ABSTRACT

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Maximo Lisandro Rivarola, Doctor of Philosophy, 2008

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Ethylene is an important regulator of plant growth, development and responses to environmental stresses. The higher plant *Arabidopsis thaliana* perceives ethylene through five homologous receptors, which negatively regulate ethylene responses. The molecular mechanism by which these receptors signal to their next downstream component remains elusive. Genetic analyses have shown that the *RTE1* locus is a positive regulator of *ETR1*. *RTE1* encodes a novel protein of unknown molecular function, and is conserved in plants, animals and some protists. The goal of this research was to analyze the mechanisms involved in the regulation of ethylene receptor signaling by *RTE1* and to enhance our understanding of the conserved cellular role of *RTE1*. Here we tested hypotheses for how *RTE1* affects ETR1 and is

specific to only ETR1, not the other ethylene receptor isoforms. We show that *ETR1* and *RTE1* gene expression patterns partially overlap and that the ETR1 receptor co-localizes with RTE1 within the cell. Moreover, *RTE1* has no effect on ETR1 protein abundance or subcellular localization suggesting other mechanisms to regulate ETR1. We provide supporting evidence that *RTE1* affects ETR1 signaling by restoring signaling of a non-functional ETR1 in an *rte1* null through changes in ETR1 conformation(s). We next addressed the question of *RTE1* specificity to ETR1. We discovered that *ETR1* is surprisingly distinct from the other four ethylene receptor genes; in that *RTE1*-dependent mutations only confer insensitivity in ETR1 and not in the other ethylene receptors when the same mutations are introduced. In contrast, the *RTE1*-independent *ETR1* insensitive mutations do give insensitivity in the closest receptor to *ETR1*, *ERS1*. Furthermore, we uncover that the ethylene binding domains are not completely interchangeable between ETR1 and ERS1. Our data point to a model in which *RTE1* specifically promotes ETR1 signaling via conformational changes in a unique way that does not occur in other ethylene receptors. These findings highlight the importance and uniqueness of ETR1 signaling conformation(s) with respect to the other ethylene receptors, as well as advance our knowledge of *RTE1* at the molecular and cellular level.

INSIGHTS INTO THE REGULATION OF ETHYLENE RECEPTOR SIGNALING BY RTE1

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

I would like to dedicate this thesis to my wife Mariana Melani and my son Matias. It is them, day after day, that are the most important thing to me. I would also like to dedicate this thesis to all my family and friends, who give me so much. I especially want to dedicate this thesis to my father who is no longer here and to my mother.

Acknowledgements

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ACC, 1-aminocyclopropane 1-carboxylic acid
ACS, ACC synthase
CAPS, Cleaved Amplified Polymorphic Sequence
CC, Coiled coil domain
cGMP, Cyclic guanosine monophosphate
Col, Columbia ecotype
CTR, Constitutive Triple Response
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
GAF, cGMP-specific and Stimulated Phosphodiesterases domain
GFP, Green Fluorescent Protein
HK, Histidine Kinase
MAPK, Mitogen Activated Protein Kinase
MAPKK, MAPK Kinase
MAPKKK, MAPKK Kinase
MS, Murashige and Skoog Medium
NBD, 2,5-norbornadiene
NR, Never ripe
PERE, Primary Ethylene Response Element
PCR, Polymerase Chain Reaction
RAN, Responsive To Antagonist
RFP, Red Fluorescent Protein
RTE, Reversion To Ethylene Sensitivity
RTH, RTE1 Homolog
TILLING, Targeting Induced Local Lesions In Genomes
Ub, Ubiquitin

Chapter 1: Introduction

Importance and history of the ethylene pathway

Plants are sessile organisms that must respond quickly to a vast array of external and internal cues such as light, gravity, temperature, pathogen attack, and hormones. The speed and accuracy of response is vital to its development and survival. The complex mechanisms by which plants perceive these signals and convert the information into physiological changes are only beginning to be understood. Plants, unlike animals, must respond to their environment through biological processes which do not involve 'running away from their predator' or 'moving away from environmental stresses'. This distinction has made plants evolve to respond rapidly. Moreoverthe ethylene pathway, as with several other pathways including the gibberellic acid (GA) pathway and light signaling are negatively regulated. Later in this section there is a more detailed explanation of the negative regulation of the ethylene pathway.

The Chang Lab is focusing on the signal transduction of the plant hormone ethylene, an alkene gas that has profound effects on plant growth and development, from seed germination to senescence. This olefin gas has a very important role in how plants respond to external stimuli such as pathogen attack or water stress, activating gene expression to cope with this new condition (Abeles, 1992). Moreover, ethylene has a great deal of importance in the agriculture field; it controls the ripening of many fruits and senescence of flowers and leaves among many other processes.

Understanding how plants utilize all the signaling pathways in concert to respond to biotic and abiotic challenges is an ultimate goal. To start this journey, we must first characterize in detail how the individual hormone signaling pathways work. The Chang lab is very active in the ethylene signaling research, especially in the upstream events of the signaling cascade. Research in the ethylene signaling pathway has elucidated an exciting and intriguing, but not yet fully understood pathway, composed of both prokaryotic and eukaryotic components in a novel combination.

Many centuries ago before anyone had knowledge of ethylene's molecular nature, people would conduct 'supernatural' rituals to change the rate of ripening of their crops. The first time natural ethylene production was used as a method to ripen fruits dates all the way back to biblical times. The prophet *Amos* described the gashing (wounding) of figs as it was known to promote stress (ethylene production) mimicking the action of the wasps when they exit pollinated fruits, and this triggered ripening. Moreover, in ancient China, they would place pressure on the top of growing bean sprouts to promote hypocotyl thickening, another ethylene response (Abeles, 1992). A common practice used in East Africa and Samoa in the past was to unknowingly use ethylene to ripen bananas by burying them in fire-warmed pits. The residual ethylene from the smoke served as the ripening compound. Not surprisingly, this phytohormone was the first gaseous molecule to have been identified to elicit responses by Dimitry Neljubov in 1901 (Neljubov, 1901). Many years have passed since then and a very intriguing pathway has emerged with novel components of unknown function and a unique combination of unlikely protein partners.

Ethylene Biosynthesis

As immobile organisms, plants must precisely and accurately monitor their environment and react accordingly through changes in their developmental and metabolic pathways. To date, several different pathways have been characterized; how plants process all these pathways to adjust their output and how all these pathways interconnect is still very much unknown and actively investigated. Ethylene plays a major role in plant development and its pathway has been very well characterized in plants. Most plant tissues have the capability to produce ethylene. Its biosynthesis has been very well studied and uses methionine as the substrate (Yang and Hoffman, 1984; Kende, 1993; Johnson and Ecker, 1998). There are three steps in the biosynthesis of ethylene; also known as the Yang cycle, in which S-adenosyl-methionine is diverted to make ACC (1-aminocyclopropane-1-carboxylic acid). First of all, methionine is turned into S-adenosyl-methionine by the Ado-Met synthetase, and next the ACC synthase (ACS) turns Ado-Met into ACC which later is turned into ethylene by the ACC-oxidase. The rate-limiting step is the production of ACC. The nine-member ACC synthase family is regulated at the transcriptional and post-translational level in *Arabidopsis thaliana*. Moreover, expression of members of the ACC synthase family is induced by different stimuli (i.e. pathogen attack or maturation), developmental signals, as well as biotic and abiotic stresses. This large gene family enables organ specific regulation of ethylene production to accelerate in a spatially and temporally specific pattern (Kanellis, 1997).

An additional regulatory mechanism in the ethylene biosynthesis pathway is the fact that another plant hormone can affect ethylene production. The regulation of

the ACC synthase5 *(ACS5)* gene is particularly instructive of how hormone crosstalk is controlled. The plant hormone cytokinin (even at low dosages) induces the triple response in an ethylene-dependent manner in four-day old seedlings grown in the dark (Cary et al., 1995). A genetic screen designed to isolate mutants that do not respond to low concentrations of cytokinin identified several mutants. One recessive mutant, cytokinin insensitive5 *(cin5)* (Vogel et al., 1998), turned out to be allelic to the dominant ethylene overproducer mutant *eto2* (Woeste et al., 1999), which encodes the *ACS5 gene*. Earlier experiments had indicated that ACS5 levels are posttranscriptionally regulated. On one hand, cytokinin treatment slightly induces its mRNA levels (less than two fold), but on the other hand, ethylene levels rise eight-fold with cytokinin treatment (Vogel et al., 1998). Analysis of purified ACS5 revealed that *eto2* plants stabilize the half-life of the protein. In a similar manner, cytokinin treatment increased the stability of ACS5 (Chae et al., 2003; Chae and Kieber, 2005).

In summary, the phenotypes of mutant *ACS5* alleles indicate that some of the effects of cytokinin can be attributed to the ethylene biosynthetic pathway, and supports earlier results that ACC synthase is the rate-limiting step for ethylene production.

The ethylene receptors

All important agronomic plants have ethylene receptors and each species gives advantages for studying the ethylene signaling pathway. For example, plants like tomato have a fruit ripening phenotype, unlike Arabidopsis, that researchers follow

and are able to address how ethylene affects different tissues inside the fruit. Nevertheless, the use of the model plant, *Arabidopsis thaliana*, has given the framework of the genetic elements and their placement on the signaling pathway map. The work performed in Arabidopsis enabled the ethylene field to advance at a pace not possible using any other plant. More importantly, the conserved nature of the pathway and its components across plant species allows for reliable translational research from Arabidopsis to other economical important plants, such as tomato, corn, and melon (Klee, 2004).

Many genes in the ethylene signaling pathway have been elucidated through genetic screens in Arabidopsis, such as the first plant hormone receptor gene, *ETHYLENE-RESPONSE1 (ETR1)* (Chang et al., 1993). These screens have focused on the ethylene response that displays the "triple response" phenotype of four-day old, dark grown seedlings (Figure 1-1). The triple response consists of inhibition of hypocotyl and root elongation, radical swelling of the hypocotyl, exaggeration of the apical hook, and proliferation of root hairs (Chen et al., 2005; Li and Guo, 2007).

