR* might function to regulate ethylene responses in a tissue-specific manner. This fits well with the hypothesis that *GR/RTE1* genes are important in the regulation of specific receptors, since ethylene receptor genes in tomato are differentially expressed in different tissues (Sato-Nara et al., 1999; Tieman and Klee, 1999). For example, it is known that floral and fruit tissues express *LeETR4* and *LeETR5*, whereas these receptors are virtually absent from hypocotyls (which exhibits no response to ectopic overexpression of *GR*) (Lashbrook et al., 1998). This is consistent with our hypothesis that *RTE1* is a

regulator of *ETR1*, but has little to no affect on the other receptors. Another hypothesis offered by Barry is that levels of *GR* expression are dependent on developmental stage or stress. For example, developing seeds are known to have high levels of *GR* expression (as is the case with *RTE1* in *Arabidopsis*). Barry speculates that a high level of *GR* may be responsible for inhibiting ethylene responses to protect the developing embryo. While this speculation is difficult to test, it is interesting since we know that *GR/RTE1* is differentially expressed through plant tissues and at various stages of development. Ectopic overexpression of *GR* has no effect on hypocotyl length and yet causes reduced ethylene sensitivity in adult fruit and flowers. Overexpression of *RTE1* causes weak ethylene insensitivity in both hypocotyl and roots, but little difference was seen between wild type and transgenic lines in an adult ethylene induced senescence analysis (data not shown). This raises the possibility that *RTE1* is regulated during developmental stages or under certain conditions so as to exert a level of control over downstream ethylene responses.

We have shown that *RTE1* is up-regulated in response to ethylene (Chapter 2), possibly as part of a feedback loop to negatively regulate ethylene responses once ethylene-response genes have been switched on. Interestingly, in *Arabidopsis*, *ETR1* and *EIN4* levels are unaffected by ethylene treatment, although *ERS1*, *ERS2* and *ETR2* do show some degree of elevated levels (Chang et al., 1993; Hua et al., 1998; O'Malley et al., 2005). Tomato ethylene receptors are all up-regulated in response to ethylene (Voesenek and Mariani, 1997; Wilkinson et al., 1997). It is interesting that levels of *ETR1* do not increase when ethylene is present, and it is plausible that *RTE1* is up-regulated in order to exert a degree of control over *ETR1*. Since *ETR1* is a key

ethylene receptor, and under wild type conditions is expressed to a higher degree than the other receptors, regulation at the transcriptional level may be inefficient. It is possible that *RTE1* may somehow regulate the *ETR1* receptor to ensure quick and efficient activation/deactivation in response to ethylene.

Interestingly, the tomato *RTE1* homolog *GR* contains an MXCXXC motif at the C-terminal end of the protein, and a MXXXM motif in one of the predicted transmembrane domains (Barry and Giovannoni, 2006), both are which are known to be copper binding motifs. Although these motifs are not conserved among other *RTE* homologs, detailed analysis of an RTE family protein alignment reveals regions of conserved His and Cys residues, which are common in metal-binding proteins. In particular, one highly conserved motif: CNCH (CXC(H)) in plant RTE proteins; and CDNCH (CXXC(H)) in animal RTE proteins, is reminiscent of a CXXC or CXXXH motif that commonly forms a binding "pocket" in metal-binding proteins. In the ETR1 receptor, copper binds to a CXXXH motif, and mutagenesis of this Cys residue to a Tyr in *etr1-1* mutants is sufficient to eliminate copper binding (Bleecker et al., 1998). It is noteworthy that the *rte1-1* allele also has a Cys to Tyr mutation at the conserved Cys^{161} residue.

The possibility that RTE1 may function as a copper chaperone to facilitate the binding of copper to the ETR1 receptor is discussed in Chapter 4.

Discussion

RTE1 homologs can be found in animals, protists and other plant genomes, suggesting that members of the *RTE* family carry out an essential, conserved function.

Since animals and protists do not contain ethylene receptors, *RTE1* may carry out a specialized function in plants, or it may act via a more general mechanism such as facilitating the binding of metal ions or regulating conformational changes. In *Arabidopsis*, only ethylene-response phenotypes have been observed in *rte1* mutants. However, sequence analysis has shown that plants contain more than one RTE-like protein, compared to animals and protists, which only have one RTE. It is therefore conceivable that the *RTE1* homolog *RTH* has a function more similar to that in animals. Further study of *RTH* is required to assess whether it has any redundancy to *RTE1*, and whether it has any involvement in regulating the ethylene-signaling pathway. Sequence analysis of *RTE1* reveals that it is a novel protein, with no known function in any organism. There are no obvious motifs present that would point towards a particular role for members of the *RTE* family. The presence of particular features, which may or may not be conserved throughout the *RTE1* homologs, may present potentially interesting insight, although much of this data should be assessed with cautious optimism. Motifs that contain only a small number of residues are often mischaracterized, and conserved Cys and His residues can be found in a variety of different protein types. The increasing number of databases available over the Internet is a great resource when analyzing a new gene or protein, but again, information should be carefully assessed. Yeast-2-hybrid analyses frequently yield false negative results (Serebriiskii et al., 2000), and localization studies are often useful only when accompanied by corresponding genetic or biochemical data. Data collected in this manner is important in its own right, but should be used to help in the design of, or as an accompaniment to additional experimental evidence.

Methods

Sequence Analysis

BLAST was performed using the NCBI website (www.ncbi.nlm.nih.gov/BLAST). For fungal genomes, BLAST was carried out using the Fungal Genome Initiative website (www.broad.mit.edu/annotation/fgi).

Transmembrane domain predictions were obtained from the Aramemnon database (http://aramemnon.botanik.uni-koeln.de/index.ep) and the hydrophobicity plot was created using the Membrane Protein Secondary Structure Prediction Server (http://split.pmfst.hr/split/4/)

Sequence alignments were carried out by using CLUSTALW (http://align.genome.jp), and a phylogenetic tree of RTE family members was created using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview/help/contents.html).

Chapter 4: RTE1: A possible copper chaperone?

Introduction

Metal ions are essential in all living organisms, and copper trafficking is critical for fundamental cellular processes in both plant and animal systems. Copper has the ability to readily lose or gain electrons, and is therefore important in many oxidative processes. It is a necessary co-factor for many enzymes in *Arabidopsis*; for example, cytochrome c oxidase (the final enzyme in the respiratory chain) and superoxide dismutase (which is important for superoxide detoxification). This oxidative property also enables disruption of the secondary structure of some proteins through oxidation of thiol bonds. Such disruption may be important in facilitating the binding of ethylene to the receptors, since it is proposed that the binding of a copper cofactor could induce a conformational change in the ethylene-binding domain (Bleecker et al., 1998). ETR1 is the only receptor so far shown to bind copper (Rodriguez et al., 1999), and candidate ligating side chains for this cofactor are the conserved Cys^{65} and His^{69} residues (Bleecker et al., 1998, Rodriguez et al., 1999). A mutation in either of these residues eliminates ethylene binding, indicating that copper is an essential cofactor (Bleecker et al., 1998). A reasonable model presented by Bleecker et al. (1998) involves the Cys^{65} and His^{69} residues in an ETR1 dimer forming a hydrophobic pocket to bind copper. Once copper is bound, ethylene is able to associate with the receptor to induce downstream responses. The necessity of copper in the ethylene-response pathway is further supported by the phenotype of the *ran1* loss of function mutants (Woeste and Kieber, 2000), which phenotypically does

not appear to have any functional ethylene receptors and is even more severe than a receptor quadruple null mutant or a strong *ctr1* constitutive loss-of-function mutant.

Although many proteins are dependent on copper, the redox reactions it is involved in can cause damage to DNA, proteins and lipids (Bremner, 1998). It is therefore essential that the cellular machinery be able to tightly regulate copper levels – through import, trafficking and export. The copper trafficking pathway has been well characterized in yeast and mammalian systems, although it is still believed that there are many components of this and other metal trafficking pathways that have yet to be discovered (Valentine and Gralla, 1997; Pena et al., 1999). Homologs of proteins involved in copper trafficking and metabolism in yeast and mammals have been identified in plants (Himelblau and Amasino, 2000). These are presumed to comprise a copper trafficking pathway in *Arabidopsis,* based on their ability to complement yeast copper mutants. Within the cell, specific transporters and chaperones function to ensure delivery to copper-dependent proteins; each protein is served by a specific copper chaperone, and the residues surrounding the copper-binding motif determine specificity (Harrison et al., 1999).

The probable copper trafficking pathway in *Arabidopsis* is shown in Figure 4-1, as compared to humans. In *Arabidopsis*, copper enters the cells through the copper transporter COPT1, where it is picked up by the copper chaperone CCH (Himelblau et al., 1998). CCH delivers copper to RAN1, possibly through direct interaction. RAN1 is most likely responsible for the transport of copper into the lumen of the Golgi, since this is the function of the homologous Menkes/Wilson protein in mammalian cells (Vulpe et al., 1993; Yamaguchi et al., 1996). RAN1 has not yet
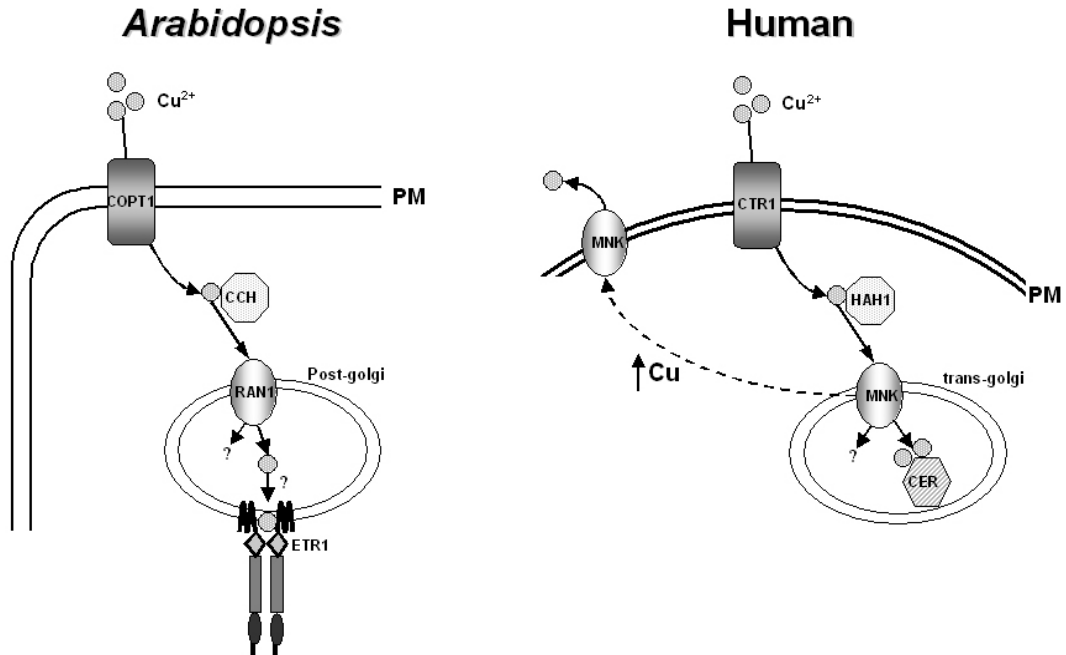
Figure 4-1. Copper trafficking pathways in *Arabidopsis* and humans.

The cell internalizes copper via a plasma membrane-bound copper transporter;

COPT1 in *Arabidopsis*, and CTR1 in humans. Once inside the cell, copper is picked

up by a copper chaperone (CCH in *Arabidopsis* and HAH1 in humans), which

delivers it to a secondary transporter: a Golgi-localized ATPase. In *Arabidopsis*, this

transporter is RAN1, which is believed to localize to the post-Golgi, although this has

not yet been shown. In humans, the transporter is MNK1, which is localized to the

trans-Golgi. In human cells, when copper levels increase, MNK is able to shuttle to

the plasma membrane, where it is involved in copper efflux. Once inside the Golgi

lumen, copper can associate with copper-dependent proteins such as ETR1 in

Arabidopsis and CER (ceruloplasmin) in humans.

been localized, and does not have the two leucine repeats present in the human MNK protein necessary for targeting and retention to the trans-Golgi, although it may contain other plant specific Golgi-targeting signals. It is believed that RAN1 localizes to the post-Golgi (Woeste and Kieber, 2000). It is likely that the copper ions have a variety of targets in the Golgi lumen. In yeast, one target for copper ions is the iron transporter Fet3 (Ferrous Transporter 3 – a multi-copper oxidase), which is dependent on the presence of copper to undergo high affinity uptake of iron (Askwith et al., 1994; Stearman et al., 1996). In human cells, copper is targeted to ceruloplasmin (Cer) – which is a Fet3 homolog. *Arabidopsis* has no known homolog of Fet3 or Cer.

Many of the copper and metal binding proteins involved in trafficking pathways have only recently been discovered and characterized, and it is believed that there is still much to learn about these complex pathways. Not all copper binding proteins exhibit the characteristic MXCXXC motif seen in MNK and RAN1-like proteins, often the presence of a conserved cysteine or di-cysteine residue(s) is sufficient for association. It is therefore likely that identification of other copper binding proteins in *Arabidopsis*, or indeed any other organism, may come through analysis of novel proteins that are found to associate with known copper-binding molecules.

We wanted to test whether RTE1 is involved in facilitating the binding of copper to the ETR1 receptor. The copper transporter RAN1 is important for the transport of copper molecules that ultimately associate with ETR1 (and probably other receptors), presumably occurring in the post-Golgi lumen (Hirayama et al., 1999; Woeste and Kieber, 2000). *ran1* mutant seedlings exhibit severe constitutive

responses indicative of there being no functional ethylene receptors (Woeste and Kieber, 2000). Although the binding of a copper molecule to the ETR1 receptor is known to be functionally essential, the molecular details surrounding this association are unknown. Whether another molecule is important for the delivery of copper to ETR1 and other receptors has not yet been shown. The sequence analysis carried out in Chapter 3 suggested that conserved Cys and His residues in RTE1 might facilitate copper binding. Since we know that a mutation in one of these Cys residues results in a loss-of-function mutant, this implies that it is functionally important. Loss of *RTE1* results in a largely non-functional ETR1 receptor, and loss of *RAN1* also impedes receptor function. Another parallel between *rte1* and *ran1* loss-of-function mutants is that *ran1* is unable to suppress the strong ethylene-insensitive mutant *etr1-3* (similar to the inability of *rte1* to suppress *etr1-1*). This raises the possibility that RTE1 may act to facilitate copper binding to the ETR1 receptor.

Results

Growth on copper or neocuproine does not affect *rte1-2* responses

The strong mutant *ran1-3* displays a constitutive triple response when grown in air, which can be partially rescued by growth on high levels of copper (Woeste and Kieber, 2000). In contrast, the weak mutants *ran1-1* and *ran1-2* appear wild type in air, but when grown on the strong copper chelator neocuproine exhibit a constitutive triple response phenotype (Fernando Rodriguez and Tony Bleecker, personal communication). Since *rte1-2* exhibits a slight constitutive response when grown in air, it was of interest to see whether this could be rescued by growth on copper, or if it

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would become more severe in the presence of the chelator neocuproine. When *rte1-2* was germinated alongside *ran1-3* control seedlings on 50μM CuSO$_4$, *rte1-2* mutants showed no difference compared to those germinated in air (Fig. 4-2A). Similarly, when grown alongside *ran1-1* and *ran1-2* seedlings on 10μM neocuproine, *rte1-2* did not exhibit any change in response (Fig. 4-2B).