Ethylene gas is readily permeable through the cell and is perceived by a family of five receptors (ETR1, ERS1, EIN4, ETR2, and ERS2) in *Arabidopsis thaliana*, which are negative regulators of ethylene response (Chang et al., 1993; Hua et al., 1995; Hua and Meyerowitz, 1998; Sakai et al., 1998; Hall and Bleecker, 2003; Qu et al., 2007). Most single receptor null mutants have a wild-type phenotype (due to redundancy), except for any single subfamily I null mutant (*ETR1* or *ERS1*), which show enhanced ethylene sensitivity (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002; Qu et al., 2007). Multiple loss-of-function receptor mutants (e.g. *etr1-7 ers1-3*)

show a constitutive ethylene response (Qu et al., 2007). On the other hand, dominant gain-of-function receptor mutants display ethylene insensitivity (e.g. *etr1-1, etr1-2, and ers1-1* and see Table 1). *etr1-1*, for example, confers strong ethylene insensitivity and fully blocks ethylene binding, whereas *etr1-2* confers weak ethylene insensitivity and has no effect on ethylene binding (Hall et al., 1999). When ethylene is present, wild type receptors are in an inactive state, allowing the response to occur (Figure 1-2).

The ATPase copper transporter *RAN1* has been shown to be genetically upstream of the receptors (Woeste and Kieber, 2000) and localized to the Golgi compartment (Dunkley et al., 2006), suggesting the likely role of *RAN1* is to deliver copper to the receptors. Furthermore, the receptors have been shown to bind ethylene (Schaller and Bleecker, 1995) with a required copper cofactor (Rodriguez et al., 1999). A *ran1* loss of function mutant seedling displays a constitutive triple response due to causing multiple receptor loss of function (Woeste and Kieber, 2000). Additional data that supports this model comes from experiments using silver. Silver is an ethylene response inhibitor that is predicted to take the place of copper in the receptors and lock the receptors in an 'ON' state of signaling (Rodriguez et al., 1999; Binder et al., 2007), rendering the receptors insensitive to ethylene. *ran1* loss of

Figure 1-1. The ethylene triple response in Arabidopsis

Seedlings were grown in the dark for four days in the presence or absence of ethylene. Seedlings were cold stratified at 4ºC for three days and then placed at 20ºC in a temperature control chamber. The main features of this response are the short and thick hypocotyl, exaggerated curvature of the apical hook, and the proliferation of the root hairs. Scale bar is 2mm.

Table 1. Allele specification of ethylene mutants

Genotype	Type of Mutation		Phenotype at 4-day dark grown assay
rte1-2	loss of function	Frame shift aa223	enhanced ethylene response
rte1-3	loss of function	stop aa57	enhanced ethylene response
rth-1	loss of function	stop aa108	similar to wild-type
etr1-7	loss of function	stop aa74	enhanced ethylene response
etr1-1	dominant, gain of function	C65Y	ethylene insensitive
etr1-2	dominant, gain of function	A102T	ethylene insensitive
ers1-3	loss of function	T-DNA	enhanced ethylene response
ers1-1	dominant, gain of function	F62I	ethylene insensitive
etr2-1	dominant, gain of function	L66P	ethylene insensitive

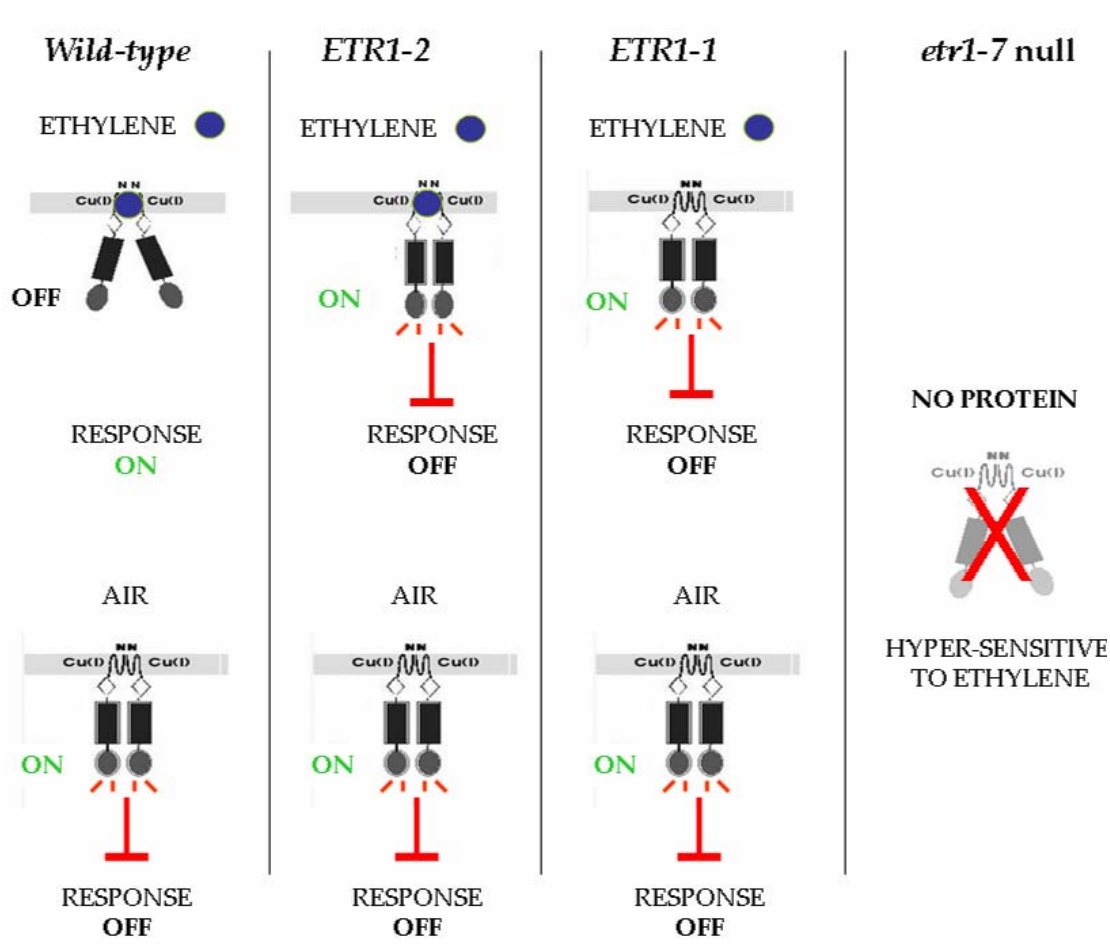


Figure 1-2. Ethylene Receptor Signaling.

Ethylene is perceived by the ethylene binding pocket of a receptor and upon ethylene binding causes conformational change(s) that turn 'OFF' the receptor signaling, allowing the ethylene response to occur. In receptor dominant gain of function mutants, the ethylene receptor is locked 'ON', either with the ability to bind ethylene (etr1-2) or not (etr1-1), and can not turn 'OFF' in the presence of ethylene. In an *etr1-7* loss of function mutant there is no ETR1 protein functional, therefore gives an ethylene hypersensitive phenotype.

function mutants are not rescued with silver, probably because silver makes its way to the receptors thru *RAN1* (Woeste and Kieber, 2000).

The ethylene receptors share sequence similarity with bacterial two-component histidine protein kinase receptors. In a typical bacterial two-component system, a sensor protein autophosphorylates in response to an external stimulus at a conserved histidine residue and then transfers the high energy phosphoryl group to an aspartate residue in the response regulator protein (Galperin et al., 2001). The ethylene receptors are divided into 2 subfamilies based on sequence similarities. In Arabidopsis, subfamily I (*ETR1* and *ERS1*) contains a conserved carboxy terminal histidine kinase domain (HK), three transmembrane domains at the amino terminus (ethylene binding domain, EBD), and a GAF domain; On the other hand, subfamily II (*ERS2, ETR2,* and *EIN4)* has a degenerate histidine kinase domain, a GAF domain, and contains four transmembrane domains at its amino terminus (EBD). Also, *ETR1* (subfamily I), *ETR2* and *EIN4* (subfamily II) have an additional receiver domain (RD) at their carboxy termini whose function has recently been suggested to be involved in an ethylene-dependent recovery growth rate (Binder et al., 2004). A cartoon of the receptors with all the domains is presented in Figure 1-3. The GAF domain was originally described as a noncatalytic cGMP-binding domain conserved in cyclic nucleotide phosphodiesterases (Charbonneau et al., 1990). Later, this domain was recognized in histidine kinases and other proteins. The role of the GAF domain in ethylene receptor signaling has yet to be determined. The five receptors have functional redundancy, yet subfamily I has a stronger effect than subfamily II in ethylene signaling (Hall and Bleecker, 2003; Qu et al., 2007). Genetic evidence

places ETR1 and ERS1 as key players in the ethylene signaling pathway; subfamily I null has a very severe ethylene response phenotype which can not be restored with any subfamily II receptor, even when the subfamily II receptors are driven by the *ETR1* promoter (Wang et al., 2003). Surprisingly, histidine kinase activity does not appear to be essential for ethylene signaling (Wang et al., 2003). Interestingly, subfamily I receptors which contain an intact (HK) motif have been shown to physically interact with CTR1 (Clark et al., 1998), the next known downstream component, which is a Raf-like serine/threonine protein kinase. The interaction with ETR1 and ERS1 is stronger than with ETR2, a subfamily II receptor which lacks the (HK) motif (Cancel and Larsen, 2002). This interaction beween a prokaryotic-type two-component histidine kinase and a eukaryotic mitogen activated protein kinase kinase kinase (MAPKKK) is not known in other signaling pathways.

Gene expression studies find the subfamily I *ETR1* receptor and the subfamily II *EIN4* receptor as constitutively expressed. On the other hand, the subfamily I *ERS1* and the subfamily II *ETR2* and *ERS2* are ethylene inducible (Ronan C. O'Malley et al., 2005). Recently, Dr. Schaller's and Dr. Klee's groups have shown that the Arabidopsis ethylene receptor ETR2 and the tomato ethylene receptor LeETR4 and LeETR6 are degraded by the 26S proteasome in a ethylene-dependent manner, respectively (Chen et al., 2007; Kevany et al., 2007). Ethylene-dependent degradation of either LeETR4 or LeETR6 results in early fruit ripening, suggesting that the control of receptor abundance increases sensitivity of plant tissues to ethylene

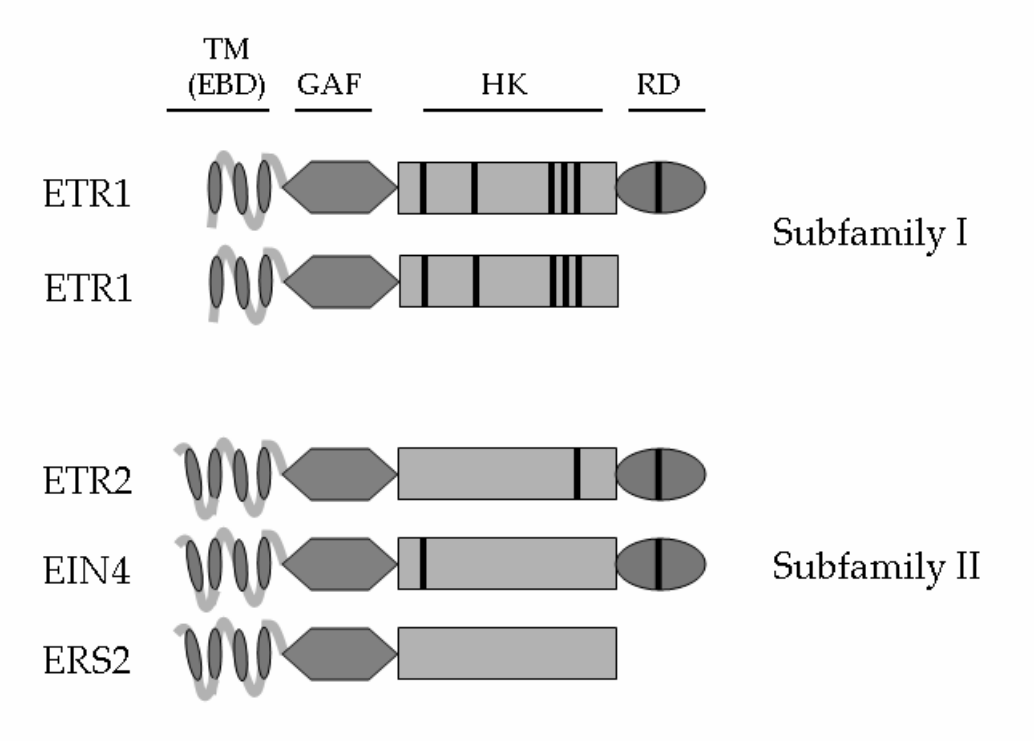


Figure 1-3. The Arabidopsis ethylene receptor family.

The ethylene receptors are divided into two subfamilies based on their gene structure/function. Subfamily I (ETR1 and ERS1) and Subfamily II (ETR2, EIN4, and ERS2) have three or four transmembrane domains where ethylene binds (ethylene binding domain, EBD), a GAF domain of unknown function, a Histidine Kinase domain (HK), and in some cases a receiver domain (RD). The conserved sequence motifs of histidine kinases and receiver domains are indicated by solid bars. Overall, Subfamily II contains four transmembrane domains and lacks many of the histidine kinases motifs.

(Kevany et al., 2007). Ongoing experiments from both groups are aimed to test whether this post-translational regulation is present for the other ethylene receptors.

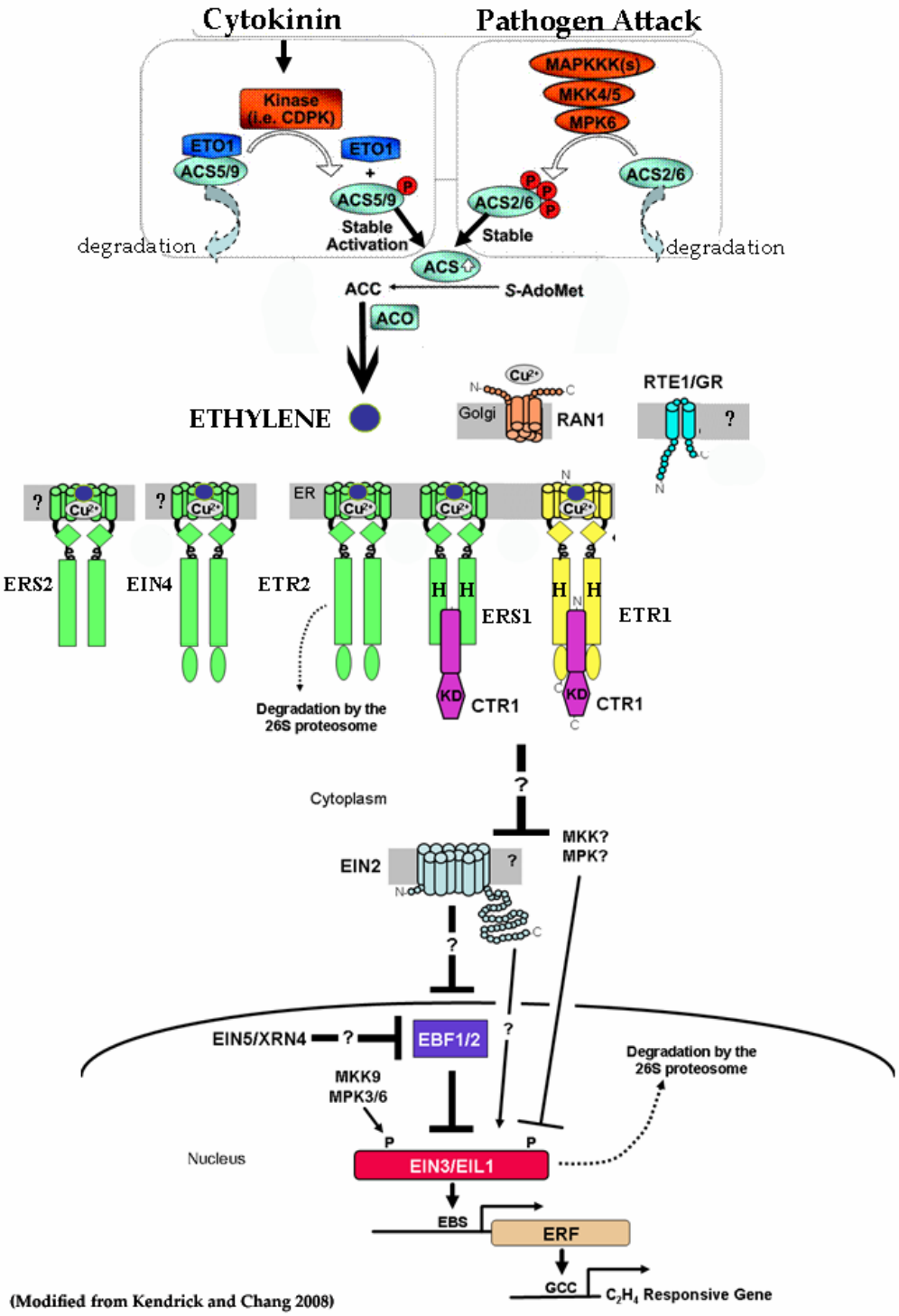
The ethylene receptor ETR1 has been shown to be localized to the endoplasmic reticulum (ER) by sucrose gradient fractionation in young Arabidopsis seedlings (Chen et al., 2002). *CTR1*, the next known downstream component of the pathway, is also a negative regulator of ethylene responses. CTR1 has also been localized to the ER, requiring the ethylene receptors for this localization (Gao et al., 2003). Whether CTR1 is always at the ER interacting with the receptors is still to be resolved. Moreover, the mechanism by which ETR1 activates CTR1 is still unclear and needs to be pursued. Recently, two publications from Klaus Harper's group and Eric Schaller's group demonstrated that the ethylene receptors interact with each other *in planta* forming heterodimers and large receptor complexes (Gao et al., 2008; Grefen et al., 2008). Both manuscripts show, either through a membrane recruitment assay (MeRA) (Grefen et al., 2008) or co-immunoprecipitation (Gao et al., 2008) experiments, *in vivo* evidence for receptor-receptor interaction. Interestingly, ETR1 interaction with subfamily II members (ETR2, EIN4, or ERS2) are more predominant than subfamily I interaction (Gao et al., 2008). This result supports the model where the subfamily II ethylene receptors require a subfamily I receptor in order to correctly signal to the downstream components (Xie et al., 2006).

Downstream components of the ethylene signaling pathway

By an unknown mechanism, *CTR1* activity represses *EIN2*, which is an N-RAMP family member metal transporter (Alonso et al., 1999). EIN2, the first positive regulator of ethylene responses in the pathway then signals to EIN3 and EIN like

proteins (EIL) when ethylene is present, which activate transcription of ethylene response factors such as ERF1. ERF1, another transcription factor, activates other effectors like pathogen related defense genes (*PDF1.2*) and differential regulated growth genes (*HOOKLESS1*) (Alonso et al., 2003). EIN3 has been recently shown to be regulated at the protein level by ethylene, an increasingly common theme appearing in plant signaling pathways. In the absence of ethylene, two F-box proteins (EBF1 and EBF2) part of a SCF complex degrade EIN3. When ethylene is present, EIN3 accumulates in the nucleus, activating the ethylene response (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004) (Figure 1-4).

Overall, the pathway so far has been linear with few "branching points". In summary, when ethylene is absent, the receptors signal to CTR1 which in turn signals to EIN2. In turn, EIN2 signals to EIN3 and EIN-like proteins to activate transcription. This pathway appears to be quiet linear, yet surprisingly, no MAPK module has been found for ethylene signaling to link CTR1 to EIN2. Contradictory data has been published on the search for the MAPK cascade (Ouaked et al., 2003; Ecker, 2004). To date, no real proof of a MAPK cascade module has been shown for the ethylene signaling pathway, yet genetic experiments show a MAPK cascade in regulating ethylene biosynthesis in the presence of external stimuli (biotic and abiotic stress) (Guo and Ecker, 2004). In any case, the novel interaction between CTR1 and ETR1 and how this combination of eukaryotic and prokaryotic-like components interacts with each other and regulates the ethylene response is of great interest.



(Modified from Kendrick and Chang 2008)

Figure 1-4. Ethylene biosynthesis and signaling pathway.

A model on ethylene biosynthesis and its signaling pathway. The figure is modified from (Kendrick and Chang, 2008). Ethylene biosynthesis is regulated by endogenous and exogenous cues. These cues in turn modify ethylene biosynthetic enzymes which delimit the amount of ethylene produced by the plant. The ethylene gas is then perceived by a family of five receptors presumably at the ER (ETR1). The receptors are inactivated by the binding of ethylene. The next downstream component, CTR1, also a negative regulator of the response, becomes inactivated by the occupied receptors. A MAPK module is proposed to act downstream of CTR1, although it has not been shown. The first of the following positive regulators of the response, EIN2, is a key player in the response yet no function has been assigned. EIN2 then activates EIN3, a transcription factor which in turn activates other transcription factors. EIN3 has also been shown to be regulated by ethylene via the 26 proteasome. Several of these transcription factors bind to primary ethylene response elements (PERE) such as ERF1 (ethylene response factor1) and up-regulate their expression.