Overexpression of *RTE1* is unable to rescue the severe *ran1-3* loss-of-function null mutant

To test whether *RTE1* would be able to compensate to any degree for the loss of the *RAN1* copper transporter, we overexpressed *RTE1* in the strong loss-of-function mutant *ran1-3*. Dark-grown *ran1-3* seedlings grown in air exhibit a strong constitutive triple response, and overexpression of *RTE1* had no effect on the hypocotyl length of seedlings grown on MS alone or on 50μM copper, with seedlings appearing phenotypically similar to *ran1-3* single mutants in both cases (Fig. 4-3). *ran1-3* mutants also display a characteristic light-grown phenotype, in which the cotyledons are significantly smaller than the wild-type and exhibit delayed opening, resulting in a 'cupped'–like phenotype. Similar to that seen in dark-grown seedlings, *ran1-3* seedlings overexpressing *RTE1* were indistinguishable from those with wild-type *RTE1* levels (Fig. 4-3).

rte1-2* reduces the ability of copper to partially rescue *ran1-3

Since *ran1-3* is a severe constitutive response mutant, and we believe that loss of *RTE1* results in a largely non-functional ETR1 receptor, it was of interest to see
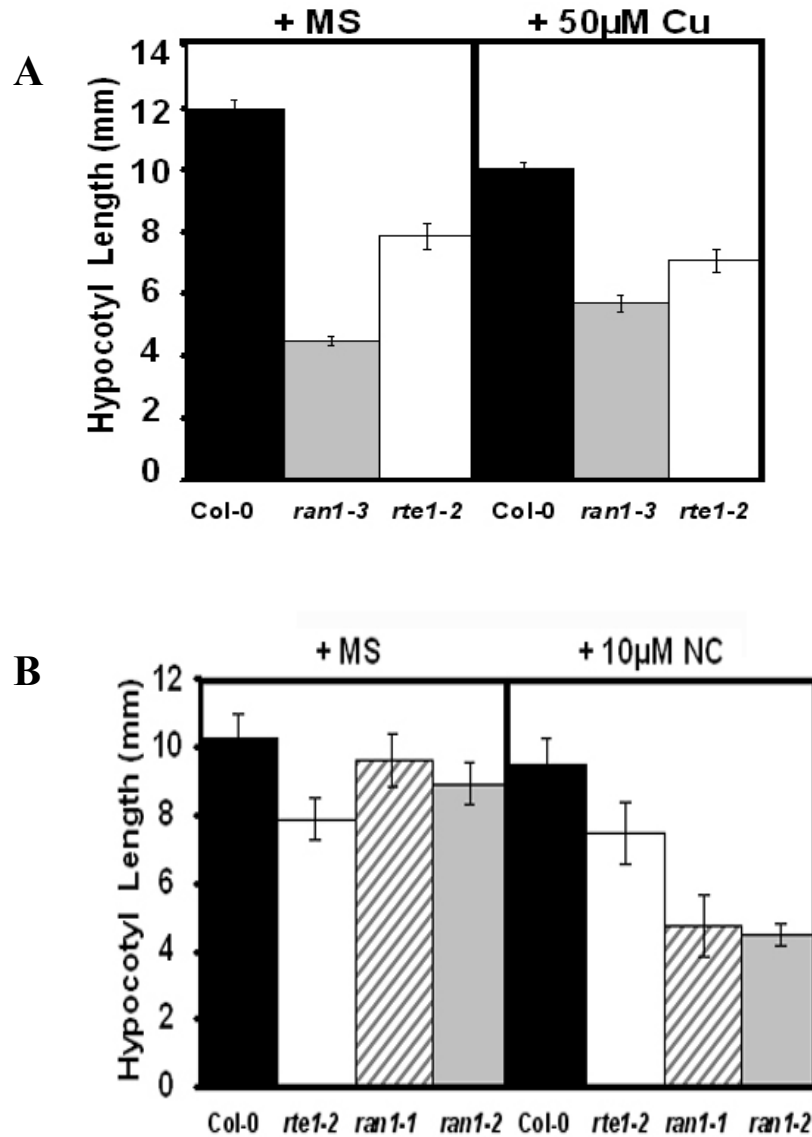
Figure 4-2. *rte1-2* is unaffected by copper and the copper chelator neocuproine.
(A) The shortened hypocotyl exhibited in *rte1-2* is not alleviated by growth on
copper, which is observed in *ran1-3* mutants. (B) Growth of *rte1-2* on the copper
chelator neocuproine (10μM) does not result in a constitutive ethylene response, as is
seen in *ran1-1* and *ran1-2* mutants. The mean +/- SE is shown in each graph for 10-
17 seedlings per genotype.
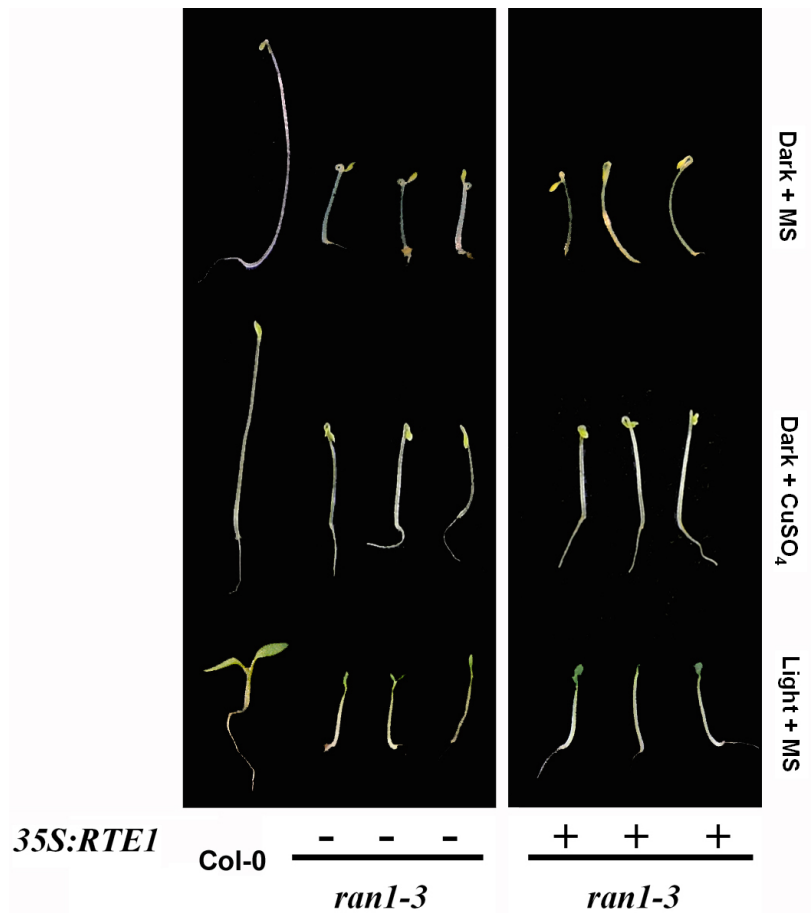
Figure 4-3. Overexpression of *RTE1* does not alleviate the constitutive response in *ran1-3* mutants.

Representative four-day-old seedlings of wild type and *ran1-3*, either untransformed (–) or stably transformed (+) with 35*S:RTE1*. Seedlings were germinated either in the dark without copper (top), dark with copper (50μM) (middle) or in the light in air (bottom).

how the loss of both genes affects responses. If *RTE1* is involved in facilitating the binding of a copper molecule to ETR1 (and possibly other receptors), loss of both *RAN1* and *RTE1* would likely result in a more severe response. As mentioned above, the severe constitutive phenotype exhibited by *ran1-3* mutant can be partially rescued by growth on high levels of copper (which is more evident in the roots of dark-grown seedlings, although the hypocotyls are also noticeably longer). If RTE1 facilitates the binding of copper to ETR1, then in the absence of RTE1, this rescue would likely be impaired. Interestingly, the *rte1-2 ran1-3* double mutant is more severe than *ran1-3* alone, and although some lengthening on copper is observed, it is not to the same degree as that seen in *ran1-3* mutants (Fig. 4-4A, B, C).

In order to test whether the more severe phenotype seen in *rte1-2 ran1-3* double mutants was not simply due to loss of functionality of the ETR1 receptor, we also created an *etr1-7 ran1-3* double null mutant. The partial rescue seen when *ran1-3* mutants are grown on high concentrations of copper is likely due to saturation of the cell resulting in a small degree of copper associating with the receptors through atypical processes. It is probable that *ETR1* is responsible for a large percentage of this atypical binding, since it is expressed to a higher degree than other receptors (O'Malley et al., 2005), and we know that loss of *ETR1* results in seedlings that are hypersensitive to ethylene. It is therefore not surprising that the *etr1-7 ran1-3* double is rescued to a lesser degree than *ran1-3* alone when grown on 50μM copper (Fig. 4-4 A, B, C). However, the *rte1-2 ran1-3* double mutant is slightly more severe than *ran1-3 etr1-7*. This result does support the possibility that RTE1 could help facilitate
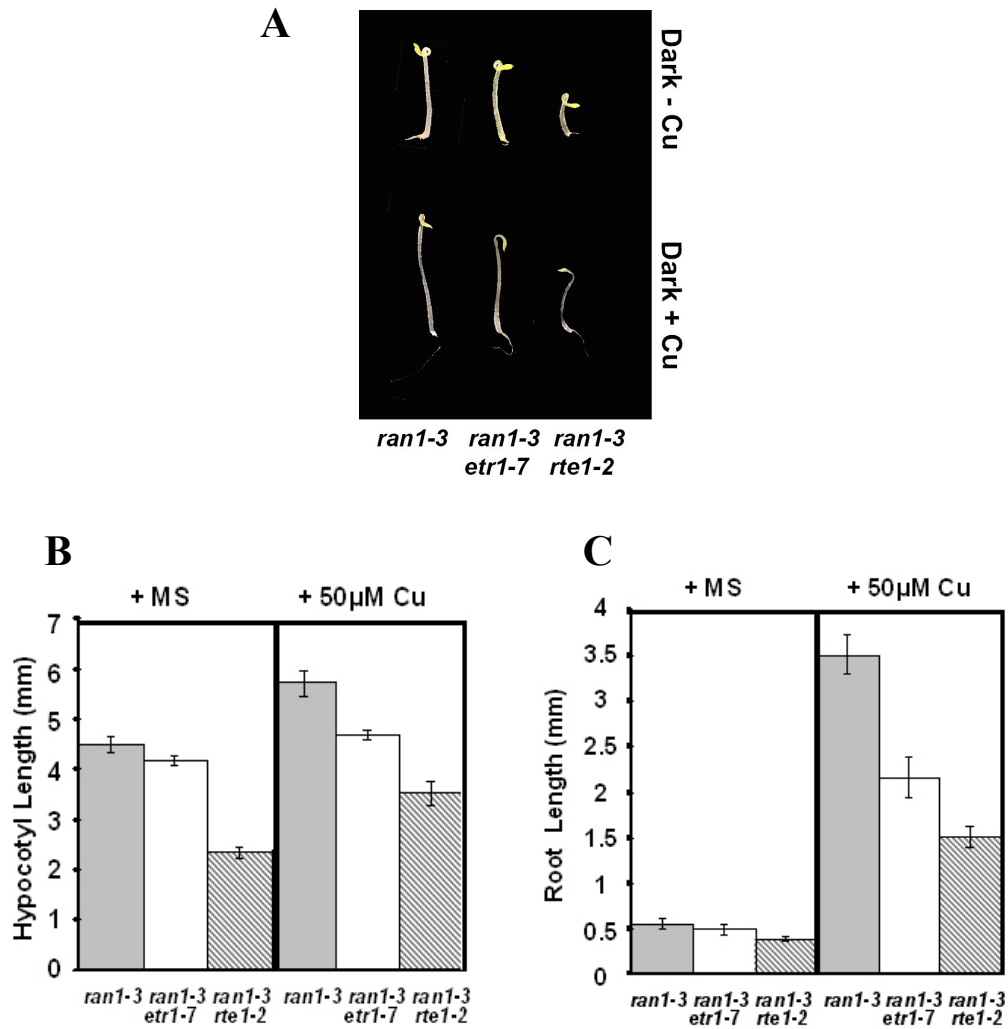
Figure 4-4. *rte1-2* enhances the phenotype of *ran1-3*.

(A) Representative four-day old dark grown seedlings of *ran1-3, ran1-3 etr1-7* and *ran1-3 rte1-2* grown in the dark with and without 50μM CuSO$_4$.

(B) & (C) Measurements of hypocotyl and root lengths for *ran1-3, ran1-3 etr1-7* and *ran1-3 rte1-2* with and without copper treatment. For each treatment, the mean +/- SE is shown for 10-15 seedlings per genotype.

binding of a copper molecule to ETR1, since if RTE1 is gone, even saturation with copper would make no difference if there is no way for it to associate with ETR1. However, additional experiments described here do not support a role for RTE1 in a copper-trafficking pathway, so it is unlikely that this is the reason *rte1-2 ran1-3* is more severe than *ran1-3* alone.

ran1-3 cannot suppress the insensitivity of the *etr1-2* insensitive mutant

Under normal wild-type conditions, copper associates with the ethylene receptors; a step that is believed to induce a conformational change essential not only for the binding of ethylene (Bleecker et al., 1998; Wang et al., 2006), but also for the receptor to be actively transmitting signals that repress ethylene responses. Loss of *RAN1* results in a severe constitutive response, indicating that the receptors are not only unable to bind ethylene, but are essentially non-functional. In *rte1* loss-of-function mutants, ETR1 is largely non-functional, presenting a possible parallel with *ran1* mutants. Although *RAN1* acts upstream of the receptors, it is interesting that *ran1-3* crossed to the strong insensitive mutant *etr1-3* retains dominant insensitivity in dark-grown seedlings (Woeste and Kieber, 2000). Earlier analysis revealed that *rte1-2* is unable to suppress the strong insensitive mutant *etr1-1*, which is interesting since *RTE1* was isolated as a suppressor of *etr1-2*. One difference between these two alleles is an ability of ETR1-2 to still bind ethylene (Bleecker et al., 1998; Wang et al., 2006), which implies that copper is also associated with the receptor, as in wild-type ETR1 receptors. A C65Y mutation in the ETR1-1 receptor inhibits ethylene binding presumably through disruption of the copper-binding pocket (Rodriguez et

al., 1999). *etr1-1* therefore confers strong insensitivity regardless of copper or ethylene binding.

We wanted to test whether suppression of *etr1-2* by *rte1* loss-of-function mutants is the result of copper deficiency, i.e. whether *etr1-2* is dependent on the copper availability/ethylene binding in order to confer insensitivity. To do this, we constructed and analyzed a *ran1-3 etr1-2* double mutant. This would also address whether loss of functional wild-type ETR1 in *rte1* mutants is due to a loss of copper from the receptor.

Analysis of *ran1-3 etr1-2* double mutants revealed that the insensitivity exhibited by *etr1-2* is not dependent on the availability of copper. *ran1-3 etr1*-2 exhibited the same degree of insensitivity as the *etr1-2* single mutant in dark-grown seedlings grown on ACC (Fig. 4-5A). Although *ran1-3* mutants exhibit a severe constitutive triple response, they are still able to exhibit a slight response to ethylene (data not shown), indicating that the receptors may retain some slight functionality. In order to ensure that residual copper was not a factor, 10μM neocuproine was also added to the media. Responses in dark-grown seedlings were phenotypically similar to *etr1-2* alone (Fig. 4-5B), thus confirming that *etr1-2* is not dependent the presence of copper to confer insensitivity.

Discussion

The presence of conserved cysteine and histidine residues, along with possible copper binding motifs (including a non-conserved MXCXXC motif present in the *Green-Ripe* homolog) raised the possibility that *RTE1* was involved in binding a
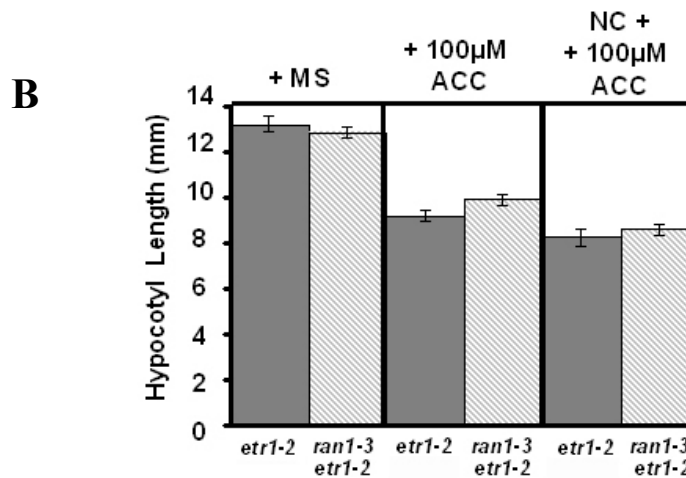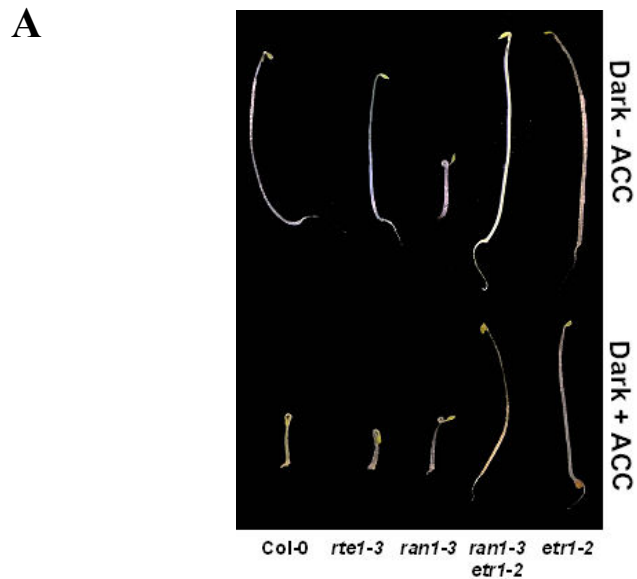
Figure 4-5. The ethylene insensitivity of *etr1-2* is not suppressed by *ran1-3*.

(A) Representative four-day old dark-grown seedlings of wild type (Col-0), *rte1-3,*

ran1-3, ran1-3 etr1-2 and *etr1-2* with and without ethylene treatment.