RTE1, a novel regulator of the ethylene response

One of the main interests in our lab is to better understand the ethylene signaling pathway at the receptor level. Previous work in our lab has shown that the locus *RTE1* (*REVERSION-TO-ETHYLENE–SENSITIVITY1*) is a negative regulator of ethylene response through the positive regulation of ETR1 signaling (Resnick et al., 2006). *RTE1* was identified as a suppressor of the ethylene insensitive receptor mutation *etr1-2*, rendering the plant sensitive to ethylene. Further genetic work by the Chang laboratory (Resnick et al. 2006) demonstrated that the nature of *rte1* suppression of *etr1-2* was likely due to loss of *etr1* function. Genetic experiments demonstrated that an *rte1* null mutant largely phenocopies an *etr1* null mutant showing similar ethylene hypersensitivity. Moreover, consistent with *RTE1* being a negative regulator of ethylene response, over-expression of *RTE1* caused partial insensitivity to ethylene (Figure 1-5). Interestingly, the suppression of *RTE1* is *ETR1* allele-specific, suppressing *etr1-2* and not the stronger ethylene insensitive allele *etr1-1*, therefore suggesting the possibility that RTE1 may affect ETR1 at the protein level (Resnick et al., 2006). All these data are consistent with *RTE1* being a regulator of *ETR1* (Figure 1-5).

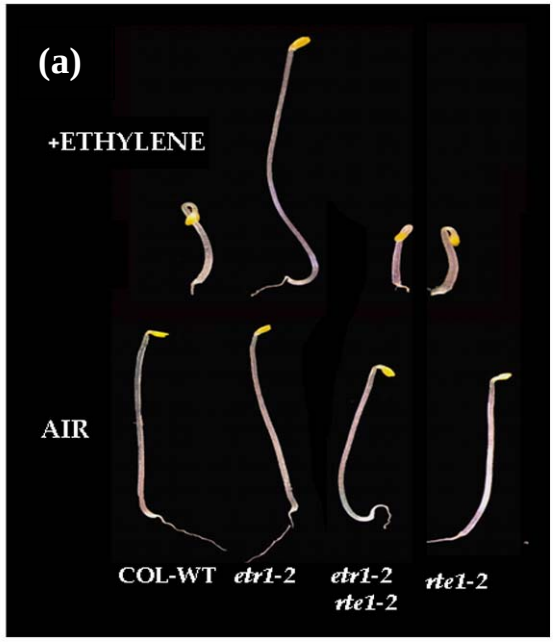
Arabidopsis *RTE1* encodes a novel integral membrane protein of 250 amino acids with three homologues in tomato, one of which has been recently shown to regulate ethylene responses. Green-ripe (*GR)*, the *RTE1* homologue in tomato was obtained as an ethylene insensitive mutant that over-expresses *GR* (Barry and Giovannoni, 2006). The ectopic expression of *GR* caused insensitivity that is consistent with the Arabidopsis *RTE1* over-expression studies (Barry and

Giovannoni, 2006; Klee, 2006). Unfortunately, there is no loss-of-function allele of *GR* and no identified target of *GR* action. Consequently, working on *RTE1* in Arabidopsis presents the best system to identify and characterize further the molecular function of *RTE1*. *RTE1* has one homologue in Arabidopsis, named *RTH* (RTE1-HOMOLOG) whose amino acid sequence shows 69% similarity (Figure 1-6 b). The *RTE* gene family is conserved in animals, plants and lower eukaryotes (except fungi) which suggests a conserved function (Figure 1-6 a) (Klee, 2006). The biochemical function of *RTE* is completely unknown in all organisms.

Our current understanding of *RTE* function comes entirely from studies in plants. *RTE1* expression is induced by ethylene (Alonso et al., 2003) and *RTH* has a similar profile as *RTE1* in the same plant tissues but with lower expression intensity and no ethylene induction (MPSS data: http://mpss.udel.edu/at and eFP browser: http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Moreover, both *RTE1* and *RTH* are up regulated in roots of seedlings where there has been nitrogen deprivation (Table 2 and NASC array browser: http://affy.arabidopsis.info/narrays.com).

How do prokaryotic-like ethylene receptors signal to eukaryotic-specific downstream components? How does *RTE1*, a very well conserved gene in animals and plants, regulate a prokaryotic-like receptor? What is the nature of this *ETR1*-*RTE1* association? These are the main questions of this thesis. This new component of the ethylene pathway (*RTE1*) is very intriguing and my thesis will try to elucidate how it is regulating the ethylene response thru ETR1. Our working hypothesis is that RTE1 specifically affects ETR1 at the protein level by affecting the protein: stability, sub-cellular localization, or the conformation(s) of ETR1 to signal correctly. We plan

to address these questions on *RTE1* function and why *RTE1* is specific to ETR1. In turn, studying *RTE1* in the context of the ethylene signaling pathway should help to shed light on the biochemical function of *RTE1* in broad biological systems. The fact that there is only one *RTE1* homolog in *Arabidopsis* (*RTH*) makes studying the role of *RTE* function in Arabidopsis quite feasible. The use of forward genetics in cloning *RTE1* and the use of reverse genetics for *RTH* and the double null *rte1 rth* will broaden our understanding of the biological role that RTE proteins play in plants and eventually in all other eukaryotic organisms.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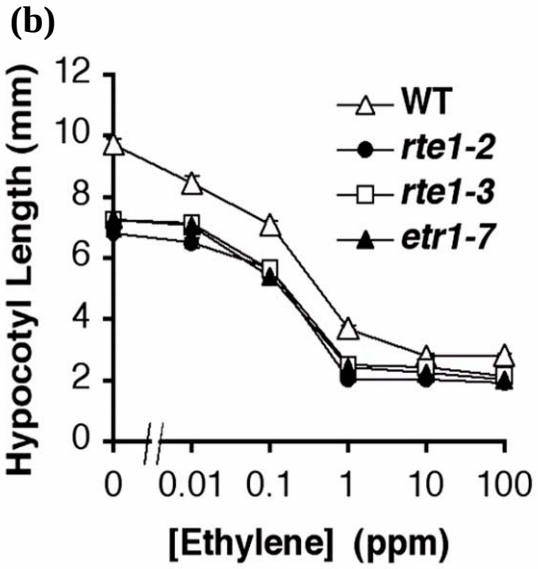
(b)
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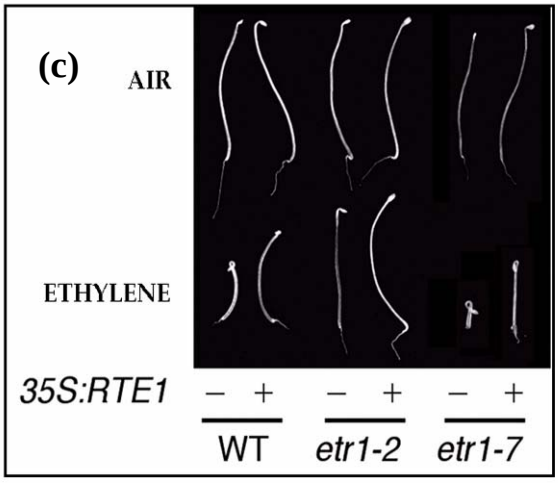
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WT
etr1-2
etr1-7

Figure 1-5. RTE1 is required for ETR1 receptor signaling.

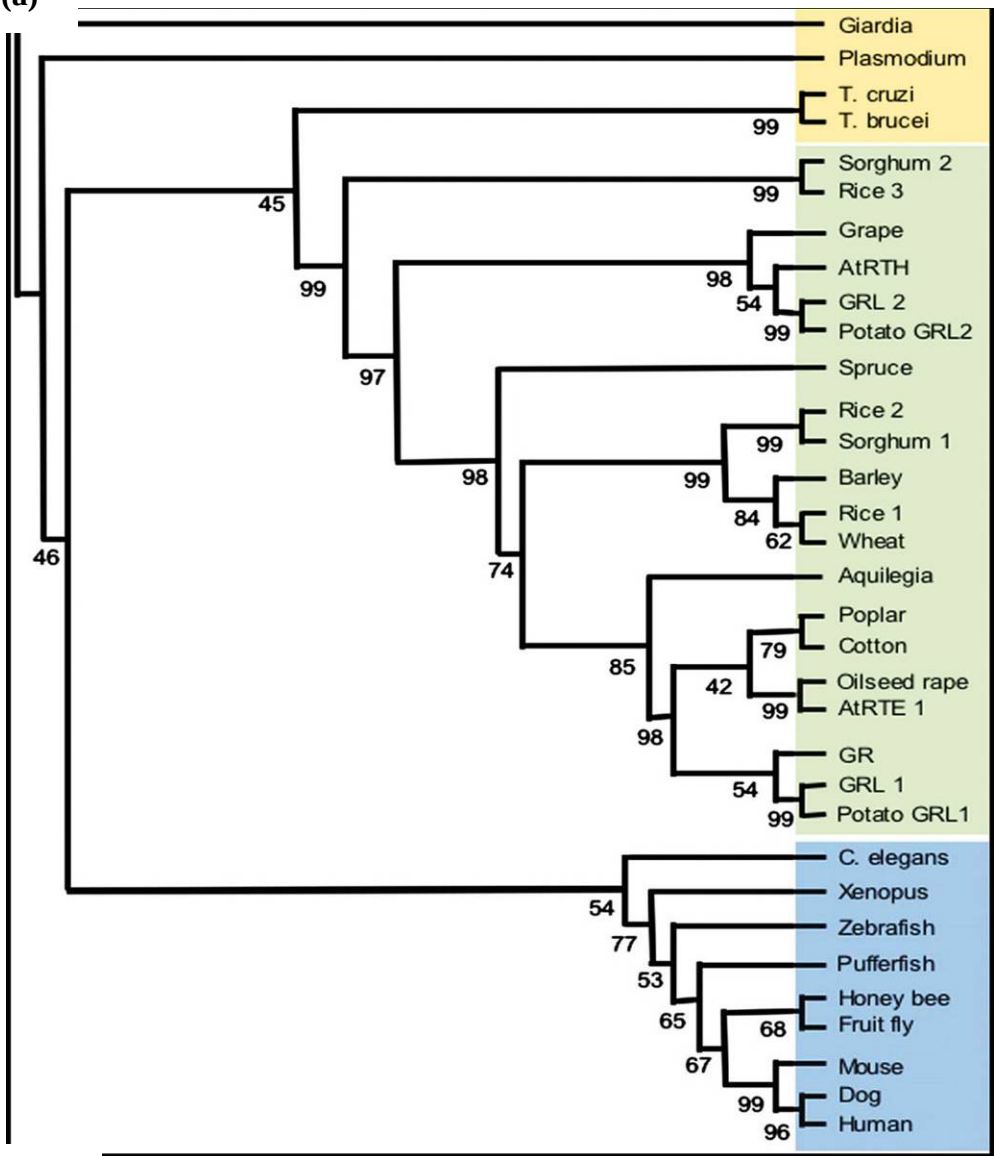
Figure is taken from Resnick et al., 2006.

(a) Comparison of 4-day-old dark-grown seedlings germinated in the presence or absence of the ethylene precursor ACC (100 μM). Representative seedlings of wild-type, ethylene insensitive *etr1-2*, suppressor line *etr1-2 rte1-2*, and single mutant *rte1-2*.

(b) Ethylene dose–response of hypocotyl length in 4-day-old dark-grown seedlings reveals similarity of enhanced ethylene sensitivity in *rte1-2*, *rte1-3*, and *etr1-7* in contrast to the wild type (WT). The mean ± SE is shown for 17–30 seedlings per genotype at each dose.

(c) Representative 4-day-old dark-grown seedlings of wild-type *etr1-2*, and the null mutant *etr1-7*, either untransformed (−) or stably transformed (+) with 35*S:RTE1* (over expression), germinated in the presence or absence of the ethylene precursor ACC (10 μM).

(a)



(b)

```
          MSRGRGVPMMDLKRSYDVEDRVVSVSIPSIIEADEADLWPLPEIDTKKSKFPCCIVWTPL 60
AtRTH     MG----------ETATDSEHRMMIG-----------LSDPMKIDPKRDRFPCCIVWTPL 38
          *.          : : * *.*::              *    :**.*:.:**********

AtRTE1    PVVSWLAPFIGHIGLCREDGVILDFAGSNFINVDDFAFGPPARYLQLDR---TKCCLPPN 117
AtRTH     PFISWLVPFIGHVGICREDGVILDFAGPNFVCVDNFAFGAVSRYIQINKEMESSRSSSSG 98
          *.:***.*****:*:************.**: **:****. :**:*:::   :. . ...

AtRTE1    MGGHTCKYGFKHTDFGTARTWDNALSSSTRSFEHKTYNIFTCNCHSFVANCLNRLCYGGS 177
AtRTH     MFNGERRYEQEEDSHEKEPTWDDALRKSTQEYQHHSYNILTCNCHSFVANNLNRLSIK-S 157
          * .   :*  :. .. .  ***:** .**:.::*::***:********** ****.   *

AtRTE1    MEWNMVNVAILLMIKGKWINGSSVVRSFLPCAVVTSLGVVLVGWPFLIGLSSFSLLLFAW 237
AtRTH     GGWNVVNLATLVLFKGRWVNKTAIVKSLLPPLIVYTIGILLGGWTFIASCSILVVLLTGW 217
            **:**:* *:::**:*:* :::*:*:**  :* ::*::* **.*: . * : :** .*

AtRTE1    FIIATYCFKNIIT- 250
AtRTH     FIIGTYCFKKLIQL 231
          ***.*****::*
```

Figure 1-6. Sequence analysis of the RTE family across different species .

(a) Phylogenetic analysis of RTE1/GR-related proteins. A non-rooted phylogenetic tree taken from Klee 2006.

(b) Amino acid sequence alignment between RTE1 and RTH of *Arabidopsis thaliana*. "*****" denotes conserved residues, "**:**" denotes conserved substitutions, and "**.**" denotes semi-conserved substitutions aligned using ClustalW2 (Larkin et al., 2007).

Table 2: Arabidopsis *RTE* family expression profiles from online gene expression repositories. Expression data for Arabidopsis *RTE1* and *RTH*

	High Abundant Expression levels
RTE1	leaves 21 root 21d inflorescence siliques (24-48h post fertilization) Nitrogen deprivation treated with ethylene treated with cytokinin
RTH	root 21d siliques (24-48h post fertilization) Nitrogen deprivation

Chapter 2: Sub-cellular co-localization of Arabidopsis RTE1 and ETR1 supports a regulatory role for RTE1 in ETR1 ethylene signaling

Introduction

According to the current model for ethylene receptor action, the receptors repress responses when ethylene is not bound, and are turned off when ethylene is bound, resulting in the activation of responses (Chen et al., 2005). The receptors have been shown to bind ethylene (Schaller and Bleecker, 1995; O'Malley et al., 2005) with the help of a copper cofactor Cu(I) (Rodriguez et al., 1999), which requires RAN1, a homolog of the Golgi-bound Menkes/Wilson P-type ATPase copper transporter in mammals (Hirayama et al., 1999; Woeste and Kieber, 2000). Yet, the molecular mechanism of ethylene receptor signaling remains unknown. .

The *REVERSION-TO-ETHYLENE SENSITIVITY1* (*RTE1*) gene was recently identified as a positive regulator of ETR1 signal transmission. *RTE1* homologs are found in plants, animals and protists, but currently the only ascribed function for *RTE1* is in ethylene signaling in plants (Barry and Giovannoni, 2006; Resnick et al., 2006). Over-expression of *RTE1* confers reduced ethylene sensitivity that is largely dependent on the *ETR1* locus (Resnick et al., 2006; Zhou et al., 2007). Similarly, over-expression of the tomato *RTE1* homolog, *GREEN-RIPE* (*GR*), confers ethylene insensitivity in tomato (Barry and Giovannoni, 2006). *rte1* does not suppress the gain-of-function allele *etr1-1*, nor does it suppress gain-of-function alleles of the four other

ethylene receptor genes, suggesting that RTE1 regulation is specific for the ETR1 receptor and is likely to occur at the protein level (Resnick et al., 2006).

Less is known about ethylene signaling at the cell biological level. Membrane fractionation studies have placed the ETR1 receptor at the endoplasmic reticulum (ER) (Chen et al., 2002). The Raf-like kinase CTR1, which is the next downstream component in the ethylene-response pathway (Kieber and Ecker, 1993; Clark et al., 1998) is recruited to the ER through physical interaction with the ethylene receptors (Gao et al., 2003). RTE1, on the other hand, has been co-localized with a marker at the Golgi apparatus (in onion epidermal cells) (Zhou et al., 2007). Sequence analyses predict that Arabidopsis RTE1 is an integral membrane protein carrying between two to four transmembrane domains (Aramemnon plant membrane protein database: http://aramemnon.botanik.uni-koeln.de/index.ep). Both RTE1 and ETR1 lack an obvious signal sequence for the secretory pathway, and there are no clear predictions of subcellular location for RTE1, RTE1 homologs or ETR1.

In order to gain a better understanding of RTE1 and its function in relation to the ETR1 receptor, we analyzed the gene expression pattern of *RTE1* and work done by lab members who obtained the subcellular localization of the RTE1 protein in Arabidopsis. We show here that *RTE1* expression is generally correlated with sites of *ETR1* expression and ethylene response. We also demonstrate that the RTE1 protein resides primarily at the Golgi and partially at the ER. In the course of this study, we found that the ETR1 receptor localizes not only at the ER as previously reported, but resides at the Golgi as well. By examining RTE1 and ETR1 simultaneously, we show that they exhibit subcellular co-localization. These findings provide cell biological

data in support of the model that RTE1 plays a role in regulating ETR1 ethylene signaling.

Results

RTE1 expression patterns in Arabidopsis

Gene array data indicate that *Arabidopsis RTE1* is expressed at detectable levels in most organs and stages and is up-regulated by ethylene (Resnick et al., 2006; Alonso et al., 2003). Based on previous gene array experiments, *RTE1* is most highly expressed in developing seeds and young siliques, with high expression also seen in seedlings and the shoot apex. Using a quantitative luciferase reporter, Zhou et al. (2007) observed expression of *RTE1* in cotyledons, leaves, the rachis and flowers, with lower expression seen in the seedling root and hypocotyl.

To further examine *RTE1* gene expression, we fused the *RTE1* promoter region (consisting of a 2.5 kb genomic DNA fragment just upstream of the *RTE1* translation start codon and including the 5' UTR of *RTE1*) with the β-glucuronidase (*GUS*) reporter gene and transformed the resulting construct into wild-type Arabidopsis plants by *Agrobacterium* infiltration (Clough and Bent, 1998). The expression pattern of *RTE1* was observed by staining for *GUS* activity in the transgenic lines. Two or more independent lines were obtained and tested in all conditions. As shown in Figure 1, there is strong expression of *RTE1* in 1- to 4-day old seedlings in the apical hook, cotyledons, root vascular tissue, root tip and root hairs, with little or no

expression in the hypocotyl. The pattern of expression was similar between dark- and light-grown seedlings (Figure 2-1 a-d). In light-grown seedlings, expression could also be seen in the apex and young leaves (Figure 2-1 d, g, i) and disappeared from the cotyledons by 10 days (Figure 2-1 i). In mature plants, *RTE1* was expressed in floral buds (Figure 2-1j), the style of mature flowers (Figure 2-1k), stems and the rachis (not shown).

To analyze the effect of ethylene treatment on *RTE1* expression, we examined the *GUS* staining pattern of etiolated seedlings germinated in the presence of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). We also examined the effect of $AgNO_3$ (an inhibitor of ethylene response (Beyer, 1976)), as well as the effect on expression when the reporter construct was transformed into the ethylene-insensitive *etr1-1* mutant background. Etiolated seedlings responding to ethylene or ACC treatment display the "triple response" phenotype, which consists of shortening and radial swelling of the hypocotyl, inhibition of root growth, proliferation of root hairs and an exaggerated apical hook, whereas seedlings treated with $AgNO_3$ or in the *etr1-1* background have longer hypocotyls and roots (Bleecker et al., 1998). Based on a qualitative assessment of GUS staining, *RTE1* transcript levels in comparison to untreated seedlings were enhanced by ACC and reduced by $AgNO_3$ and *etr1-1*, although the pattern itself was unaltered by the treatments (Figure 2-1 l-w). These results suggest that there is a negative feedback mechanism in ethylene signaling through the regulation of *RTE1* expression, since *RTE1* is a negative regulator of ethylene responses.