(B) Measurements of hypocotyl length for *etr1-2* and *ran1-3 etr1-2* grown on MS,

100μM ACC and 10μM neocuproine + 100μM ACC. For each treatment, the mean

+/- SE is shown for 10-20 seedlings per genotype.

copper molecule. It is likely that at least one of the conserved cysteine residues is functionally important since the *rte1-1* loss of function allele is the result of a point mutation at cys[161]. This hypothesis fits with our initial genetic data: loss of *RTE1* results in a non-functional ETR1 receptor, as does loss of the copper transporter RAN1. However, further genetic analysis indicated that *RTE1* is unlikely to be involved in facilitating the binding of an essential copper cofactor to the ETR1 receptor. *rte1-2* seedlings grown in the dark on either copper or the copper chelator neocuproine displayed no significant change in either hypocotyl or root lengths. If RTE1 were important for facilitating copper binding to ETR1, it would be likely that a certain degree of alleviation would be exhibited when *rte1-2* was grown on a high concentration of copper. However, it is possible that alleviation even on high doses of copper cannot occur when RTE1 is absent if it is essential for copper binding to ETR1. This is supported in the *rte1-2 ran1-3* double mutant, which is more severe than *ran1-3* alone, and when grown on a high concentration of copper exhibits much weaker rescue of hypocotyl and root lengths. However, since *ETR1* is a key receptor in the ethylene-signaling pathway, we also see that loss of this receptor alone is sufficient to partially inhibit rescue of *ran1-3* by excess copper. The finding that *rte1-2 ran1-3* double mutants are alleviated to an even lesser degree than *etr1-7 ran1-3* mutants might indicate that *RTE1* does to some extent regulate the other ethylene receptors. Since loss of both *RAN1* and *RTE1* together results in such a severe response, it does support the possibility that RTE1 is involved in a copper trafficking pathway. However, additional genetic and biochemical analyses did not reinforce this

hypothesis. This phenotype might also be considered additive, indicating that perhaps *RTE1* and *RAN1* act in parallel pathways upstream of *ETR1*.

Overexpression of *RTE1* in the *ran1-3* null mutant was unable to rescue the severe constitutive phenotype seen in *Arabidopsis* seedlings, even when an excess of copper is added to the media. Since we know that overexpression of RTE1 in both wild-type and null mutants confers weak insensitivity, if this was due to RTE1 activating more receptors through an increase in copper loading, then we would expect to see some degree of rescue of the *ran1-3* phenotype when *RTE1* is overexpressed. This indicates that overexpression of *RTE1* is unlikely to confer insensitivity through an increase in copper loading to ETR1.

The hypothesis that *RTE1* might be involved in facilitating copper binding was based on sequence analysis and a possible parallel between *rte1* and *ran1* loss of-function mutants. *rte1-2* mutants cannot suppress the strong insensitive mutant *etr1-1*; and similarly, *ran1-3* cannot suppress the insensitivity of *etr1-3* (which has similarity to *etr1-1*). However, *rte1-2* can suppress the insensitivity of *etr1-2*, which is a weaker mutant than *etr1-1*, and retains copper and ethylene binding capabilities (whereas *etr1-1* does not). Our hypothesis centered upon the speculation that *etr1-2* might require copper to be associated with the receptor in order to confer insensitivity. We know that *etr1-1* is unable to bind copper, so insensitivity in this case is copper-independent. Wild-type ETR1 receptors are non-functional in both *ran1* and *rte1* loss-of-function mutants; and ETR1-2 receptors are also non-functional when *RTE1* is absent. However, our results showed that *etr1-2* mutants are still insensitive in a *ran1-3* background, i.e. when no copper is available for ETR1-2, it is still able to

confer insensitivity. This indicates that our model in this case is incorrect, and combined with other data described here implies that *RTE1* is most likely not involved in facilitating the binding of copper ions to the ETR1 receptor.

In addition, some biochemical analyses were carried out to help assess whether RTE1 can associate with copper molecules, or is likely to be involved in the copper-trafficking pathway (described in Appendix A). This includes flame atomic absorption spectrometry and a complementation analysis of the yeast copper trafficking mutant Δccc2. The results of these experiments also pointed away from a copper-binding role for *RTE1*.

Materials and Methods

Plant Growth Conditions

Plant strains and growth conditions were as described in Chapter 2. For the copper response assays, seedlings were germinated on MS medium containing 50 M copper sulfate ($CuSO_4$) or 10 M neocuproine as appropriate.

Transgenic Constructs and Plant Transformation

For *RTE1* overexpression in a *ran1-3* background, constructs and transformation protocol was as described in Chapter 2. Adult plants to be transformed were confirmed to be heterozygous for the *ran1-3* mutation by genotyping.

Homozygous *ran1-3* plants are lethal at the rosette stage, so are not viable for transformation. Positive transformants were selected using BASTA and were genotyped for *ran1-3*. Five independent T3 transgenic lines homozygous for CaMV

35S:*RTE1* and heterozygous for *ran1-3* were analyzed. Seedlings exhibiting a *ran1-3* phenotype were examined (segregating as three-fourths of the total population), and selected using BASTA to confirm the presence of *35S*:*RTE1*.

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 (the *ran1-3* mutation encodes Gly759–Arg (Woeste and Kieber, 2000)). F_1 plants confirmed by genotyping to be heterozygous for *ran1-3* were allowed to self, and double mutants were identified in the F_2 generation by genotyping. For both *rte1-2 ran1-3* and *etr1-2 ran1-3* double mutants, lines are maintained as heterozygous for the *ran1-3* locus, due to adult lethality in both cases.

Genotyping markers for *etr1-2* and *rte1-2* are as described in Chapter 2. The *ran1-3* allele was detected by CAPS, using the primers (5'-CTCAAGGAACTACTG TG-3') and (5'- CTACGGAGACCTTCCAC'3') to amplify a DNA fragment that is cleaved by the restriction enzyme *Hph*I if the fragment if from *ran1-3*, but not from the wild-type RAN1 allele.

Chapter 5: *RTE1* may be important for the regulation of signal transmission within the ETR1 receptor

Introduction

The genetic analyses described in Chapter 2 implicate *RTE1* as a regulator of *ETR1*: loss of *RTE1* in an *etr1-2* ethylene-insensitive background results in a wild-type phenotype, and experimental data indicates that this is probably due to the ETR1 receptor being largely non-functional. This suggests a close regulatory relationship exists between *ETR1* and *RTE1* – but the full nature of this relationship and the functional role of *RTE1* are still unknown.

A recent study carried out by Wang et al. (Plant Cell, in press) indicates that a complex mechanism involving both ethylene binding and conformational changes are responsible for regulating signal transmission within the ETR1 receptor. Multiple residues in the transmembrane region of the receptor were shown to be essential for functionality of the receptor, some forming an ethylene-binding pocket, and others being required for an essential conformational shift that results in signal transmission to the 'transmitter domain' (comprising the GAF domain through the receiver domain) (Fig. 5-1). When the transmitter is 'on', the receptor is actively signaling to repress ethylene responses. Inactivation of the transmitter allows signaling to downstream components to progress, and ethylene-response genes to be transcribed. This data supports a model where the receptor passes through a 'quasi-stable' state occurring after ethylene binding but before receptor inactivation. Under normal conditions, when no ethylene is bound, the transmitter domain of the receptor is 'on'.

ETR1 ethylene binding domain

Important for ethylene binding

Important for conformational changes

Unspecified importance, but possibly important for conformational changes

Important for transmission of signal to the cytoplasmic 'transmitter' domain
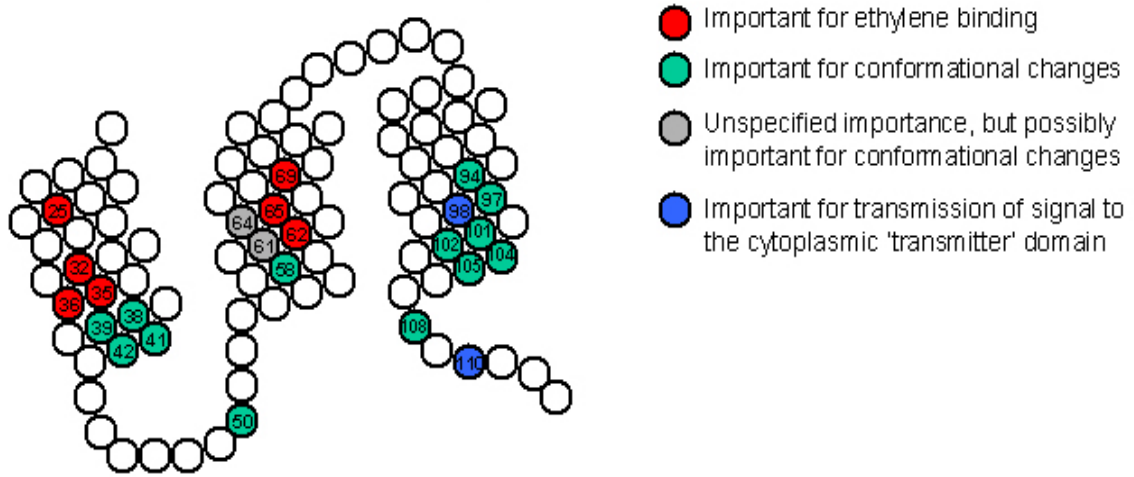
Figure 5-1. The ethylene-binding domain of ETR1.

Representation of the transmembrane helices that comprise the ethylene-binding domain of the ETR1 receptor. Four classes of residues as designated by Wang et al. (2006) are shown, represented by different colors.

Mutations in residues that form the ethylene-binding pocket inhibit ethylene binding, thus causing insensitivity. These residues are annotated as red in Figure.5-1. Ethylene binding triggers conformational changes that ultimately turn the transmitter 'off'. Mutations in any residue important for this conformational change lock the receptor in an 'on' state, since the transmitter cannot be inactivated, and so also results in ethylene insensitivity. These residues are annotated as green in Figure 5-1. Two additional residues are also important for the activation/inactivation of the transmitter domain; mutations in these two residues cause the receptor to be non-functional (in a persistent 'off' conformation), indicating that when the ethylene-binding region cannot regulate the transmitter domain, its natural state is to be 'off '. These residues are annotated as blue in Figure 5-1.

The close relationship between copper binding, ethylene binding and conformational changes paints a complex picture of how the N-terminal transmembrane region of ETR1 transmits a signal to the C-terminal cytoplasmic 'transmitter' domain. As a regulator of *ETR1*, these factors may be important when assessing how *RTE1* is likely to function.

One question that has remained is why *rte1-2* is able to suppress the ethylene-insensitive mutant *etr1-2*, but not *etr1-1*. The differences between these two alleles may provide insight into how RTE1 regulates ETR1. There are two main differences between these two alleles. Firstly, the *etr1-2* encoded receptor retains the ability to bind ethylene, but *etr1-1* encodes a mutation in the ethylene-binding pocket that prevents ethylene binding. Secondly, *etr1-2* is a weak insensitive mutant, exhibiting some responsiveness to high concentrations of ethylene, whereas *etr1-1* is completely

ethylene-insensitive (Hall et al., 1999). Assessing which of these differences are important determinants in whether *rte1-2* suppresses the insensitivity may help in elucidating the molecular function of *RTE1*. In order to address this, it is important to look at additional insensitive alleles and whether they are suppressed by *rte1-2*. The study by Wang et al. provides us with not only a variety of *etr1* insensitive alleles, but also detailed information regarding those alleles; including strength of insensitivity, ethylene-binding capabilities, and a working hypothesis depicting the roles played by individual residues.

Sub-cellular localization of RTE1 may also help shed light on the mechanism through which RTE1 acts to regulate the ETR1 receptor. Since RTE1 is a membrane-bound protein, we would expect it to co-localize with ETR1, which appears to be localized to the endoplasmic reticulum (Chen et al., 2002). This would support regulation of ETR1 via a physical interaction. However, if RTE1 is involved in the post-translational modification or localization of ETR1, it might localize to the Golgi apparatus, where modifications including the binding of copper are believed to occur. If RTE1 is shown to localize to a different membrane than the ER or Golgi apparatus, then how RTE1 acts to regulate ETR1 might need to be re-assessed.

Results

rte1-3* is able to suppress the double mutant *etr1-2 ran1-3

The double mutant *etr1-2 ran1-3* was initially created to study whether loss of copper from the ETR1-2 receptor affected its ability to confer ethylene insensitivity. This was described in Chapter 4, and indicated that the dominant insensitivity

observed in *etr1-2* mutants is copper independent. Since this mutant has reduced

copper availability, it also follows that ethylene binding is impaired in this mutant –

since copper is essential for the association of ethylene with the receptor. By

inhibiting the ability of ethylene to bind to ETR1-2, we can assess whether this is an

important difference between the *etr1-1* and *etr1-2* insensitive mutants with regards

to suppression by *rte1* mutants. Interestingly, the *rte1-3 etr1-2 ran1-3* triple mutant is

phenotypically similar to *ran1-3*, indicating that *rte1-3* can still suppress *etr1-2* even

when no copper or ethylene is associated with the receptor (Fig. 5-2A) (it also

confirms that loss of *RTE1* results in a non-functional ETR1-2 receptor). This result is

interesting since it was expected that *etr1-2 ran1-3* would act like *etr1-1*, because the

weak insensitivity exhibited by *etr1-2* was thought to be due to the ability of *etr1-2* to

still bind ethylene. However, when grown alongside *etr1-1*, it is apparent that the

ran1-3 etr1-2 double mutant is not like *etr1-1*: even when ethylene binding is

inhibited, *etr1-2* acts like a weak insensitive mutant (Fig. 5-2B). This indicates that

etr1-2 probably confers weak ethylene insensitivity as the result of a conformational

change in the receptor that disrupts inactivation of the ETR1 transmitter.

rte1-2 is able to suppress both weak and strong insensitive *etr1* mutants

In order to obtain a clearer picture of how *RTE1* regulates *ETR1*, and whether

suppression by *rte1* is likely to be dependent on the conformation of the receptor, we

next assessed whether *rte1-2* could suppress other *etr1* insensitive alleles. We tested

eleven insensitive mutant transgenes, the result of point mutations created through

Figure 5-2. *rte1-3* **is able to suppress the** *etr1-2 ran1-3* **double mutant.**

(A) Comparison of four-day old dark grown seedlings grown in the presence and absence of 100μM ACC. Representative seedlings of *ran1-3 etr1-2 rte1-3* are shown, which exhibit the triple response on ACC. In contrast, the *ran1-3 etr1-2* exhibits insensitivity. Representative seedlings of control genotypes are also shown: Col-0*; rte1-2; ran1-3; etr1-2; etr1-1; and rte1-3 etr1-2.* (B) The *etr1-2 ran1-3* double mutant does not exhibit strong insensitivity to 100μM ACC, as is seen in *etr1-1*. The mean +/- SE is shown for 12-19 seedlings per genotype.

A

Dark – ACC

Dark + ACC

Col-0   rte1-3   ran1-3   ran1-3   etr1-2   etr1-1   rte1-3   ran1-3
                          etr1-2   etr1-2   etr1-2
                                                                    rte1-3

B

+ MS   + 100μM ACC

Hypocotyl Length (mm)

etr1-2   etr1-2   etr1-1   etr1-2   etr1-2   etr1-1
ran1-3   ran1-3
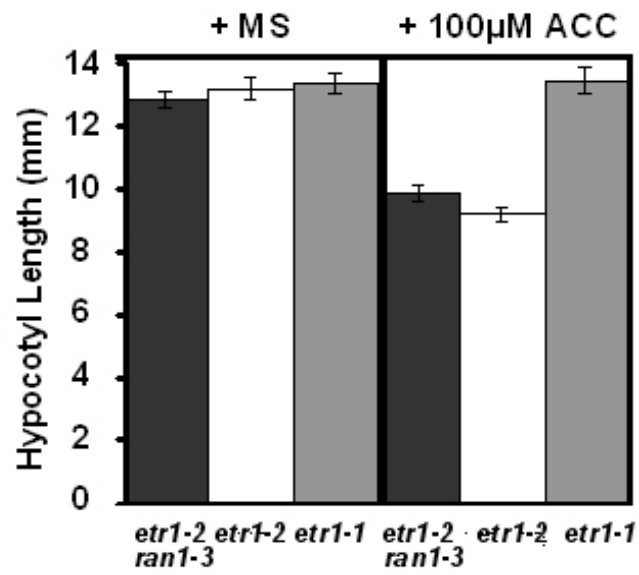
site-directed mutagenesis (a gift from Dr. Tony Bleecker's lab). All eleven mutants

carried point mutations in the ethylene-binding transmembrane region, where

conserved residues were replaced with alanine. Mutants were selected that were

representative of both weak and strong mutants, exhibiting varying degrees of

ethylene-binding capabilities, as assayed by Wang et al. (2006) in a transgenic yeast

system. Eight strong mutants (D25A, Y32A, E38A, F58A, T94A, T101A, L105A,

I108A) and three weak mutants (F61A, L64A and M104A) were tested. Including

etr1-2 (A102T) and *etr1-1* (C65Y), a total of nine strong and four weak mutants were

tested for suppression by *rte1- 2* (Fig. 5-3). According to Wang et al., three of the

residues tested are important for the formation of the ethylene-binding pocket, eight

for conformational changes, and two have unspecified functions (Fig. 5-1). In the

previous study of these mutants, transgenes were transformed into an *etr1 etr2 ein4*

triple receptor null mutant to test for rescue of *etr1* as well as degree of insensitivity

(Wang et al., 2006). In order to assess the insensitivity of these mutants in a wild-type

background, and to confirm the degree of insensitivity, we transformed these

transgenes into Col-0 as well as *rte1-2* plants. The degree of insensitivity for each

mutant transgene was measured on 10μM and 50μM, or 50μM and 100μM ACC,

depending on whether they were previously annotated as being weak or strong

mutants. All lines were measured on 0μM ACC (MS alone) (Table 5-1). Transgenic

mutants were considered to be strong mutants if the response on the highest dose of

ACC was more than 75% of that seen on MS alone (Tables 5-1, 5-2). Mutants were

considered to be suppressed by *rte1-2* if they measured less than half that of the
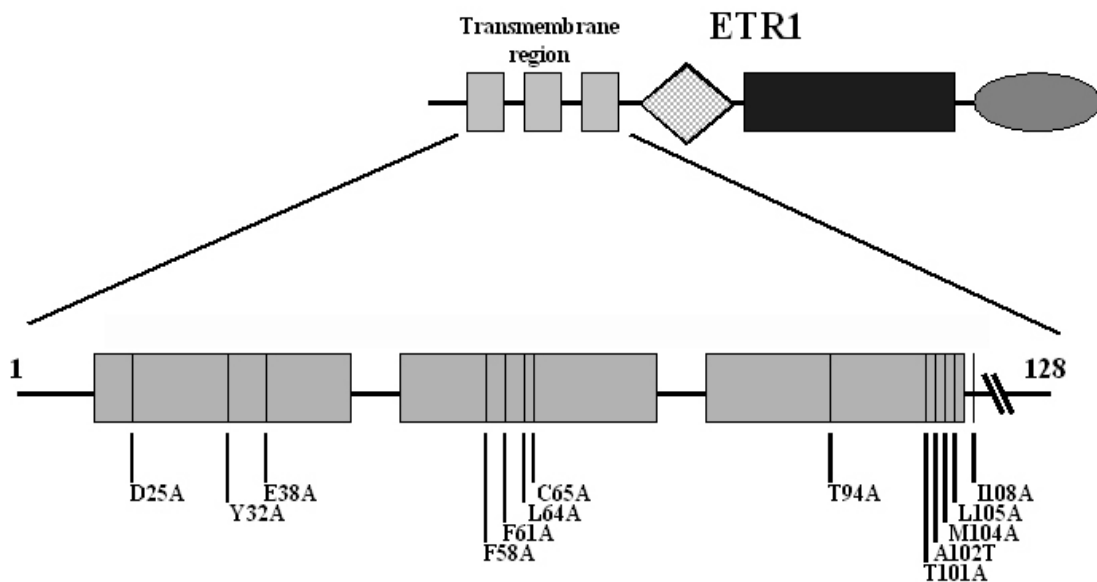
Figure 5-3. Positions of mutations tested for suppression by *rte1-2*.

A representation of the three transmembrane regions within the ETR1 receptor. Bars

represent the approximate location of mutations that were transformed into wild type

and *rte1-2* mutants to test for insensitivity and suppression. In addition to C65Y (*etr1-*

1) and A102T (*etr1-2*), 11 transgenes were tested that contained alanine substitutions

in the residues shown.

Table 5-1. Hypocotyl lengths of dark-grown seedlings on MS with and without different doses of ACC.

Average Hypocotyl Length (mm)

Genotype	MS	10µM ACC	50µM ACC	100µM ACC
Col-0	11.44 +/- 0.61	4.08 +/- 0.51	4.00 +/- 0.40	3.98 +/- 0.45
rte1-2	7.95 +/- 0.62	2.40 +/- 0.30	2.51 +/- 0.27	2.25 +/- 0.23
ers1-10	12.58 +/- 0.81	9.59 +/- 0.97	7.04 +/- 0.67	6.42 +/-0.79
etr1-1	12.19 +/- 0.97	12.51 +/- 0.64	12.83 +/- 0.97	12.53 +/- 0.77
etr1-2	11.39 +/- 1.19	9.67 +/- 0.75	8.98 +/-0.63	8.41 +/- 0.34
rte1-2 etr1-2	8.33 +/- 0.78	3.94 +/- 0.53	3.27 +/- 0.47	3.06 +/- 0.34
rte1-2 etr1-1	11.50 +/- 1.31	11.63 +/- 1.02	11.61 +/- 1.02	11.24 +/- 0.94
D25A Col-0	11.32 +/- 0.91		10.01 +/- 1.34	9.88 +/- 1.32
D25A *rte1-2*	9.50 +/- 1.43		8.51 +/- 1.09	7.48 +/- 1.31
Y32A Col-0	11.49 +/- 0.71		12.24 +/- 1.54	11.82 +/- 1.17
Y32A *rte1-2*	10.15 +/- 1.10		5.39 +/- 0.59	4.89 +/- 0.63
E38A Col-0	10.84 +/- 1.26		10.34 +/- 1.10	9.78 +/- 1.08
E38A *rte1-2*	8.86 +/- 1.08		4.39 +/- 0.67	2.61 +/- 0.70
F58A Col-0	10.60 +/- 1.51		9.64 +/- 1.10	9.69 +/- 1.56
F58A *rte1-2*	8.33 +/- 0.81		4.32 +/- 0.47	4.12 +/- 0.48
F61A Col-0	10.21 +/- 0.91	8.61 +/- 0.96	7.20 +/- 1.10	
F61A *rte1-2*	9.53 +/- 0.94	3.17 +/- 0.49	3.08 +/- 0.54	
L64A Col-0	10.49 +/- 1.15	7.47 +/- 0.91	6.45 +/- 0.76	
L64A *rte1-2*	9.38 +/- 0.93	3.83 +/- 0.63	3.11 +/- 0.89	
T94A Col-0	12.32 +/- 1.09		12.42 +/- 1.29	12.51 +/- 1.23
T94A *rte1-2*	11.50 +/- 0.99		11.69 +/- 1.06	11.68 +/- 1.03
T101A Col-0	10.97 +/- 1.18		11.21 +/- 1.23	10.84 +/- 1.21
T101A *rte1-2*	10.83 +/- 0.82		11.15 +/- 1.37	10.11 +/- 0.92
M104A Col-0	10.84 +/- 0.91	8.23 +/- 1.12	7.70 +/- 0.96	
M104A *rte1-2*	9.79 +/- 1.22	3.45 +/- 0.54	3.62 +/- 0.37	
L105A Col-0	10.37 +/- 0.96		11.06 +/- 1.09	10.04 +/- 1.14
L105A *rte1-2*	10.13 +/- 1.08		10.22 +/- 1.64	8.98 +/- 1.79
I108A Col-0	10.15 +/- 0.82		9.58 +/- 1.08	9.61 +/- 1.22
I108A *rte1-2*	9.94 +/- 0.83		8.01 +/- 1.10	7.79 +/- 0.83

Lengths are representative of the mean of 2-4 independent transgenic lines +/- SD. Standard deviation is representative of all individuals measured across independent lines.

Table 5-2. Summary of the effects of different *etr1* mutations in wild type and

rte1-2.

Mutation	Ethylene Binding *	Strength of mutant **	Suppressed by *rte1-2*? [§]
E38A	155%	Strong (90%)	Yes (27%)
etr1-2 (A102T)	150%	Weak (74%)	Yes (36%)
Y32A	<5%	Strong (100%)	Yes (41%)
F58A	110%	Strong (91%)	Yes (42%)
F61A	20%	Weak (70%)	Yes (42%)
M104A	90%	Weak (71%)	Yes (47%)
L64A	110%	Weak (61%)	Yes (48%)
D25A	0%	Strong (87%)	No (76%)
I108A	70%	Strong (94%)	No (81%)
L105A	50%	Strong (97%)	No (89%)
etr1-1 (C65Y)	0%	Strong (100%)	No (90%)
T94A	110%	Strong (100%)	No (93%)
T101A	50%	Strong (99%)	No (93%)

* Approximate degree of ethylene binding compared to wild-type (Wang et al., 2006).
** % of hypocotyl length on MS relative to that on the highest dose of ACC (Col-0 background).
§ % of hypocotyl length in *rte1-2* relative to that in Col-0 background on the highest dose of ACC.

equivalent transgene in a Col-0 background, on the highest dose of ACC (Tables 5-1, 5-2). Control measurements were taken of Col-0 alone, *rte1-2, rte1-2 etr1-2*, the strong insensitive mutant *etr1-1*, and the weak insensitive mutants *etr1-2* and *ers1-10,* all of which responded as expected (Table 5-1 and Fig. 5-4A).

rte1-2 was able to suppress six out of the eleven additional insensitive *etr1* mutant transgenes analyzed (Table 5-2 and Fig. 5-4B). When we include *etr1-2* and *etr1-1* to our results, this totals seven suppressed and six non-suppressed insensitive *etr1* mutants. Interestingly, mutants shown to be suppressed constituted both weak and strong mutants, exhibiting wide variations in ethylene-binding capabilities, from close to zero ethylene binding (Y32A) to more than 150% binding (*etr1-2* and E38A).

rte1-2 was found to suppress mostly mutated residues predicted to be important for conformational changes (E38A, F58A, A102T (*etr1-2*) and M104A), as well as two with unspecified function (F61A, L64A), and one predicted to be a part of the ethylene-binding pocket (Y32A). D25A and C65Y (*etr1-1*) are also predicted to be important for formation of the ethylene-binding pocket and are not suppressed by *rte1-2*. These mutants are unable to bind ethylene, and result in strong ethylene insensitivity. T94A, T101A, L105A and I108A are also strong ethylene-insensitive mutants that are not suppressed by *rte1-2*, and are implicated as being important for conformational changes, since they can all bind ethylene to some degree (Wang et al, 2006 and Table 5-2). In addition, *rte1-2* was unable to suppress the mutation I35F in the Landsberg *erecta* background, as exhibited during mapping of the *rte1* locus (Resnick et al., 2006). In Col-0, an I35A mutation causes strong ethylene insensitivity and eliminates ethylene binding (Wang et al., 2006), implicating I35 as an important

Figure 5-4. Effects of *etr1* transgenes on wild-type and *rte1-2* seedlings.

(A) Measurements of the hypocotyl length of six control genotypes: Col-0, *etr1-1*, *etr1-2*, *ers1-10*, *rte1-2* and *rte1-2 etr1-2* grown for four days in the dark on MS with or without different doses of ACC. (B) Graphic representation of the effect of *etr1* mutant transgenes on Col-0 and *rte1-2*. Hypocotyl lengths of four-day old dark-grown seedlings on 50μM ACC are shown. All hypocotyl lengths are representative of those shown in Table 5-1.
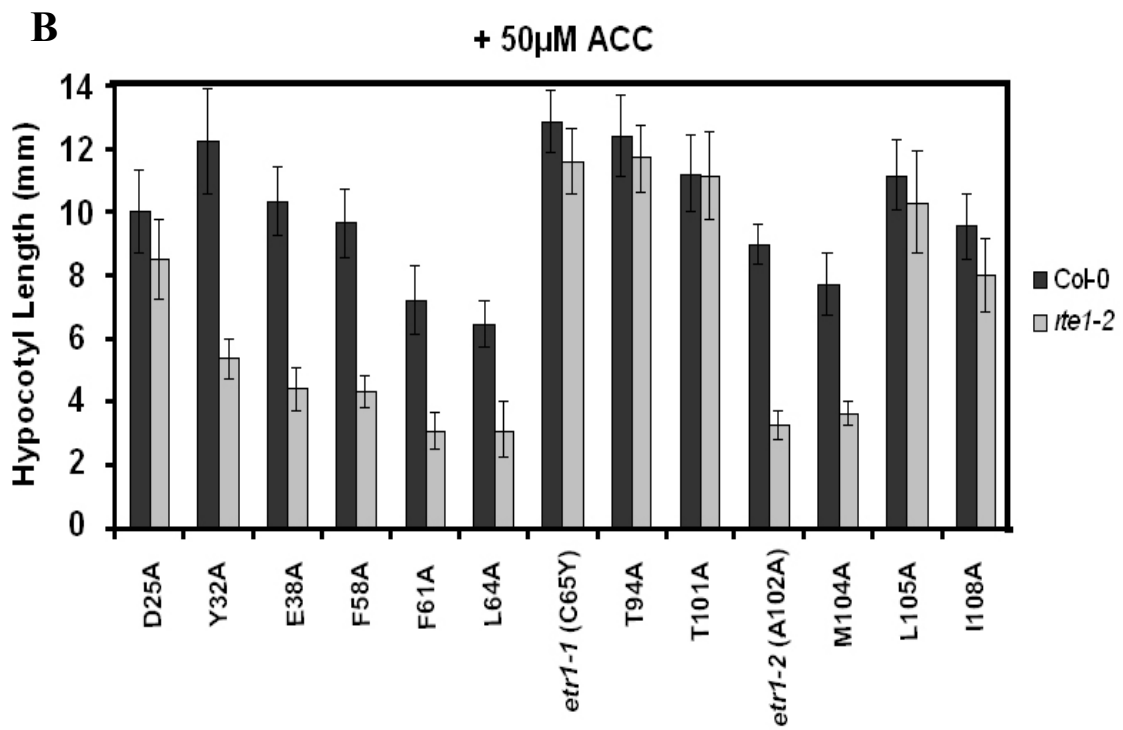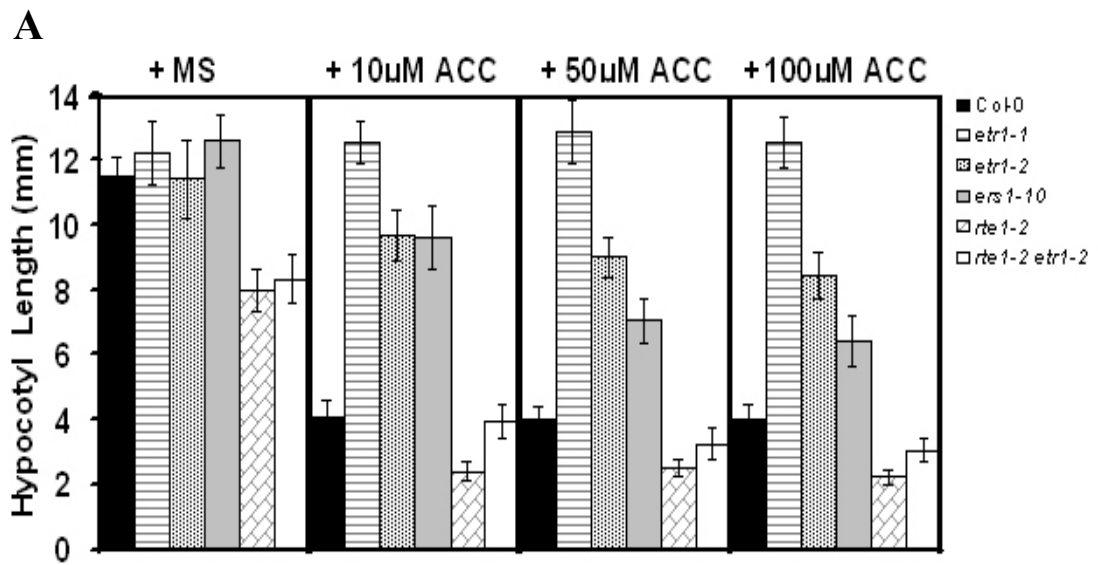
constituent of the ethylene-binding pocket.

Suppression by *rte1-2* therefore does not fall into one tidy category. Rather, *rte1-2* is able to suppress both weak and strong mutants, some of which retain the ability to bind ethylene, and others that have greatly decreased binding capabilities. These mutants span the 'red' (ethylene-binding), 'green' (conformational) and gray (uncharacterized) categories as defined by Wang et al. (2006) (Fig. 5-1). Residues within these categories that are suppressed are depicted as purple in Figure 5-5.

RTE1 probably localizes to the endoplasmic reticulum

Suppression of multiple *etr1* insensitive alleles by *rte1-2* raises the possibility that *RTE1* regulates *ETR1* through a physical interaction. It is known that ETR1 localizes to the endoplasmic reticulum (Chen et al., 2002), and if RTE1 is also shown to be present in this membrane, it would support this hypothesis.