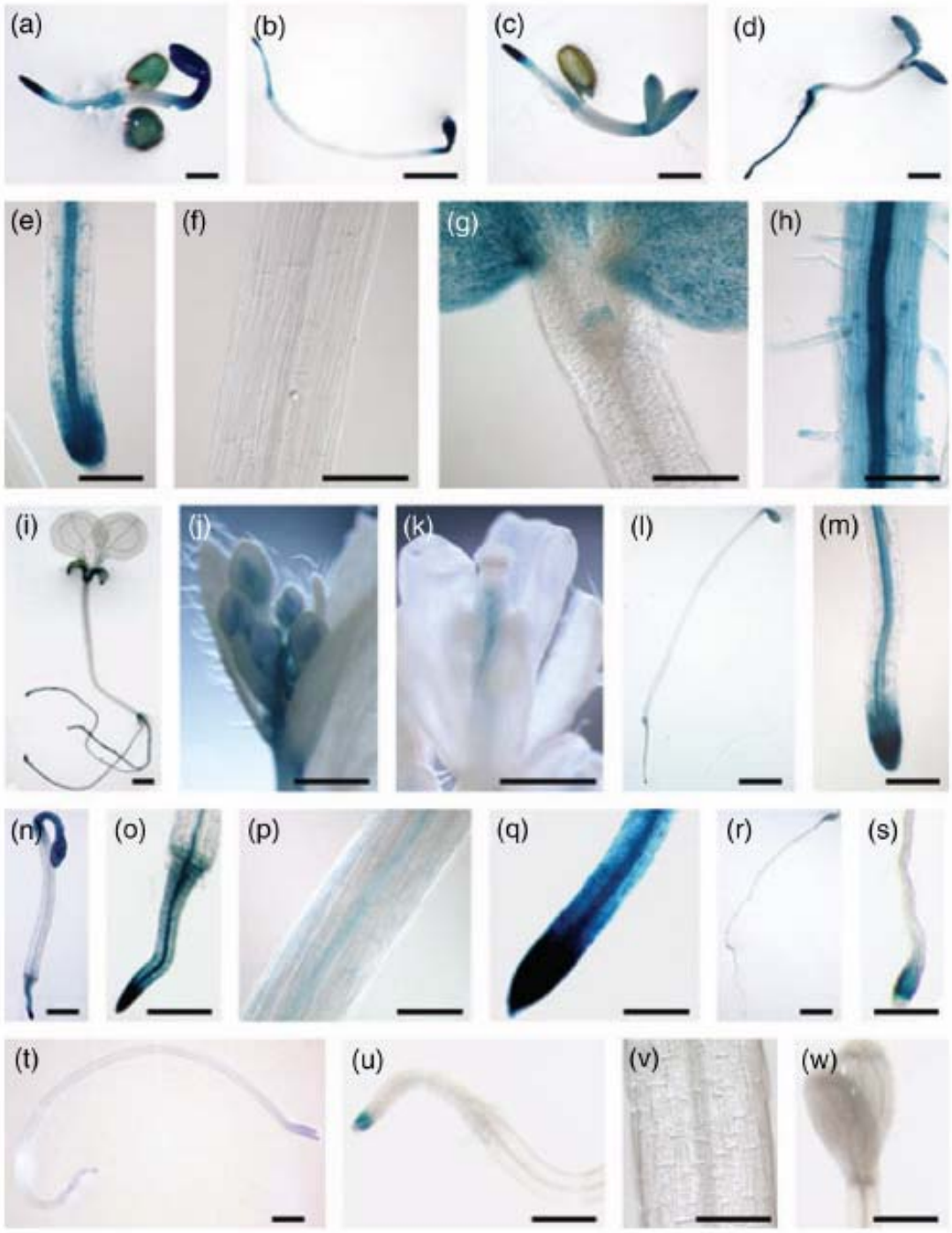

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)
(n)
(o)
(p)
(q)
(r)
(s)
(t)
(u)
(v)
(w)

Figure 2-1 *RTE1promoter*::GUS expression patterns.

Representative GUS expression is seen in the following wild-type tissues: **(a)** cotyledons, apical hook and root of a 1-day-old dark-grown seedling; **(b)** cotyledons, apical hook and root of a 3-day-old dark-grown seedling; **(c)** cotyledons and root of a 1-day-old light-grown seedling; **(d)** cotyledons, root and shoot apex of a 3-day-old light-grown seedling; **(e)** vascular tissue and the root tip of a 3-day-old dark grown seedling; **(g)** cotyledons and shoot apex of a 3-day-old light grown seedling; **(h)** root, including root hairs, of a 3-day-old light-grown seedling; **(i)** developing leaves and roots of a 10-day-old light-grown seedling; **(j)** floral buds; **(k)** style of mature flower. **(f)** No expression is detected in the hypocotyl of a 3-day-old dark-grown seedling. **(l–w)** Representative 4-day-old dark-grown seedlings subjected to various treatments: **(l)** no treatment; **(m)** root (close-up) of seedling with no treatment; **(n)** germinated on medium containing 100 μM ACC; **(o)** root (close-up) of seedling germinated on medium containing 100 μM ACC; **(p)** hypocotyl (close-up) of seedling germinated on medium containing 100 μM ACC; **(q)** root tip (close-up) of seedling germinated on medium containing 100 μM ACC; **(r)** germinated on medium containing 10 μM AgNO3 (an inhibitor of ethylene response); **(s)** root of seedling grown on medium containing 10 μM AgNO3, showing weak expression at the root tip; **(t)** etr1–1 seedling with no treatment; **(u)** etr1–1 seedling root, showing weak expression at the root tip; **(v)** etr1–1 seedling hypocotyl (close-up), showing no detectable expression; **(w)** etr1–1 seedling cotyledons, showing no detectable expression. Scale bars = 1 mm in (a–d,i–l,n,o,r–u,w) and 100 μm in (e–h,m,p,q,v).

Localization of ETR1-5xMyc in Arabidopsis root hair cells

RTE1 has been shown to be localized mainly to the Golgi compartment in root cells by another lab member (Dong et al., 2008). Subsequently, we were interested in investigating whether ETR1 and RTE1 share similar sub-cellular localization. The low expression levels of the ETR1 receptor have made the use of a fluorescent tag on ETR1 protein not possible for sub-cellular localization studies. To increase the sensitivity for detection of ETR1, we used a 5xMyc epitope tag fused at the carboxyl-terminus of ETR1. The ETR1-5xMyc fusion was expressed under the control of the native *ETR1* promoter region (comprising 3.2 kb upstream of the *ETR1* translation start site) (Figure 2-2a). We determined that the ETR1-5xMyc fusion construct possessed wild-type ETR1 activity by transforming it into the *etr1 etr2 ein4* triple null mutant, which has a constitutive ethylene-response phenotype. We examined five independent transgenic lines and found that the triple null phenotype was rescued to the less severe *etr2 ein4* double null mutant phenotype (Figure 2-2b). In all subsequent analyses with the ETR1 protein, the ETR1-5xMyc construct was stably transformed into either the *etr1-7* null mutant or wild-type plants.

To ensure that the ETR1-5xMyc fusion protein was intact in the transformed lines, we isolated protein from the transformed plants and visualized the protein on a western blot using an anti-Myc antibody. The results consistently showed a single band of the correct monomer size on a western blot of a denaturing PAGE gel (Figure 2-2c). There was also a single band of the predicted molecular weight for the dimer under non-denaturing conditions (data not shown). Occasionally, a single non-

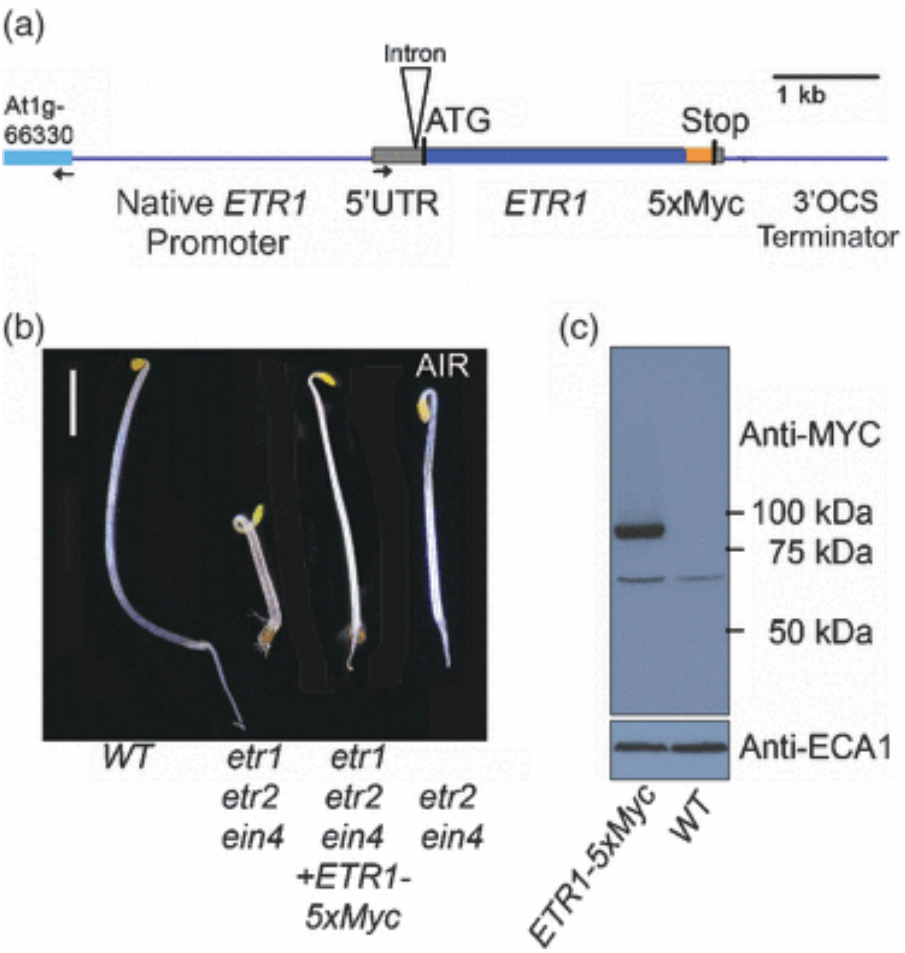

(a)
Intron
ATG
Stop
1 kb
At1g-
66330
Native ETR1
Promoter
5'UTR
ETR1
5xMyc
3'OCS
Terminator
(b)
AIR
WT
etr1
etr2
ein4
etr1
etr2
ein4
+ETR1-
5xMyc
etr2
ein4
(c)
Anti-MYC
100 kDa
75 kDa
50 kDa
Anti-ECA1
ETR1-5xMyc
WT

Figure 2-2. Function and detection of the ETR1 receptor fused with an epitope tag (5xMyc).