To visualize the sub-cellular location of RTE1, we fused Red Fluorescent Protein (RFP) to both the N and C terminus of RTE1, using the native promoter and terminator of *RTE1*. To test for functionality of both chimeric proteins, we looked for complementation of the *rte1-3 etr1-2* double mutant, which should exhibit ethylene insensitivity in dark-grown seedlings if the construct is functional. The RFP-RTE1 (NT-tag) construct exhibited a greater degree of insensitivity than RTE1-RFP (CT-tag) (Fig. 5-6A), indicating that this construct is largely functional, and an accurate localization analysis can be carried out by visualizing this protein at a cellular level. Stable transgenic lines expressing RFP-RTE1 were created, and crossed to stable lines expressing a GFP marker to the endoplasmic reticulum to enable co-localization
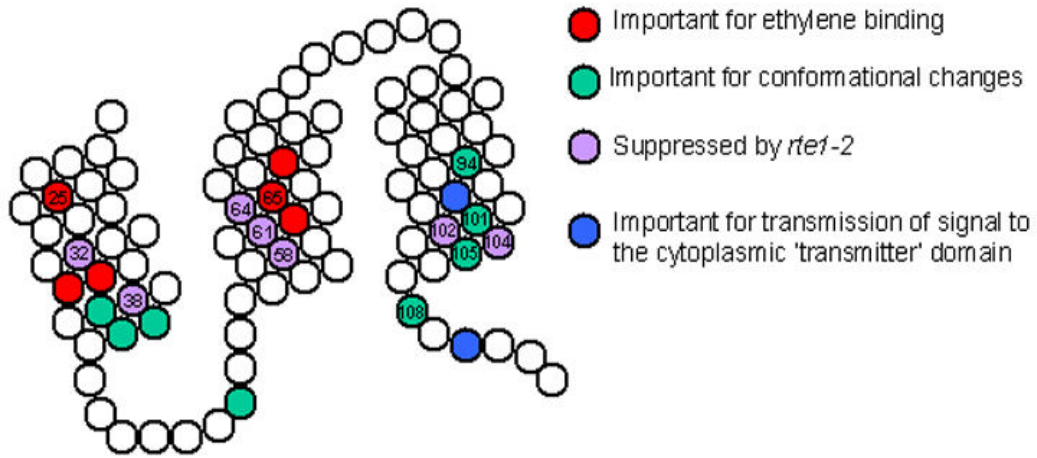
Figure 5-5. Categorization of residues in the ETR1 ethylene-binding domain important for signal transmission.

A representation of the three transmembrane domains of ETR1 depicting the categories of residues important for signal transmission as defined by both the study carried out by Wang et al. (2006) and from the *rte1-2* suppression analysis. Residues depicted in purple are suppressed by *rte1-2* and are predicted to be important for the *RTE1*-dependent regulation of ETR1. Residues that are not numbered were not tested for suppression by *rte1-2*.

Figure 5-6. RFP-RTE1 is a biologically functional protein that probably localizes to the endoplasmic reticulum.

(A) *RFP-RTE1* is able to significantly complement the *etr1-2 rte1-3* double mutant, since it confers ethylene insensitivity to 100μM ACC. *RTE1-RFP* is not able to complement to the same degree, indicating that the construct is likely to be less functional. Representative four-day old dark-grown seedlings on 100μM are shown. (B) Co-localization of RFP-RTE1 in root cells with an ER-GFP marker. Individual imaging of the marker construct and RFP-RTE1 are shown, along with an overlap of both.

of these two proteins. From preliminary confocal microscopy analysis of root cells from F_1 seedlings it does appear that RTE1 localizes to the endoplasmic reticulum (Fig.5-6B). This preliminary analysis was carried out with the help of Dr. Chun-Hai Dong, who will continue with this work to confirm localization, using additional markers to assess whether RTE1 is also likely to be present in other membranes.

Discussion

Elucidating how *RTE1* functions to regulate *ETR1* is important, not only to help us understand its role in the ethylene-signaling pathway, but also on a larger scale to elucidate the function of *RTE1* homologs.

The question as to why *rte1-2* could suppress the insensitivity of *etr1-2* but not *etr1-1* addressed the possible mechanism of how RTE1 might function. Since loss of *RTE1* is also believed to affect wild-type ETR1 receptors (as discussed in Chapter 2), the properties exhibited by the ETR1-1 receptor makes it somehow 'immune' to suppression by *rte1-2*. ETR1-1 differs from ETR1-2 in that ETR1-2 retains the ability to bind ethylene and is a weaker mutant, exhibiting a small degree of responsiveness to high concentrations of ethylene. By assessing the suppression of additional *etr1* insensitive mutants, we were able to carry out a more detailed analysis of the properties exhibited by suppressed and non-suppressed alleles.

Interestingly, *rte1-3* was still able to suppress the insensitivity exhibited by the *ran1-3 etr1-2* double mutant, indicating that *rte1-3* can still suppress *etr1-2* even when no copper or ethylene is associated with the receptor. In the *ran1-3 etr1-2* double mutant, the ability to bind copper and ethylene has been inhibited (since *ran1-*

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3 prevents the delivery of copper to the receptor), and so in this regard it has similarity to the *etr1-1* mutant. However, this double mutant still only conferred weak insensitivity, since it still exhibited a response to high doses of ethylene compared to *etr1-1*. This indicates that the weak insensitivity conferred by *etr1-2* is due to the conformation of the receptor, not its retained ability to bind ethylene. The weak response to ethylene that is seen in a *ran1-3 etr1-2* double mutant is therefore likely due the ability of the other receptors to function weakly: i.e., an incomplete 'gain-of-function' with regards to transmission of the insensitive signal to the other receptors. It is believed that the receptors interact with one another (Wen and Chang, unpublished), and there is some evidence to suggest that multiple receptors are present in a large ER-associated complex (G.E. Schaller, personal communication). This is probably how dominant (gain-of-function) insensitivity can occur from a single point mutation in just one of the receptors. It is likely that point mutations resulting in insensitivity cause conformational changes in the receptor that lock it in an 'on' state, which is then conferred to the other receptors through a physical interaction. It is therefore reasonable that certain conformations can cause weak insensitivity due to an incomplete conference to the other receptors: i.e. the remaining wild-type receptors retain some signaling capability.

Recent work by Wang et al. (2006) (which is described at the beginning of the chapter) revealed that mutations in different residues of the ethylene-binding domain confer varying degrees of ethylene insensitivity. Wang speculates that insensitivity caused by mutations that do not disrupt ethylene binding is due to the conformation of the receptor. In these cases, ethylene binds, but the receptor fails to undergo a

conformational change necessary for the receptor to inactivate. We can further this speculation with regards to the other (wild-type) receptors, which seemingly remain 'on' if one of the receptors is locked in such a conformation, i.e. if one receptor is locked in an 'on' conformation, the receptors are all 'on' (thus conferring ethylene insensitivity). However, there is some variability, since certain weak conformations such as that exhibited by ETR1-2 allow the other receptors to function to some degree. It is therefore of interest to look closely at the mutations in addition to *etr1-2* that are suppressed by *rte1-2*.

As represented in Table 5-2, *rte1-2* is able to suppress both weak and strong mutants, and suppression appears to be largely independent of ethylene-binding capabilities. By comparing Figure 5-1 with 5-5, we can separate out the residues that are suppressed, along with their functional importance as speculated by Wang et al. Four of the seven alleles suppressed by *rte1-2* are clearly defined by Wang et al. as being important for conformational changes within the ethylene-binding domain. Another two are of undefined function, and just one is believed to have a direct role in ethylene binding. Since suppression by *rte1-2* appears to be independent of ethylene binding, and occurs primarily in alleles with mutations in conformationally important residues, it suggests a possible role for *RTE1* in regulating structural changes within the ETR1 receptor. Wang postulates that upon ethylene binding, a conformational shift occurs in the ethylene-binding domain that is essential for transmission of signal to the transmitter domain, resulting in inactivation of the receptor. Mutations in any of the residues annotated in green in Figure 5-1 do not prevent ethylene binding, but inhibit the conformational change from taking place,

and thus 'lock' the receptor in what would normally be a 'quasi-stable' state. The degree of insensitivity conferred by these mutations varies, probably due to partial conformational changes and retention of some signaling ability in the other receptors.

The ability of *rte1-2* to suppress some but not all of the *etr1* insensitive alleles indicates there may be an additional class of residues in the ethylene-binding domain: conformational changes in these residues upon ethylene binding are sensed by RTE1, resulting in its inactivation. It is possible that *RTE1* shuts off at the point at which the conformational change occurs within the receptor, just before the transmitter is inactivated. Certain residues within the ethylene-binding domain may interact with RTE1, perhaps physically, conferring a signal for RTE1 to turn off once ethylene has bound. This adds an additional 'quasi-stable' state that the ETR1 receptor passes through just after ethylene binding, where ethylene is bound, and a conformational change takes place to inactivate RTE1 in order for signaling to progress to the transmitter. Mutations in residues that confer this signal to RTE1 cause insensitivity since RTE1 remains active, which prevents ETR1 from turning 'off'. Loss of *RTE1* function in this case suppresses the insensitivity since ETR1 has already undertaken the conformational change necessary for the 'off' signal to be transmitted. However, the receptor is still unable to function normally since RTE1 is absent and the receptor is mutated, preventing the receptor from reverting back to its active signaling state. We therefore observe a phenotype similar to an *etr1* loss-of-function mutant. Assuming that this hypothesis is correct, three classes of residues are likely to exist:

- Class A: Residues involved in ethylene binding. When mutated, ethylene cannot bind, thus locking the ETR1 receptor in state I (strongly ON), and insensitivity cannot be suppressed by *rte1-2*.

- Class B: Residues involved in conformational changes once ethylene has bound. Mutations in these residues prevent a full conformational change from occurring, even though the receptor may still bind ethylene. This locks the receptor in state II, (strongly ON), even though ethylene can bind. Insensitivity cannot be suppressed by *rte1-2*.

- Class C: Residues involved in turning RTE1 off: When mutated, the receptor is locked in state III ('ON'). Mutations in these residues prevent RTE1 from being turned off, thus preventing the full conformational change from occurring. In this case, insensitivity is RTE1-dependent (i.e. insensitivity can be suppressed by loss of *RTE1*).

A possible model incorporating a passage through these three states before the receptor can be fully inactivated in shown in Figure 5-7. In this model, RTE1 acts as a kind of 'molecular chaperone'; when RTE1 is 'on', conformational changes are inhibited, but inactivation of RTE1 allows ETR1 to shift fully into its inactive state (State IV). One possible analogy is that RTE1 acts like a 'spring-loaded' protein, such that it regulates ETR1 by holding it in a specific conformation until ethylene has bound and the conformational change in the ethylene-binding domain has taken place. The inactivation of RTE1 is the final step in the process before the transmitter domain is inactivated and the receptor proceeds to State IV (off). Such a high degree of
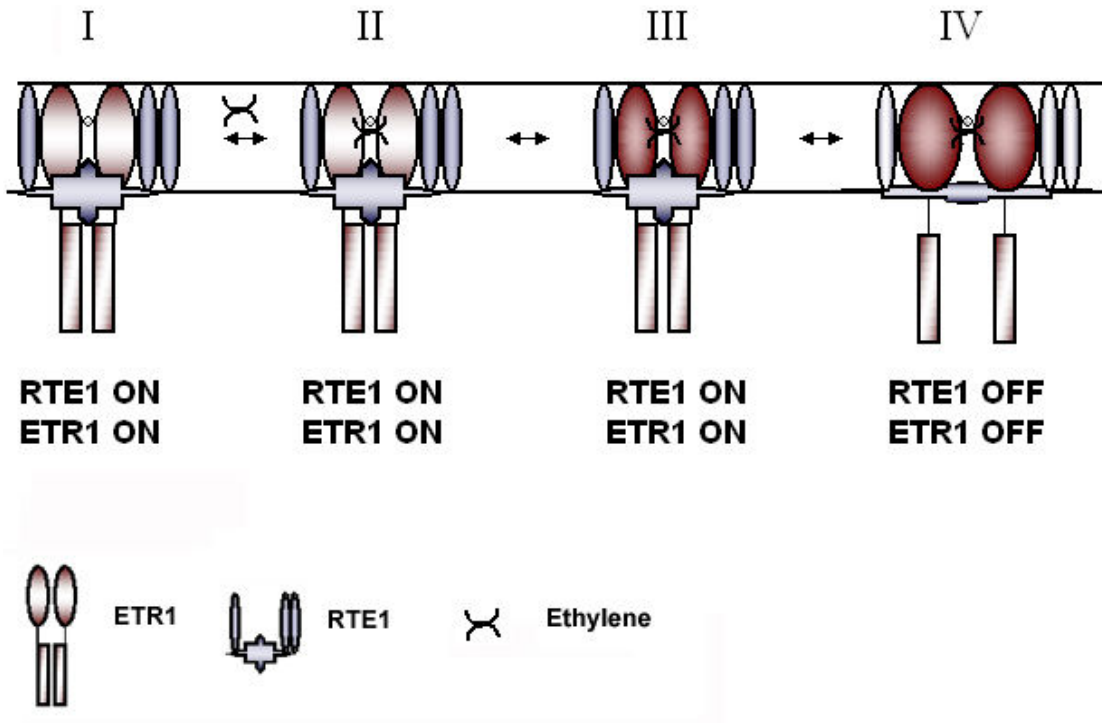
Figure 5-7. Possible model of RTE1 function.

RTE1 may act as a type of 'molecular chaperone' for RTE1, regulating the ability of the ethylene-binding domain to signal to the transmitter domain. When ETR1 is in state I, no ethylene is bound, RTE1 is on, and the receptor is signaling to repress responses. Association of ethylene with the receptor causes transition to state II: a quasi-stable state where ethylene is bound but the receptor is still on. A conformational shift at this stage, probably induced through resides including T94, T101, L105, I108 allow transition of state III. This state is also a quasi-stable state where the receptor is in a conformation consistent with being 'off', but the concomitant change in the transmitter domain cannot occur until RTE1 inactivates. Once this has occurred, signaling by the transmitter is inactivated, and the ETR1 receptor can no longer signal to repress ethylene responses (i.e. ETR1 is 'off').

regulation may prove to be important for the 'fine-tuning' of the ethylene-signaling pathway.

In addition to the three residue classes discussed is a fourth class, which is also described by Wang et al., and can be designated as class D. These residues are directly implicated in signaling to the transmitter domain (annotated as blue in Fig. 5-1), and when mutated, result in an *etr1* loss-of-function phenotype (Wang et al., 2006). This directly indicates that when signal transmission between the ethylene-binding domain and the transmitter domain is impaired, *ETR1* becomes a loss-of-function mutant. A mutation in any of the 'green residues' results in a gain-of-function insensitive phenotype, and the same can be said for any of the newly designated 'purple' residues, indicating that these residues are not directly involved in signaling by the transmitter. However, an *rte1* loss-of-function mutant results in a phenotype similar to that of an *etr1* loss-of-function mutant, indicating that it is potentially an important component of signaling through the transmitter domain. Once ETR1 loses the ability to regulate the transmitter domain, it becomes locked in state IV (off). Mutations in Class D residues result in this state, and it is possible that loss of *RTE1* has a similar effect.

One question remains with this hypothesis: why is *rte1-2* is able to suppress the Y32A mutation? Y32 is believed to be important for the formation of the ethylene-binding pocket, since the Y32A mutation prevents ethylene binding and confers strong insensitivity (i.e. we would expect Y32A to cause ETR1 to be locked in CS1). It is possible that Y32A is involved in both binding ethylene and signaling to RTE1. Alternatively, Y32A may primarily be involved in signaling to RTE1, but

this particular mutation also impedes ethylene binding, perhaps through obstruction of the binding pocket. It is also of note that the ethylene-binding assays were performed in a yeast system (Wang et al., 2006), and so it is possible that some of the calculated ethylene-binding percentages may not be fully representative of actual *in-vivo* binding in *Arabidopsis*. If this is the case, then Y32A may be incorrectly categorized.

In analyzing the suppression data collected in this study, we have taken into account the characteristics of the suppressed mutations with regards to extent of insensitivity, ethylene-binding capabilities, and the category designated to them by Wang et al. One additional point of consideration is the possible three-dimensional structure of the ETR1 ethylene-binding domain, and where these residues might fall within it. If RTE1 interacts directly with ETR1, and that occurs via the 'class C' residues (mutations in which are suppressed by *rte1-2*), then we might expect them all to lie on the same face of the protein. Since we do not yet have a 3D model of the ETR1 ethylene-binding domain, we are unable to do a detailed analysis on this level, but this will hopefully be a future possibility.