(a) Diagram of the Myc epitope (5xMyc)-tagged ETR1 construct driven by the native ETR1 promoter region. Shown are the promoter region, which includes a small portion of the flanking gene in the genome (light blue), the *ETR1* 5' UTR (gray) with native intron, the *ETR1* coding sequence (dark blue), the 5xMyc epitope translational fusion (orange) and 3' OCS terminator. Arrows indicate the direction of transcription.

(b) Rescue of the *etr1-7* null mutation in the Arabidopsis triple receptor null mutant (*etr1-6 etr2-3 ein4-4*) by ETR1–5xMyc. Representative 4-day-old dark-grown seedlings in air (no ethylene treatment) show that ETR1–5xMyc rescues the *etr1-6* mutation, alleviating the constitutive triple response and restoring the triple mutant to the *etr2-3 ein4-4* double null phenotype. Scale bar = 2 mm.

(c) Western blot showing the intact ETR1–5xMyc monomer isolated from the microsomal membrane fraction of Arabidopsis seedlings run on denaturing PAGE and detected by an anti-c-myc antibody. ETR1–5xMyc transformed into *etr1-7* gives a predominant band of approximately 80 kDa (left lane), which is absent in the untransformed wild-type (right lane). A non-specific band of lower molecular weight is detected in both samples. ECA1, an ER-membrane protein (Liang *et al.*, 1997), was used as a loading control.

specific background band of smaller size was detected, whether or not the samples carried the ETR1-5xMyc protein (Figure 2-2c).

Given that the anti-Myc antibody detected an intact fusion protein, we proceeded with immunohistochemistry of root hair cells of plants that had been stably transformed with ETR1-5xMyc. The GFP-HDEL and ST-GFP marker constructs (used above) were transformed into the ETR1-5xMyc lines to generate separate lines expressing ETR1-5xMyc with each marker. Notably, substantial co-localization of ETR1-5xMyc was observed with the Golgi marker (Figure 2-3a) and partial co-localization was also seen with the ER marker (Figure 2-3b). No signal was observed in root hair cells of untransformed seedlings that were fixed and treated in parallel with the anti-Myc antibody, indicating that the background band seen in the western blot was not detected by this method (data not shown).

Co-localization of RTE1 and ETR1 in Arabidopsis root hair cells

Next, we examined whether RTE1 co-localizes with the ETR1 receptor. To obtain Arabidopsis plants harboring both RFP-RTE1 and ETR1-5xMyc, we crossed the individual transformants (above) together and allowed the resulting F_1 to self-pollinate to produce F_2 seeds. Whole roots from 4-day old F_2 seedlings were analyzed by immunohistochemistry using a monoclonal anti-Myc antibody. The immunolocalization of ETR1-5xMyc, as well as RFP fluorescence from RFP-RTE1, was viewed in root hair cells by confocal laser scanning microscopy. As shown in Figure 2-4, co-localization of RTE1 and ETR1 was observed. No signal was detected in root hair cells for approximately half of the untransformed seedlings, which were

segregating in the F2 population in this experiment, indicating the absence of non-specific background signal from this method (data not shown).

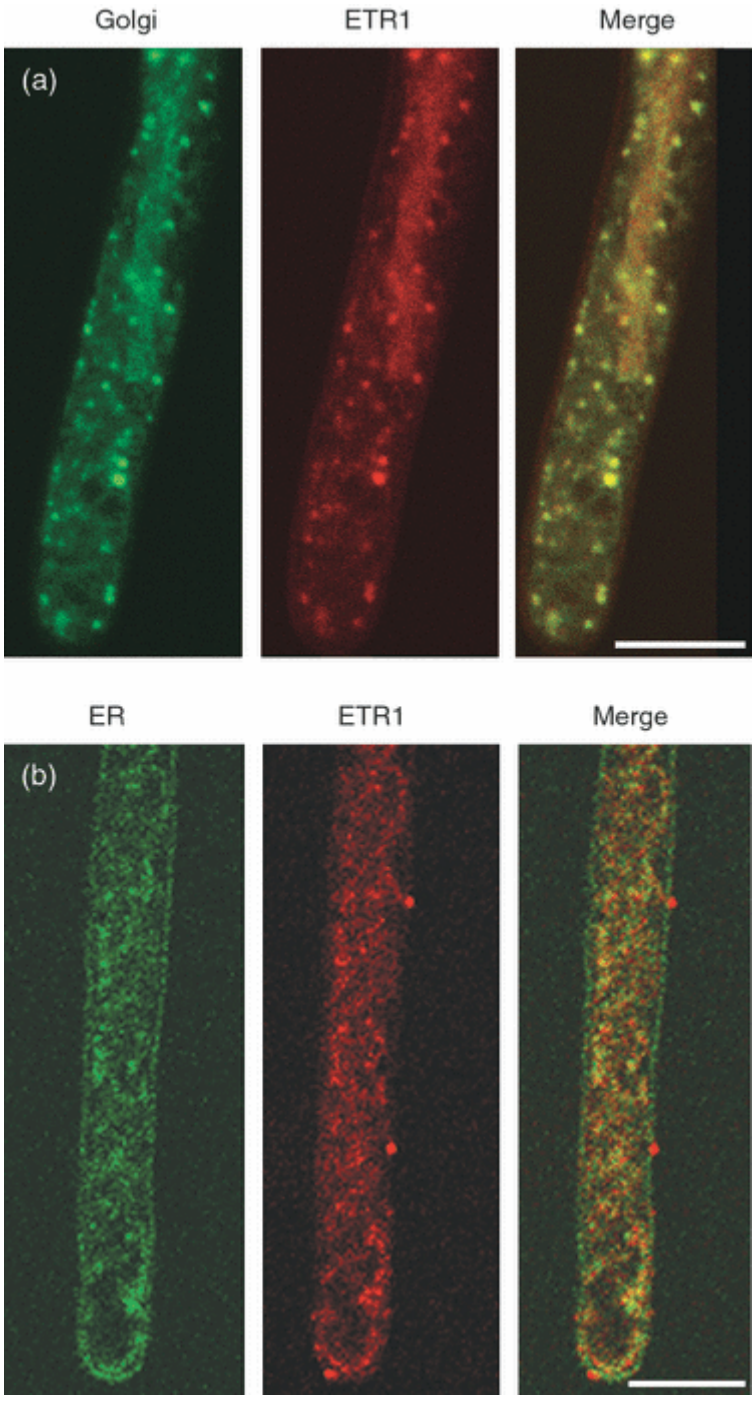

Golgi
ETR1
Merge
(a)
ER
ETR1
Merge
(b)

Figure 2-3. Localization of ETR1–5xMyc at the Golgi apparatus and ER in Arabidopsis root hair cells.

Representative root hair cells viewed by confocal laser scanning microscopy.

(a) Root hair cell of a 5-day-old light-grown seedling expressing both ST–GFP (Golgi) and ETR1–5xMyc, visualized by immunohistochemistry using an anti-c-myc antibody.

(b) Root hair cell of a 5-day-old light-grown seedling expressing both GFP–HDEL (ER) and ETR1–5xMyc, visualized by immunohistochemistry using an anti-c-myc antibody.

Scale bars = 10 μm.

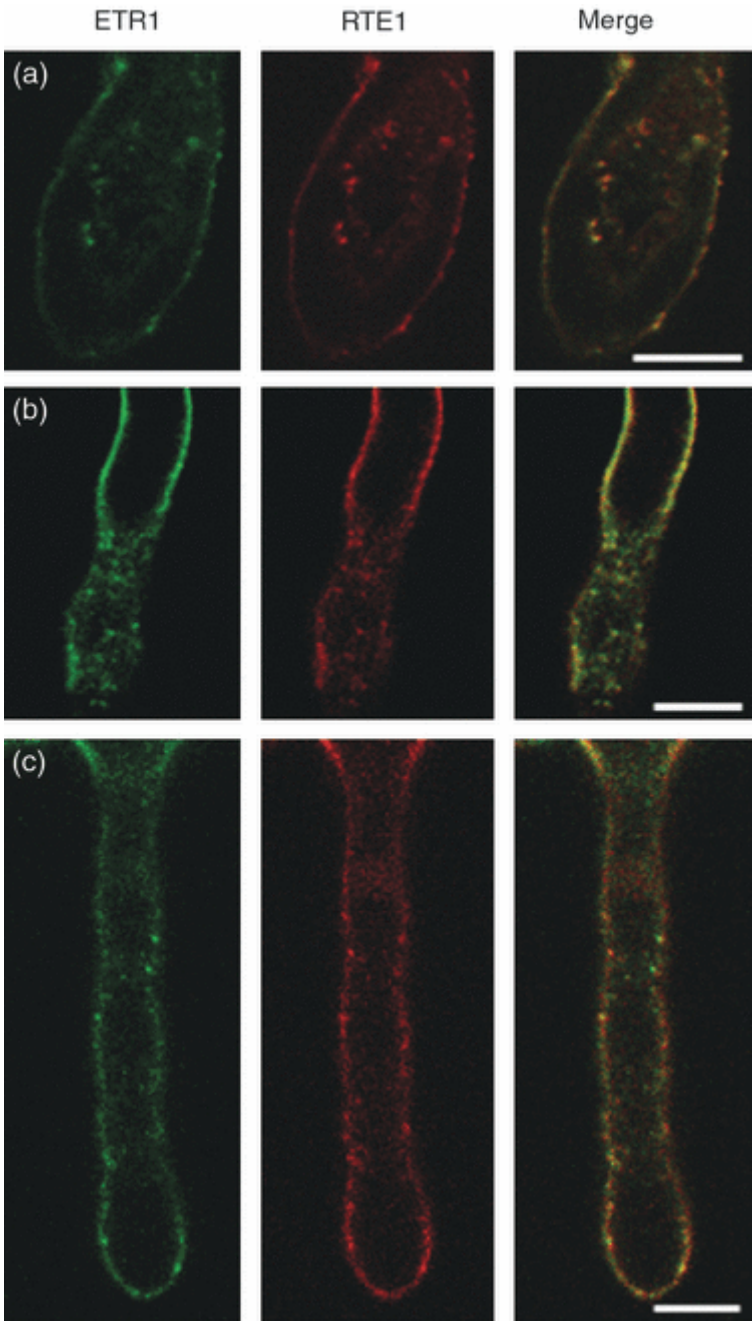

ETR1
RTE1
Merge
(a)
(b)
(c)

Figure 2-4. Co-localization of RTE1 and ETR1 in Arabidopsis root hair cells.