So does this model fit with previous findings? We know that ETR1 appears to be non-functional in an *rte1* loss-of-function mutant, and this model does indicate that when ETR1 cannot be regulated by RTE1, the receptor is essentially non-functional. We also saw that over-expression of RTE1 resulted in partial ethylene-insensitivity. Since ETR1 acts as a negative regulator of the pathway, when ETR1 is on, ethylene responses are inhibited. One possibility is that ETR1 protein levels are present at a steady-state level, with the rate-limiting factor being the levels of RTE1 protein. We

know that ETR1 levels are not up-regulated by ethylene (Chang et al., 2003), which may support this theory. If RTE1 levels are increased, what we might be observing is the result of an increased number of 'active' ethylene receptors, and consequent increase of receptors under various states of equilibrium; such that there are more receptors at the intermediate states I, II or III, resulting in partial insensitivity to ethylene. We also know that RTE1 levels are up-regulated by ethylene (Chapter 2 and Alonso et al., 2003). Since *RTE1* is a negative regulator of the ethylene-signaling pathway, this may seem counter-intuitive. However, this is not unique. The two F-box proteins EBF1 and EBF2 also act as negative regulators of the pathway, promoting the degradation of EIN3, and both are up-regulated by ethylene (Potuschak et al., 2003). This indicates that there is a negative feed-back mechanism modulating ethylene responses, possibly to ensure a rapid activation of the pathway once ethylene levels diminish.

Since the proposed mechanism for RTE1 action described here includes a likely physical interaction between RTE1 and ETR1, we would expect both RTE1 and ETR1 to localize to the same membrane. Preliminary localization indicates that RTE1 is likely to be present in the ER, which supports a possible functional interaction between RTE1 and ETR1. Although ETR1 localizes to the ER (Chen et al., 2002), it also probably shuttles through the Golgi since it contains integral glycosylation sites, (and this is also the likely site of association with the copper cofactor). Additional analysis therefore needs to be carried out to assess whether RTE1 also localizes to other membranes. Although this supports the possibility of a

physical interaction occurring between RTE1 and ETR1, additional research is required to demonstrate this at the molecular level.

Materials and Methods

Plant Strains and Growth Conditions

Plant strains and growth conditions were as described in Chapter 2.

Genetic analysis

To create the triple mutant *ran1-3/+ rte1-3 etr1-2*, the double mutant *ran1-3/+ etr1-2* (Chapter 4) was crossed with the double mutant *rte1-3 etr1-2* (Chapter 2). F_1 plants confirmed by genotyping to be heterozygous for *ran1-3* were allowed to self-fertilize, and triple *rte1-3 etr1-2 ran1-3/+* plants were identified in the F_2 generation. Genotyping markers for *rte1-3* and *etr1-2* are as described in Chapter 2, and those for *ran1-3*, as described in Chapter 4.

Transgenic Constructs and Plant Transformation

All mutant *etr1* transgenes were kindly donated by Dr. Tony Bleecker and were created as described in Wang et al., 2006. The plasmid PZP211 containing individual *etr1* transgenes were transformed into Col-0 and *rte1-2* plants by the floral dip method (Clough and Bent, 1999) using *Agrobacterium tumefaciens* strain GV3101. Transformed T_1 individuals were selected on MS plates containing Kanamycin (100μg/mL). Five to eight independent transgenic T_2 lines were screened on MS plates with and without ACC, with a total observation of between 100-200

seedlings per line. Using ImageJ, measurements were taken from three to four of these lines, with 15-25 seedlings measured from each line.

For RTE1 localization, we amplified the *RFP* coding sequence from the vector pDSRed2-C1 (a gift from Dr. Biao Ding at Ohio State University). For incorporation at the N-terminus of RTE1, primers (5'-CCTAGGATGGCCTCCTCCGAGAACGTC-3') and (5'-GCTAGCTCTAGATCCGGTGGATCCCGG-3') were used to amplify *RFP*, eliminating the STOP codon and incorporating an *Avr*II restriction site at the 5' end and a *Bmt*I restriction site at the 3'end. For incorporation at the C-terminus of RTE1, primers (5'-CCTAGGATGGCCTCCTCCGAGAACGTC-3') and (5'-GCTAGCTTATCTAGATCCGGTGGATCC-3') were used to amplify *RFP*, incorporating an *Avr*II restriction site at the 5' end and a *Bmt*I restriction site at the 3'end. Amplification was carried out using PCR and the resulting fragments were cloned into pGEMT-easy, and designated pGEM-NTRFP and pGEM-CTRFP, accordingly.

Total genomic DNA was isolated from *Arabidopsis* wild-type plants as described in Chapter 2. For the C-terminal tag, primers (5'-CCTAGGTTGGATGATGTGATCACCATCG-3') and (5'-CCTAGGAGTAATTATGTTCTTAAAACAGTAAC-3') were used to amplify the region encompassing the 5'UTR through the end of the *RTE1* coding region, eliminating the STOP codon, and incorporating flanking *Avr*II restriction sites. Primers (5'-GCTAGCAGCAGTATGAGAGAAAT-3') and (5'-GCTAGCTCACTGTTGGTACAACTTTGTGG-3') were used to amplify the region

encompassing the 3'UTR of *RTE1,* incorporating flanking *Bmt*I restriction sites.

Fragments were amplified using PCR, cloned into pGEMT-easy; and designated

pGEM-CTPC and pGEM-CTUTR respectively. The fragments were released in

succession and ligated into the respective *Avr*II and *Bmt*I sites of pGEM-CTRFP.

For the N-terminal tag, primers (5'-CCTAGGTTGGATGATGTGATCACCATCG-

3') and (5'-CCTAGGTTTTAGATTCCTAATCACACAAGAC-3') were used to

amplify the region encompassing the 5'UTR, incorporating flanking *Avr*II restriction

sites. Primers (5'-GCTAGCATGTCACGTGGAAGAGGAGTTCC-3') and (5'-

GCTAGCTCACTGTTGGTACAACTTTGTGG-3') were used to amplify the region

encompassing the coding region of *RTE1* and including the 3'UTR, and incorporating

flanking *Bmt*I restriction sites. Fragments were amplified using PCR, cloned into

pGEMT-easy, and designated pGEM-NTUTR and pGEM-CTCT respectively. The

fragments were released in succession and ligated into the respective *Avr*II and *Bmt*I

sites of pGEM-NTRFP. The composite fragments for both the C-terminal and N-

terminal tags fused to RFP were released with *Not*I and ligated into the *Not*I site of

pMLBart. Transformation into *Arabidopsis* adult plants was carried out as described

in Chapter 2. For expression of each transgene in an *rte1-3 etr1-2* background, five

independent T_2 lines were analyzed for insensitivity on 50μM ACC.

The control GFP marker was a gift from Dr. Chris Hawes (Oxford Brookes

University, U.K.). pVKH18EN6-mGFPer contains an N-terminal *Arabidopsis*

chitinase signal sequence and a C-terminal HDEL tag fused to *GFP* to target the

endoplasmic reticulum. Plants were transformed as described (above). Transformants

were selected by growing seedlings on MS containing 250μg/mL Hygromycin. To

observe co-expression of *RFP-RTE1* with the ER marker, T_1 heterozygous lines were crossed (carried out by Dr. Chun-Hai Dong). Of the resultant F1 population, One fourth were analyzed for the presence of both RFP-RTE and the marker transgene.

Chapter 6: Insight, conclusions and future directions: what have we learned, what can we conclude, and where do we go from here?

The importance of a PhD project has been emphasized to me on several occasions. It cannot be summarized as just one point, and in fact contains multiple aspects that may vary between individuals. Scientifically, I think most would agree that the prime objective is to significantly advance your scientific field, and on a broader level, to advance science as a whole. I would like to believe that my work on *RTE1* has made a considerable contribution to the field of ethylene biology, and also that it will have an impact on other fields, as additional members of the *RTE* family are discovered and studied.

When people look back on my PhD research papers and thesis, what will they find? In this chapter, I will discuss important conclusions, questions raised, and what future directions might be taken to further the study of *RTE1*.

A clearer insight into the ethylene-signaling pathway

It is fair to say that the ethylene-signaling pathway is more complex than initially thought. As more components have been identified, the pathway has become more fascinating, with many unique features and plenty of unknowns. It was originally predicted that the pathway would be similar to other eukaryotic pathways that contained both prokaryotic and eukarytotic components, such as the HOG1

pathway in *S. cerevisiae* (Posas et al., 1996). However, as more is learned about the ethylene-signaling pathway, it is apparent that it has many unique characteristics. The many questions that remain regarding unknown components, interactions and mechanisms indicate that genetic screens for ethylene-response mutants are not yet saturated. By using different parameters in genetic screens it may be possible to 'tease out' new components in the pathway. *RTE1* was isolated as a suppressor of the *etr1-2* insensitive mutant, and through the work described in this thesis has been characterized as a negative regulator of the ethylene-signaling pathway. On a more detailed level, it was found to act as a positive regulator of the ETR1 receptor, being largely required for ETR1 to be functional. We have therefore uncovered just one more of the unknowns, although the exact details of what *RTE1* does and how it functions is by no means a complete story. More work is still required to build on what we now know, to create a clear picture of RTE1's involvement in the ethylene-signaling pathway.

Conclusions, questions, speculations

Through the process of analyzing why loss of *RTE1* would suppress the insensitivity conferred by *etr1-2*, it became apparent that an *rte1* loss-of-function mutant largely phenocopies an *etr1* loss-of-function mutant. Perhaps the biggest conclusion that can be drawn from this thesis is that *RTE1* is a positive regulator of *ETR1*. However, questions still remain as to whether *ETR1* is completely dependent on *RTE1* in order to be functional, and whether *RTE1* is likely to regulate other receptors to any degree. Another important question is why *ETR1* seems to require a

specific regulator, while other receptors probably do not. We also cannot rule out a role for RTE1 in regulating responses outside of ethylene signaling.

The retention of ETR1 protein in *rte1* mutants may be significant

One interesting result is that the *rte1-3 ers1-3* double mutant does not phenocopy the *etr1-7 ers1-3* double mutant, as might be expected (Chapter 2, Fig. 2-4). The *etr1-7 ers1-3* double is known to be a strong null mutant, exhibiting a severe constitutive triple response in dark-grown seedlings (G.E. Schaller, personal communication). In contrast, another subfamily I 'null' mutant, *etr1-7 ers1-2*, where the *ers1-2* mutation is a T-DNA insertion in the promoter region (Zhao et al., 2002; Hall and Bleecker, 2003) is not as severe, and exhibits a very weak constitutive triple response phenotype. This double mutant has been described as 'leaky', and highlights the requirement for both receptors to be completely non-functional in order for a strong constitutive response to be observed. It is intriguing that loss of both subfamily I receptors results in such a strong constitutive ethylene response, more so than loss of all three subfamily II members, which still results in a viable adult plant. One possible reason for this is touched on briefly in Chapter 1, and outlines likely receptor differences with regards to their affinity for CTR1 – which physically interacts with the receptors to elicit downstream responses. It is theorized that CTR1 has a higher affinity for type I receptors over type II (Gao et al., 2003; Guo and Ecker, 2004), and therefore in an *etr1 ers1* null mutant, a large portion of the signaling CTR1 population is missing. Since *CTR1* is a negative regulator of the pathway, when it is absent, ethylene genes are activated and we observe a strong

constitutive response. This may be the reason we see such a strong response in the double subfamily I null. In the *rte1-3 ers1-3* double mutant, although it is predicted that for the most part ETR1 is non-functional, the fact remains that ETR1 is still physically present, and may still be bound to CTR1, which could retain some partial activity. One possible experiment would be to look at the amount of CTR1 in the soluble versus membrane-bound portion of an *rte1-3 ers1-3* cellular extract, similar to the assay carried out by Gao et al. (2003), which shows a large increase in soluble CTR1 in the *ers1-2* null mutant. It is also interesting that an *etr1-7 ers1-3* double mutant exhibits a more severe response than wild-type seedlings grown on the highest dose of ethylene. One possible reason for this is speculated by Wang et al (2006), in fitting with the hypothesis that ETR1 passes through a quasi-stable state between ethylene binding and inactivation of the receptor. If this model is correct, then in a wild-type situation, when ethylene is present, there will always be a portion of the receptors that have ethylene bound but remain 'on', creating a state of equilibrium between the two receptor states, possibly to ensure that the seedling does not become so severely impaired that it cannot grow. In contrast, when both *ETR1* and *ERS1* are gone, there is no state of equilibrium between these two states, and we have complete loss of both members of subfamily I. In an *rte1* mutant, ETR1 protein is probably still present, although largely non-functional. It is feasible that ETR1 retains enough functionality that the same degree of severity is not observed (especially considering the 'leaky' *etr1-7 ers1-2* mutant does not exhibit a constitutive response). It is also possible that in an *rte1* mutant, the ETR1 receptor retains the ability to bind ethylene, although not to respond to it. This could potentially chelate a significant portion of

free ethylene away from associating with the other receptors, and therefore prevent such a severe response as that seen in the subfamily I null mutant.

RTE1 may be part of a large receptor complex

Although not much is known about the interactions between the five ethylene receptors, it is likely to be a complex relationship. The receptors have been shown to form homodimers (Schaller et al., 1995), but there is no evidence that they form heterodimers. Even if heterodimers do not form between receptors, communication between them appears to occur (Wen and Chang, unpublished). The ETR1 receptors are known to form a large receptor complex, incorporating ETR1, CTR1 and other unknown components (Gao et al., 2003; G.E. Schaller, personal communication). It is quite possible that other ethylene receptors also associate with, or are an integral part of this complex. If this is the case, then in an *rte1* mutant, the association of an ETR1 dimer with another receptor dimer may present an entirely new question regarding the functionality of these receptors. A single residue substitution in one of the receptors is sufficient to confer dominant gain-of-function insensitivity in the plant. In an *rte1* null mutant, it is likely that the ETR1 protein is still present (Rivarola and Chang, unpublished), although it is largely non-functional. The presence of a 'defective' ETR1 receptor may be conveyed to the other receptors through direct interaction, and may also affect their signaling capabilities. If this is the case, what we really might be seeing is a significant loss of *ETR1* function, along with slight loss of function in the other receptors due to cross-talk between them, such that the appearance is equal to that of an *etr1* null mutant. This would explain why we do not see a severe response

in the *rte1 ers1* double null, and also why *rte1-2* is unable to suppress dominant insensitive mutations in other receptors, since they would be only indirectly affected by the loss of *RTE1*.

It is also feasible that in an *etr1* null mutant, protein levels for other receptors are up-regulated to compensate for this loss. In an *rte1* mutant, this may not occur since the protein is most likely still physically present (Rivarola and Chang, unpublished). This might help explain the phenotype seen in the *rte1-2 ran1-3* double mutant, which when grown on copper is not alleviated to the same degree as the *ran1-3 etr1-7* mutant. If this is the case, then the *ran1-3 etr1-7* mutant has more receptors exhibiting partial functionality due to the addition of copper than *rte1-2 ran1-3*. It would be of interest to look at the levels of other receptors in an *etr1* versus an *rte1* null mutant. It is also possible that *RTE1* acts in a parallel pathway to *RAN1* to regulate ETR1, since the loss of both genes has a phenotypically additive effect.

It is possible that RTE1 also regulates other receptors

At this point, although it appears that *RTE1* specifically regulates *ETR1*, we cannot rule out that it may directly affect other ethylene receptors. *rte1-2* is unable to suppress insensitive mutations in other receptors, indicating that it is likely to be specific to *ETR1*. However, a detailed suppression analysis of *rte1-2* with a wide variety of *etr1* insensitive mutants shows that some mutants are not suppressed. *rte1-2* was found to suppress all weak *etr1* insensitive mutants, but not the weak insensitive mutant *ers1-10*, indicating that *RTE1* is unlikely to regulate *ERS1*. However, all mutants tested in other receptors conferred strong insensitivity, since

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weak insensitive mutants in these receptors have not been identified. In order to confirm that *RTE1* does not regulate other receptors, it may be necessary to engineer mutations that confer weak insensitivity. Mutations in conserved residues similar to those described in Chapter 5 would likely have a similar affect in other receptors, and it would be interesting to see if they are suppressed by *rte1-2*.

RTE1 may regulate other, non-ethylene related pathways

Overexpression of *RTE1* conferred weak insensitivity in *Arabidopsis* seedlings, consistent with the hypersensitivity observed in *rte1* null mutants, and with *RTE1* acting as a positive regulator of *ETR1*. While overexpression is more pronounced in seedlings expressing ETR1, there is still noticeable insensitivity in *etr1* null mutants. This may be indicative of ectopic effects, or may imply that *RTE1* does indeed regulate other pathways in *Arabidopsis*. For example, it is possible that *RTE1* regulates other pathways involved in hypocotyl elongation, since *rte1* loss-of-function mutants are shorter than wild type. This shortening is assumed to be solely due to loss of *ETR1* function, but could conceivably be the result of severe impairment of *ETR1* along with inhibition of another unrelated pathway involved in hypocotyl elongation. This would fit with the phenotype seen in *etr1* null mutants overexpressing *RTE1*, which even without ethylene treatment are longer than *etr1* controls, but are not as long as wild-type seedlings overexpressing *RTE1*. Although other, non-ethylene related phenotypes were not immediately apparent in *rte1* mutants, a more detailed analysis could be carried out to assess this more thoroughly.

RTE1 may be important for ethylene signaling in all green plants

The possibility of _RTE1_ being involved in other pathways is a reasonable

speculation. It would be intriguing if the only role of _RTE1_ were to regulate _ETR1_,

especially since _RTE1_ has family members in such a wide variety of other organisms,

including animals, which do not carry ethylene receptors. However, plant genomes

have been shown to have multiple _RTE1_-like genes, whereas animal genomes have

only one. Interestingly, despite commonly utilizing two-component regulators,

prokaryotes do not contain an _RTE1_ homolog, indicating that _RTE1_ is not required for

regulation of two-component receptors. Homologs of both _RTE1_ and _ETR1_ can be

found in the moss _Physcomitrella patens_, suggesting that Bryophytes are capable of

ethylene-signaling, and require RTE1. This is not surprising since it is believed that

plant hormones are also essential in lower plants. It is possible that _RTE1_ and _ETR1_

first appeared together in the single celled green algae _Chlamydomonas_, since partial

homologs of both proteins can be found in the incomplete sequence data available. In

contrast, no _RTE1_ homolog is apparent in Cyanobacteria, whereas an _ETR1_ homolog

in _Synechocystis_ is present and has the ability to bind ethylene (Rodriguez et al.,

1999). Interestingly, there is no evidence that this primitive receptor homolog is

involved in initiating a downstream signaling cascade, whereas in _Chlamydomonas_

there is evidence for an ethylene biosynthetic pathway and AP2-EREBP family of

transcription factors (Ravanel et al., 1998; Maillard et al., 2005; Shigyo et al., 2006;

http://chlamytfdb.bio.uni-potsdam.de/v1.0/fam_mem.php?family_id=AP2-EREBP),

suggestive of the existence of at least some form of ethylene-signaling pathway. It is

therefore feasible that perhaps _RTE1_ is the 'missing link' between the ability of the

receptor to just bind ethylene and the ability for this action to illicit a downstream signaling response. This fits with our model of *RTE1* acting as a positive regulator of the *ETR1* receptor.

If *RTE1* acts as a molecular chaperone, as speculated in Chapter 5, it is possible that plants evolved with additional RTE proteins to specifically regulate prokaryotic two-component proteins, which so far have not been identified in animals. In *Arabidopsis*, *ETR1* has similarity to two-component response regulators, with all the components necessary for functional histidine kinase activity (this activity has been demonstrated *in vivo* (Gamble et al., 1998)). However, this activity is not required for ethylene signaling, indicating that the protein has evolved a novel signaling mechanism. The downstream involvement of CTR1, a eukaryotic raf-like MAPKKK, which is believed to physically interact with the receptors (Clark et al., 1998) supports this. And so we come back to the fact that the ethylene-signaling pathway is a complex one, where standards are broken and new precedents are set. So maybe it is not so surprising that *RTE1* may indeed act as a specific regulator of *ETR1*, the key receptor in a complicated signaling pathway. *RTE* genes evolved in eukaryotes, perhaps as molecular chaperones that are not necessarily specific to just one receptor or protein. In the case of *RTE1*, a duplication event may have presented a protein that could positively regulate a prokaryotic-like protein, which was itself evolving a novel mechanism of signaling. A study of *RTH*, the homolog of *RTE1* in *Arabidopsis* may reveal a more generalized function, perhaps comparable to that of *RTE* family members in animal systems.

Addressing the RTE1 'molecular chaperone' hypothesis

If RTE proteins do act as molecular chaperones, they may have evolved to specifically regulate certain protein types, but may all act in a similar manner. This hypothesis is a relatively recent one, based upon the data collected from the suppression analysis described in Chapter 5. The model suggests a role for RTE1 in facilitating conformational changes within the receptor. One possibility to look at this further would be to assess the temperature sensitivity of these mutants. A change in temperature may prevent suppression by *rte1-2* due to an altered conformation of the receptor; or alternatively, mutants previously not suppressed may exhibit a triple response under altered conditions. This would support the hypothesis and model, and may provide more detailed information regarding the relationship between *RTE1* and *ETR1*. In addition, in order to ascertain whether RTE1 and ETR1 do in fact functionally interact, assays such as split GFP or split-ubiquitin could be carried out. Other members of the Chang lab have already begun to carry out these experiments.

While the model presented in Chapter 5 supports the data presented, it is by no means a confirmed hypothesis. Other possible functions for *RTE1* have been considered over the years since we obtained its protein sequence. These hypotheses have not been tested, but are worth discussion, and possibly even further analysis. The final section of this final chapter discusses these alternative hypotheses.

Alternative Hypothesis 1: RTE1 is involved in the ubiquitination pathway

Recent analysis revealed more than 1300 genes in *Arabidopsis thaliana* believed to be involved in the ubiquitin/26S proteasome pathway (Vierstra, 2003),

which is now known to be the main proteolytic pathway in eukaryotic systems. Through this system, not only are proteins specifically targeted for degradation, but also it is now believed that it may be an important post-translational regulatory mechanism within the cell (Vierstra, 2003). Since there are so many genes believed to be important in this pathway, it can be assumed that there is a high degree of specificity involved the regulation of its proteins. A basic description of the ubiquitin/26S proteasome pathway is shown in Figure 6-1 and is as follows: ubiquitin molecules must first be activated, conjugated, and then ligated to the target protein. These three steps are the first part of ubiquitin-mediated degradation, and there are many difference enzymes involved in these steps alone. Following ubiquitination of the target, proteins are recognized for degradation by the proteasome.

There are examples of membrane bound proteins that are involved in the ubiquitin/proteasome pathway. For example, UBC6 is a ubiquitin-conjugating enzyme that in yeast is known to be anchored to the ER membrane. There is also some evidence that the proteasome is involved in extraction of ER membrane proteins for degradation (Mayer et al., 1998), and therefore it may be recruited to the membrane by ER-bound proteins. It is possible therefore, that ETR1 – a membrane bound protein shown to be at the ER membrane (Chen et al., 2002) – could potentially be regulated through ubiquitin-mediated degradation.

Interestingly, protein deubiquitination is reversible, a process that is now believed to be an important regulatory mechanism in protein degradation (Kim et al., 2003). Deubiquitinating enzymes (DUBs) are able to remove ubiquitin molecules from proteins targeted for degradation. DUBs fall into two main distinct groups:
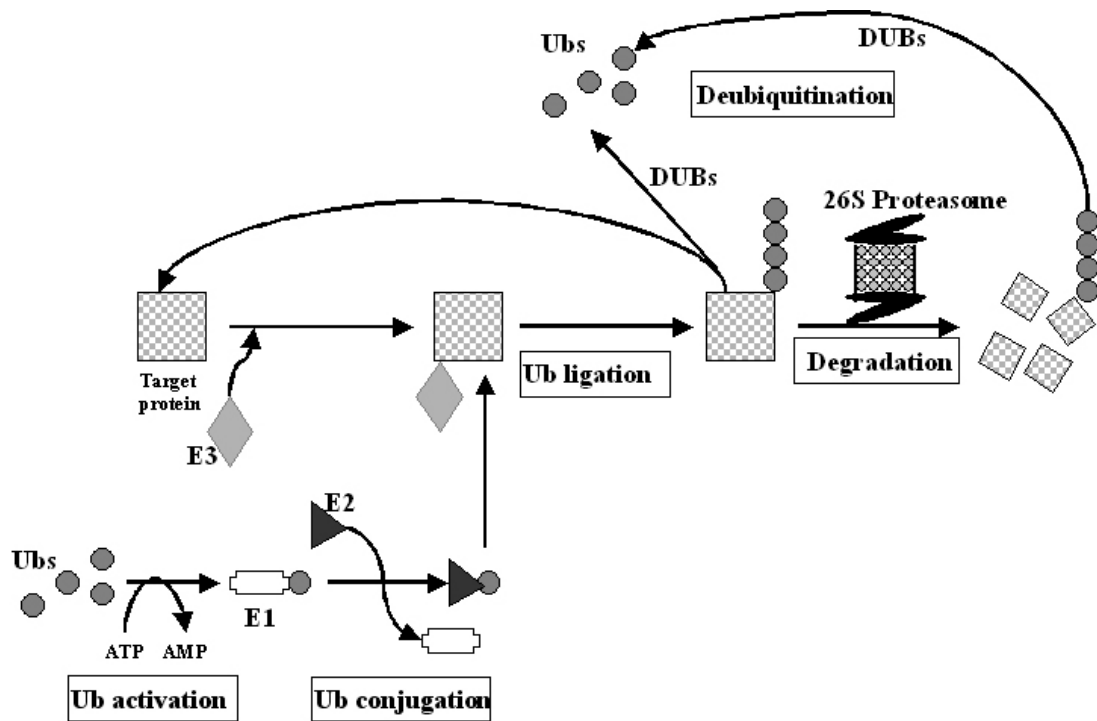
Figure 6-1. Ubiquitination and deubiquitination in the 26S proteasomal pathway
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Proteins are targeted for degradation by the covalent attachment of multiple ubiquitin

molecules. The attachment of ubiquitin is mediated by a multi-enzyme system,

comprised of E1, E2 and E3 enzymes. E1 enzymes are responsible for Ub activation

through C-terminal adenylation, which enables it to be transferred to the E2 Ub-

conjugating enzyme. E2 recruits an E3 Ub-ligating enzyme (which also has a role in

substrate recognition), to facilitate ligation of the ubiquitin molecule onto the target.

Additional ligation of Ub molecules create a poly-Ub chain, thus targeting the protein

for degradation by the 26S proteasome. DUBs (deubiquitinating proteins) inhibit

protein degradation by removing ubiquitin molecules from the target protein.

Ub-specific processing proteases (UBPs), and Ub C-terminal hydrolases (UCHs).

Two new families were also recently documented: Otubain and JAMM families

(Balakirev et al., 2003; Evans et al., 2003; Ambroggio et al., 2004; Hershko, 2005),

which play a similar role in the ubiquitin pathway, but are not related to the UBP or

UCH family. Otubain family members represent a group of putative cysteine

proteases; JAMM proteins are so called due to the presence of a conserved JAMM

(Jab1/Pad1/MPN-domain metallo-enzyme) domain, and are functional

metalloproteases. Both are believed to act in a deubiquitination pathway. Cysteine,

histidine and aspartate residues are functionally essential for members of the DUB

family: all UBPs and UCHs require these conserved residues to form an essential

"catalytic triad". DUBs have conserved histidine and cysteine residues, and JAMM

family members contain conserved histidine and aspartate residues as a part of their

metal binding motif. *RTE1* has some similarity to the UCH and Otubain families of

DUBs. RTE1 contains highly conserved cysteine, histidine and aspartate "catalytic

triad" residues, although the aspartate and cysteine residues are not separated by 2

amino acids as they are in UCH and Otubain proteins. UCH proteins are relatively

small (20-30kDa usually), indicating that RTE1, at ~27kDa is consistent with the size

of some DUB enzymes.

Some DUB proteins contain a Ub-interaction motif (UIM) (or UIM-like), or a Ub-

associated (UBA) (or UBA-like) domain, both of which require the presence of

amino acid with specific properties as well as conserved residues (Hofmann and

Bucher, 1996; Hofmann and Falquet, 2001). RTE1 contains a relatively conserved

region with some similarity to the UIM motif found in DUB proteins. This motif

consists of **∅-xx-A-xxx-S-xx-Ac**, where ∅ indicates an aromatic amino acid, and Ac indicates an acidic amino acid. This region is highlighted in Figure 6-2. Families featuring this UIM display a high degree of conservation of alanine and serine residues within the motif, whereas RTE family members only show full conservation at the alanine residue, although almost all display a serine residue within one amino acid of the specified motif location. Not all members display an acidic amino acid at the end of the motif, although there is a glutamic acid residue at this location that is conserved among approximately 50% of *RTE* family members.

The possibility of *RTE1* being involved in the ubiquitin-proteasome pathway first surfaced following the results of a yeast-2-hybrid analysis carried out by Dr. Chi-Kuang Wen, a former post-doc from the Chang lab, which indicated that RTE1 may interact with the PAG1 subunit from the 20S complex of the 26s proteasome. This interaction may seem implausible, since RTE proteins are presumed to be membrane bound, whereas the proteasome is a fully soluble cytoplasmic protein, which should not need to be recruited to the membrane. However, as previously mentioned, there is some evidence of the proteaosome being recruited to the ER for membrane extraction. It is important to remember that yeast-2-hybrid experiments conducted using membrane bound proteins often give 'false positive' interaction results, so this result would need to be backed up by other experimental evidence to support it.

Although there is no evidence of DUB proteins being membrane bound, other proteins involved in the ubiquitination pathway have been localized to the ER. Two ubiquitin-conjugating (E2) enzymes, UBC6 and UBC7 have been found to localize to the ER (Sommer and Jentsch, 1993; Biederer et al., 1996), and along with the sec61

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AtRTE1    117 NMGGHTCKYGFKHTDFG..TARTVDNALSSSTRSFEHKTYNIFTCNCHSFVANCLNRICY
riceRTE1  108 NLATHVCERSYKHAEAG..TAISVDDALQLGMRSFGHKFYNLFTCNCYSFVANCLNRLAY
AtRTH      96 SSGMFNGERRYEQEEDSHEKEPTVDDALRKSTQEYQHSYNILTCNCHSFVANNLNRLSI
riceRTE2   69 .........CYKLLEP..EGASTWDLALRGVQLFQHRGYSLFTCNCHSFVVNNLNRLFY
fishRTE    88 .............VYAS..GSNAWDTAVHDASEEYKHRMHNLCCDNCHSHVAMALNLMRY
humanRTE   93 .............VYAS..GPNAWDTAVHDASEEYKHRMHNLCCDNCHSHVALALNLMRY
flyRTE     87 .............MVG...GSYAWDEAVSKASVLYGTRIHNIFCDNCHSHVATALIYMRY
wormRTE    76 .............VEG...GAEVHDRAVQDASDTYKTRTHNLICDNCHSHVALALNKMRY
```

Figure 6-2. RTE1 has weak similarity to the UIM motif in DUB proteins.

The similarity to the UIM motif ($\varnothing$-xx-A-xxx-S-xx-Ac) is highlighted by a box in the above sequence ($\varnothing$ indicates an aromatic amino acid, and Ac indicates an acidic amino acid). The greatest degree of similarity can be observed in AtRTH, although it is not strongly conserved throughout other RTE family members.

transposon in yeast, the proteasome itself has been found to be important in membrane extraction of ER proteins – membrane extraction is essential for proteasomal degradation (Mayer et al., 1998). Taking these facts together, the possibility of a functional interaction between the proteasome and RTE1 may not be so unlikely, although it is worth noting that we do not know whether RTE1 does indeed localize to the ER.

With regards to *RTE1* genetic data, this hypothesis could fit with our findings. If RTE1 is involved in the de-ubiquitination of ETR1 to prevent degradation, when RTE1 is absent or non-functional, ETR1 would be targeted for degradation and the resulting phenotype would be similar to that of the *etr1* null mutant. This is what we see in an *rte1* loss-of-function mutant, where the resultant phenotype is reminiscent to that of the *etr1-7* null mutant. Certain insensitive mutants such as *etr1-1* may not be subject to the same degree of regulation by ubiquitination/proteasomal degradation, possibly due to changes in folding or localization.

Although it has not been shown that ethylene receptors are regulated through the ubiquitin-proteasome pathway, it is possible that they are post-translationally regulated in this way. It is noteworthy that ETR1 levels appear to be unaffected by increased levels of ethylene (Chang et al., 1993), possibly indicating that there is no change in protein stability through RTE1. However, slight increases in protein levels may be difficult to observe. Preliminary unpublished data from the Chang lab indicates that ETR1 protein levels are unchanged in *rte1* mutants, suggesting that RTE1 probably does not regulate ETR1 in this way.

Experimentally testing the Ub-pathway hypothesis

Since the protein levels of ETR1 appear to be unchanged in *rte1* mutants (Rivarola and Chang, unpublished), the possibility of RTE1 being involved in the ubiquitination pathway seems unlikely. However, if closer analysis is deemed appropriate, an in-vitro ubiqutination assay could be carried out to assess possible changes ETR1-Ub levels (Mudgil et al., 2004). In addition, an experiment that may give useful information even outside of this hypothesis is a detailed analysis of some of the conserved residues in RTE1. In particular, RTE1 contains conserved Cys, His and Asp residues that could potentially comprise a 'catalytic triad' within the protein sequence, as described above. We already know that one of the cysteine residues is functionally important for RTE1; a Cys-Tyr mutation at residue 161 causes loss-of-function (i.e. the *rte1-1* allele). RTE1 contains seven other candidate residues that are highly conserved and may comprise important catalytic residues: this includes two additional cysteine residues, two histidine residues and three aspartate residues. It would be interesting to carry out site directed mutagenesis on these residues (to a non-reactive Alanine residue), and assess functionality by looking for complementation of an *rte1-3 etr1-2* double mutant, which will be reverted back to insensitivity if the RTE1 is functional. If mutations in all three residue types are unable to rescue the *rte1-3 etr1-2* phenotype, this would support the role of a catalytic triad in RTE1. If this is not the case, but we still see loss-of-function from mutations in some of the residues, then we have still learned more about which residues are functionally important for RTE1.

Alternative Hypothesis 2: RTE1 is an 'RER'-like protein

To look for remote homologs or proteins with some similarity to RTE1, a sequence analysis was carried out using an HMMR-search. HMM-search utilizes hidden Markov modeling to find proteins that may be biologically related (Karplus et al., 1998). When RTE1 along with several RTE homologs were analyzed, proteins with the most likely similarity are members of the RER (**R**etrieval to the **ER**) family, with expectation-values (E-values) of between e-26 to e-3 (members of the RTE family have e-values ranging from e-144 to e-95). Although the E-values are a useful indicator of the significance of similarity between proteins, it is still just a statistical guide. In general, the lower the E-value, the more significant the similarity between the query sequence and the sequences extracted from the database. An E-value of 1 or higher usually indicates that the proteins are not related. Since the values observed between the RTE and RER families are less than 1, and some are significantly low enough to suggest a possible relationship, closer analysis is warranted to assess whether RTE1 may be involved in retrieving or retaining proteins to/in the ER. Many proteins that are localized to the endoplasmic reticulum (ER) contain a C-terminal targeting sequence: most commonly a KDEL/HDEL sequence, di-lysine (KKXX) or di-arginine (RR) motif; that is responsible for retrieving the protein to the ER (Munro and Pelham, 1987). 'Retrieval' to the ER is necessary since often proteins undergo modifications that require them to be transferred to the Golgi apparatus, in which case they must then be sent back to the ER. However, some proteins known to localize to the ER do not contain these sequences, and instead are retrieved back to the ER by RER proteins. RER proteins were first identified in yeast (Sato et al., 1995) via a

screen in which Sec12p – an important ER protein, was mislocalized to the Golgi.

RER proteins have been identified in *Arabidopsis*, humans, mice *C. elegans*, *Drosophila* and rice, and are also present in yeast and other fungi (whereas there is no *RTE* representative present in yeast). However, it is noteworthy that *Saccharomyces cerevisiae* only contains one RER protein, whereas higher eukaryotes contain multiple copies. *Arabidopsis* contains at least three RER proteins, which were identified based on their homology to yeast RER1 (Sato et al., 1999). The Rer1 protein in yeast is a Golgi-localized protein with four transmembrane domains, and is responsible for the correct localization of several proteins to the ER. In plants, several types of ER membranes have been defined, implying that the mechanism for ER protein sorting and retrieval is more complex (Staehelin, 1997). Many ER-localized plant proteins contain a KDEL-like motif in the C-terminus to ensure correct localization. HDEL, KDEL and RDEL have all been shown to be sufficient localization signals (Staehelin, 1997), although thus far, no plant ER protein has been identified that contains the KKXX motif that can be found in other organisms.

Three RER1 proteins have been characterized in *Arabidopsis* (Sato et al., 1999), but there is still a great deal that is not known about the molecular mechanisms of ER localization in plant cells.

AtRER proteins are highly expressed in roots, although expression is low in stems and seedlings (Sato-Nara et al., 1999). Although there are high RER levels in floral buds, there is very little in siliques, which is where RTE1 is most highly expressed. RER proteins are of similar size to RTE proteins – being approximately 200 amino acid residues in length, and are localized to the Golgi apparatus. They

contain four transmembrane regions, with a predicted cytoplasmic C-terminal tail.

The C-terminus is the most conserved region, and has been shown to be essential for

RER function, since deletion of 25 residues from the yeast Rer1p results in a loss-of-

function mutant (Sato et al., 2001). RTE1 also has a region of high amino acid

conservation at the C-terminal end. We know that this region is important for RTE1

function, since the *rte1-2* loss-of-function mutant is the result of a frameshift

mutation, where the last 27 residues are replaced with 15 incorrect residues.

Although there are no obvious motifs present in RER proteins, there is a semi-

conserved "GKKKY" and a "YIPL" sequence at the C-terminal end. GKKKY

contains a di-lysine-like motif, and YIPL is known to be important for recognition in

some proteins (Sato et al., 2001). In both of these cases, the Tyr residue has been

shown to be important for localization to the Golgi (Sato et al., 2001). Interestingly,

the three *Arabidopsis* RER proteins known to rescue the yeast *Rer* null mutant do not

contain these conserved GKKKY or YIPL sequences, although the Tyr residue is

conserved in both cases, supporting this being a key residue in RER localization and

function. RTE1 does not contain either GKKKY or YIPL motifs, or any other known

localization sequences. Several highly conserved Tyr residues are present, but this is

not enough to draw any conclusions. With regards to sequence similarity, the highest

E-value between RTE and RER is 4.3e-26, which compares mouse RTE with mouse

RER. Out of 184 residues analyzed in an alignment, 26 residues are the same (14%),

but an additional 116 residues are similar (64%), to make 78% of the residues having

similar or equal properties between the two proteins. However, a ClustalW alignment

of RTE1 with three AtRER proteins does not give a strong alignment (Fig. 6-3A),

with the level of similarity between these proteins ranging from 9.5 – 12%. A phylogenetic tree also indicates that the two families are not closely related (Fig. 6-3B).

An important question is whether this hypothesis fits with our genetic data. If RTE1 were essential for the retention or retrieval of ETR1 to the ER, when RTE1 is non-functional, ETR1 would be likely be mis-localized, and would therefore probably be non-functional. It is possible that *etr1-2* and some other insensitive mutants also are dependent on localization to the ER to induce dominant insensitivity, and so may also be mis-localized in an RTE1 loss-of-function mutant. A question is raised with regards to *etr1-1* and other strong insensitive mutants not suppressed by *rte1* mutants. Perhaps since it is a strong mutant, the localization of the receptor is less important when compared to *etr1-2*, which is a weak mutant. This is all speculative, but the hypothesis of RTE1 functioning as a protein that may be important to ensure ETR1 is localized to the ER is not one we can yet rule out.

Experimentally testing the RER hypothesis

One possibility to test whether RTE1 is able to retrieve/retain proteins to the ER is to carry out a complementation analysis in yeast. AtRER1, *2* and *3* all are able to complement the yeast *rer1-2* mutant to varying degrees (Sato et al., 1999). Rescue of the mutant can be observed using a 'halo' assay (Sato et al., 1999): Sec12p is a protein that is localized to the ER, but is mis-localized to the Golgi in *rer* mutants. By creating a Sec12p-Mfα1p (mating factor α) fusion protein, if Sec12p goes to the Golgi, α-factor is released, causing a halo when grown on selective media due to
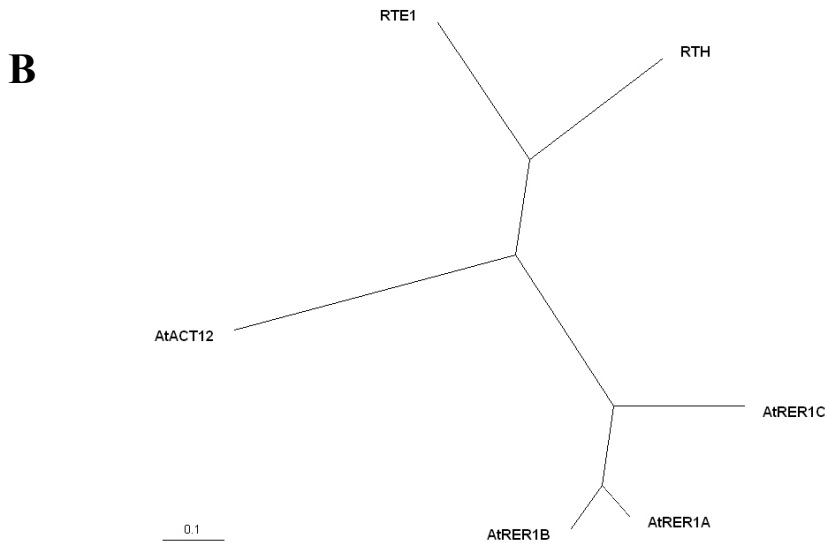
Figure 6-3. Comparison of AtRER proteins and RTE1.

(A) ClustalW alignment of AtRER1, AtRER2, AtRER3 and RTE1 protein sequences.

(B) Unrooted phylogenetic tree of the same proteins as in A, with the addition of

RTH, and AtACT12 (actin 12) as a control reference point.

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growth inhibition. If RTE1 is able to retrieve Sec12p to the ER, less of a halo will be observed.

Conclusion

Our work on *RTE1* reveals the existence of a novel negative regulator of the ethylene-signaling pathway, acting as a positive regulator of the ETR1 receptor. The presence of homologs in a wide variety of organisms indicates that members of the *RTE* family carry out an essential, conserved function. Although we cannot definitively say how *RTE1* functions at the molecular level, we have made significant strides in ascertaining its importance in ethylene signaling and specific regulation of the ETR1 receptor. We have created a strong foundation to help us understand the broader role of RTE proteins in other organisms, and can therefore be confident that through this work we have made a significant impact on the field of biology.

Appendix A

This Appendix describes several experiments that either produced limited conclusions or yielded negative results. They have little impact on the overall conclusions of this thesis, however it is worthwhile noting that such experiments were carried out.

Creating an Antibody to RTE1

Creating a good antibody to the RTE1 protein offers the opportunity to study several aspects of the biology of RTE1. For example, we would be able to confirm that the *rte1-3* null allele is a true null, where no RTE1 protein can be detected. An antibody is also useful for localization analysis: immunolocalization, immunoprecipitation, and sucrose-density gradients are all techniques that can be used to ascertain the location of a protein under varying conditions and in different genetic backgrounds. Antibodies are useful to assess protein levels in other mutants – such as ethylene-insensitive mutants such as *etr1-2* and *etr1-1*, or the etr1 null mutant *etr1-7*, and also to see whether levels of the mutant RTE1-2 protein differ from wild-type RTE1 under various conditions or in different genetic backgrounds. Wild-type plants exposed to ethylene for five hours revealed that *RTE1* transcript levels are up-regulated by ethylene (Fig. 2-1B)**,** and affymetrix gene expression arrays also showed that RTE1 levels are four times higher when ethylene is present (Alonso *et al.*, 2003). A western blot carried out with a reliable antibody could be used to back this up these findings.

One interesting question is whether RTE1 is under the control of the transcription factor *EIN3*. EIN3 is a nuclear transcription factor that regulates a transcriptional cascade and ultimately the expression of *ERF1* and other ethylene responsive genes (Chao *et al.*, 1997). *ein3-1* mutants are largely insensitive to ethylene, since *ERF1* and other genes are not transcribed (Chao *et al.*, 1997). If *RTE1* were also under the control of *EIN3*, protein levels would be affected in *ein3-1* mutants, such that when ethylene is present, levels of RTE1 would not increase as seen in wild type plants.

Experiments to assess the levels and localization of RTE1 under different conditions and in different backgrounds, along with an analysis of RTE1 in *ein3-1* mutants were important points to be addressed with the use of an antibody against RTE1. Rabbit antibodies were raised against a synthetic peptide of RTE1 (IEADEADLWPLPE(C)) that would prevent non-specific binding to *RTE* homologs such as RTH. The antisera was extensively tested over a series of 8 bleeds and an affinity purification using the synthesized peptide. Western blots were performed on RTE1 protein extracted from both *Arabidopsis* tissue and *Saccharomyces cerevisiae*, which was transformed with *AtRTE1* for the purpose of protein expression. We also attempted to show the absence of RTE1 protein in the *rte1-3* null mutant, as well as the slight difference in molecular weight between RTE1-2 (26.49kDa) and wild-type RTE1 (27.93kDa). Although the RTE protein expressed in yeast cells could clearly be detected, there was unfortunately a high degree of non-specific binding in plant tissue extracts, such that this antibody could not be utilized for localization or expression studies in plant cells.

Yeast Mutant Analysis: Does RTE1 alter the growth rates of yeast copper mutants?

The copper trafficking pathway is highly conserved between mammalian, yeast and plant cells (Fig 4-1). Yeast mutants deficient in various components of the copper trafficking pathway can be complemented by the relevant homolog from either mammalian or plant systems. We obtained yeast mutants deficient in different components of the copper trafficking pathway (a gift from Dan Kosman's lab), and wanted to see whether expression of *RTE1* in these mutants would affect growth under various conditions. Although there is no *RTE1* homologue in yeast, if RTE1 has a conserved copper binding function, it might have some impact on the growth rate of yeast copper mutants. The yeast strain we tested was deficient in Ccc2 - a copper transporting ATPase that transports copper into the Golgi. The reporter for this assay was fet3, which is an iron oxidase that requires copper as a cofactor for iron uptake. Δccc2 mutants grow at a reduced level in synthetic complete media, but growth is even further reduced in iron deficient media due to the lack of copper available for incorporation into Fet3 (Askwith *et al.*, 1994, Silva *et al.*, 1995). Wild-type cells are able to grow in iron deficient media since Fet3 is able to "scavenge" for iron, but they can only do this if copper is successfully trafficked into the Golgi. Incorporation of either RAN1 or the MNK protein restores the growth of Δccc2 mutants, and out aim was to see whether RTE1 could fully or partially rescue the growth of this mutant in iron deficient media

Our analysis indicated that the growth rate of the Δccc2 mutants were unaffected by the presence of RTE1, indicating that RTE1 is unable to compensate

for any of the copper trafficking proteins upstream of ccc2 in this copper trafficking

pathway (data not shown). This does not rule out a role for RTE1 in copper binding,

but does indicate that it is unlikely to carry out this role in yeast. This may not be a

great surprise, since yeast does not contain an RTE1 homologue.

Testing the copper-binding capabilities of RTE1

Another technique that we utilized to assess whether RTE1 is likely a copper

binding protein was flame atomic absorption spectroscopy.

Flame atomic absorption is useful to measure the concentration of copper and

other metals in a sample, and can help ascertain whether RTE1 is likely to be

involved in the binding of copper. This technique was used to help show that ETR1

binds a copper cofactor (Rodriguez et al., 1999): when wild-type ETR1 was

expressed in *Saccharomyces cerevisiae* and purified, it was found to contain six-fold

higher concentration of copper when compared to the mutant ETR1-1 protein, which

is unable to bind copper. Our aim was to carry out a similar experiment using RTE1

expressed in yeast, to see whether this would result in an increased level of copper

when compared to yeast not expressing RTE1. Since RTE1 contains conserved

regions of Cys and His residues, our hypothesis centered on these being potentially

important metal-binding residues, especially since the *rte1-1* loss-of-function mutant

contains a Cys-Tyr mutation at the conserved Cys^{161} residue. A proposed follow-up

experiment was to express this RTE1-1 mutant protein in yeast to see if this would

decrease the copper-binding capabilities of the protein. We carried out this flame

atomic absorption spectroscopy experiment on several occasions, using slightly

different protocols, and two independent flame atomic absorption spectrometers. This work was carried out with the help of Marko Jovanovic. Results did not indicate any significant reproducible differences in the copper concentration of yeast containing RTE1 versus those that did not. In addition there was no indication that RTE1 is involved in binding other metal ions.

Materials and Methods

Protein analysis

The RTE1 antisera was produced in rabbits by Alpha Diagnostic International (http://www.4adi.com/) and raised against the synthetic peptide [IEADEADLWPLPE(C)], which was also synthesized by Alpha Diagnostic. For testing of the antibody, RTE1 protein was extracted from the yeast strain LRB520 as described (Clark *et al*., 1998). Protein were separated out by SDS-PAGE on 12% precast gels (Biorad laboratories), and blotted to Immobilon-P PVDF membranes (Millipore). Western blots were carried out as per Clark *et al*., 1998. For separation of RTE1-2 protein compared to RTE1 wild type, a gradient gel of 4-20% was used. Membranes were probed with the RTE1 antisera at concentrations ranging between 1/200 and 1/5000 to obtain optimal signal. To normalize protein loading concentrations, a BSA protein assay was carried out.

Yeast expression and purification

For *RTE1* expression in the yeast strain LRB520, the *RTE1* cDNA sequence was released from the pBLUESCRIPT vector using flanking *Eco*RI sites and ligated

in the yeast expression vector pYCDE2, which carries the *ADH* Promoter (Hadfield et al., 1986). Transformation into LRB520 was carried out as described (Chen *et al.*, 1992). Positive transformants were selected for on synthetic complete (SC) media plates –tryptophan (trp). pYCDE2-RTE1 was also transformed into the yeast copper mutant Δccc2 (selection on SCM –trp –leucine (leu)), and the corresponding wild-type strain 2098 (selection on SCM-trp). Growth rates of yeast in iron deficient media were assayed as per Payne and Gitlin, 1998. As a control, the pYCDE2 vector alone was also transformed into each yeast strain.

Flame atomic absorption was carried out on yeast cells as previously described (Rodriguez et al., 1999).

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