Representative root hair cells viewed by confocal laser scanning microscopy.

(a–c) Three root hair cells of 5-day-old light-grown seedlings expressing both ETR1–5xMyc and RFP–RTE1. RFP–RTE1 is visualized by fluorescence and ETR1–5xMyc is visualized by immunohistochemistry. Scale bars = 10 μm.

Discussion

Previous genetic analyses have indicated that Arabidopsis *RTE1* is a positive regulator of ETR1 ethylene receptor function (Resnick et al., 2006; Zhou et al., 2007). In this paper, we advance our understanding of RTE1 and ETR1 function at the cell biological level, providing data that support and enhance the genetic model.

The GUS reporter analysis of the *RTE1* promoter revealed that *RTE1* has discrete and specific expression patterns, some of which can be correlated with sites of *ETR1* expression (Grefen et al., 2008) and ethylene response. *RTE1* is strongly expressed in the seedling apical hook, root tip and root hairs – all cells that are linked to ethylene-inducible rapid cell division and/or cell elongation (Dolan, 2001; Raz and Koornneef, 2001; Ortega-Martinez et al., 2007). While *RTE1* shows little or no expression in the hypocotyl, the hypocotyl is derived from cells that have passed through the apical hook (Raz and Ecker, 1999) where *RTE1* expression is high. *RTE1* is also expressed in developing leaves, young cotyledons, stems, rachis and style. The *RTE1* expression pattern partly overlaps with expression of the *ETR1* receptor gene, which has been detected by in situ hybridization in etiolated seedlings (Hua et al., 1998; Raz and Ecker, 1999), although *ETR1* expression is higher in the hypocotyl and weaker in the apical hook in 2- and 3-day old seedlings (Raz and Ecker, 1999; Grefen et al., 2008). *ETR1* is also expressed in stems and leaves, and in the locules of anthers, developing carpels and ovules (Hua et al., 1998). Unlike *ETR1* expression, which is not ethylene induced (Hua et al., 1998), *RTE1* expression is enhanced upon ethylene treatment and reduced when ethylene signaling is blocked, suggesting a

mechanism of negative feedback on the response pathway. The ethylene-enhanced expression we observed is consistent with array data indicating that exposure to ethylene results in a four-fold increase in *RTE1* transcript levels (Alonso et al., 2003) as also seen in RNA blots (Resnick et al., 2006). These findings, showing that *RTE1* is expressed preferentially at several important sites for ethylene response and that expression is responsive to ethylene, are consistent with *RTE1* having a regulatory role in ethylene signaling.

The RTE1 protein was visualized by Dr. Chunhai Dong in living Arabidopsis cells (protoplasts, root cells and root hair cells) using the *RTE1* native promoter and a red fluorescent protein tag. He found that RTE1 is localized predominantly at the Golgi apparatus and partially at the ER. We do not rule out the possibility of a small amount of RTE1 localization at the vacuole, based on the examination of protoplasts co-expressing RFP-RTE1 and a vacuole marker (Dong et al., 2008). The ER is one of the major components of the endomembrane system, closely connected with the Golgi apparatus and vacuoles (Hawes and Satiat-Jeunemaitre, 2005). Dr. Chunhai Dong found that there was no obvious localization of RTE1 at the plasma membrane, peroxisome, mitochondrion or plastid organelles. In addition, he did not detect any alteration in the subcellular localization of RTE1 when the seedlings were treated with ethylene, consistent with the findings of Zhou et al. (2007). Zhou et al. (2007) showed that a CaMV 35S-driven GFP-tagged RTE1 fusion was localized at the Golgi in onion epidermal cells.

Interestingly, we found that the ETR1 receptor is localized primarily at the Golgi, and partially at the ER, in Arabidopsis root hair cells. Chen et al. (2002)

previously reported localization of ETR1 at the ER, but did not rule out the possibility of Golgi localization. ER-localization was based on the co-fractionation of ETR1 and an ER marker by sucrose density-gradient centrifugation, in which the Golgi-containing fractions showed a similar, but slightly broader distribution, than that of the ER fractions (Chen et al., 2002; Chen et al., 2007). The ER and Golgi fractions exhibited the same shift from higher to lower density in the absence of Mg^{2+}, indicating that the ER and Golgi are not easily resolved by this method (Chen et al., 2002; Chen et al., 2007). There are known structural and functional links between the ER and Golgi, and in fact, a continuum between the ER and Golgi has been proposed by Hawes and Satiat-Jeunemaitre (2005). Conceivably, ETR1 is differentially localized depending on the stage or type of cell, thus yielding different results depending on the cell types examined. For the previously published sucrose-density gradient centrifugation, protein was extracted from plants grown in liquid culture containing predominantly green tissue. Additionally, leaf cells were examined by immunoelectron microscopy, but again the results did not rule out the possibility of ETR1 localized at the Golgi (Chen et al., 2002). In the study presented here, the ETR1-5xMyc fusion was localized by immunohistochemistry of intact root hair cells. Although the 5xMyc-epitope tag could potentially lead to artifacts, the ETR1-5xMyc construct was able to rescue an *etr1* null mutation and the ETR1-5xMyc fusion protein was seen in western blots as an intact band of the expected molecular weight. There was no distinct background signal detected by immunofluorescence microscopy of root hair cells, even though in western blots a single faint band was occasionally detected whether or not the plants carried the ETR1-5xMyc construct.

Localization of ETR1 at the Golgi presents an interesting modification to our current understanding of ethylene receptor signaling, but is consistent with the overall model of ethylene signaling. Due to the solubility of ethylene in aqueous and lipid environments, ethylene should be readily perceived by receptors residing at either organelle (Abeles et al., 1992). The receptors require a copper cofactor in order to bind ethylene (Rodriguez et al., 1999), and it is believed that this copper is delivered by RAN1 (Hirayama et al., 1999; Woeste and Kieber, 2000). RAN1 is a homolog of the mammalian Menkes/Wilson P-type ATPase copper transporter, which has been localized (in mammals) at the Golgi membrane and delivers copper to the lumen (Petris et al., 1996). RAN1 is similarly localized at the Golgi in plants (Dunkley et al., 2006), then copper could be directly supplied to the Golgi-associated ETR1 receptor, providing a cell biological link between RAN1 and ethylene receptor signaling. Another possible connection is the fact that certain ethylene-induced responses require Golgi-specific functions, thereby associating ETR1 and RTE1 with a site of ethylene response. For example, cell wall synthesis is required for the processes of cell elongation and expansion, which occur in certain responses to ethylene, such as at the apical hook and in root hair elongation. The components for cell wall synthesis are produced at the Golgi (Lerouxel et al., 2006), and thus the regulation of these processes by ethylene could involve co-localization of ETR1 and RTE1 with components in the Golgi, in a manner similar to what Chen et al. (2005) has proposed for ER-localized ethylene receptors. Not all ethylene receptors may be localized at the ER or Golgi. ETR2 (Chen et al., 2007) and the melon ethylene receptor CmERS1 (subfamily I) have been localized to the ER using sucrose density gradient

fractionation (Ma et al., 2006), but tobacco NTHK1 (subfamily II) appears to localize at the plasma membrane (PM) (Xie et al., 2003), and unpublished work suggests that tomato NEVER-RIPE (subfamily I) may reside at the PM as well (Klee and Tieman, personal communication 2002).

The subcellular co-localization of RTE1 and ETR1 supports the possibility that RTE1 promotes ETR1 signaling through physical interaction with ETR1. Whether a physical interaction occurs between these proteins is currently under investigation. If RTE1 acts directly on ETR1, then RTE1 might serve as a molecular chaperone or cofactor for ETR1, or affect the membrane trafficking or stability of ETR1. Alternatively, RTE1 could exert an indirect effect, such as altering the conformation of ETR1 via changes to the membrane or to other proteins or changes in the status of copper. If the other ethylene receptors in Arabidopsis prove to be localized primarily to other tissues or membranes relative to RTE1, then co-localization with ETR1 might be an underlying basis for the specificity of RTE1 for ETR1. Differential tissue localization of ethylene receptors has been postulated for the non-global ethylene effects of *GR* over-expression in tomato (Barry and Giovannoni, 2007). Further insight into the connections between RTE1 and ETR1 should advance our understanding of the basis for RTE1's regulation of, and specificity for, the ETR1 receptor in ethylene signaling.

Experimental Procedures

Plant growth and transformation

Arabidopsis thaliana plants (ecotype Columbia (Col-0), unless noted) were grown in soil under 16-h light/8-h dark in a controlled environment chamber at 20^{o}C under white fluorescent light. For seedling growth, seeds were sown on MS plates containing 0.8% agar. After stratification for 3 days at 4^{o}C, the seeds were incubated at 20^{o}C either under continuous light or in the dark for the indicated lengths of time. Transgenic plants were generated by the floral dip infiltration method mediated by *Agrobacterium tumefaciens* (Clough and Bent, 1998) strain GV3101. To select for transformed plants, we used either hygromycin (250 mg/L) or Basta (0.1% Finale^{TM} sprayed onto seedlings), depending on the binary vector used.

The triple response assay was performed as described (Resnick et al., 2006) using the stated concentrations of ACC or $AgNO_3$ in the medium.

Construction of *RTE1*$_{promoter}$ and *ETR1-5xMYC* reporter fusions

To construct the *RTE1*$_{promoter}$-GUS fusion, a DNA fragment containing the *RTE1* promoter region (2,485 bp upstream from the *RTE1* start codon, which includes the intron located in the *RTE1* 5'UTR) was PCR-amplified from Arabidopsis wild-type genomic DNA using the following primers;

5'-GGATGATGTGATCACCATCG-3' and

5'-TTTTAGATTCCTAATCACACAAGAC-3'

The PCR product was cloned into the pCR8/GW/TOPO TA Cloning plasmid vector (Invitrogen) and verified by nucleotide sequencing. Using the Gateway recombination system (Invitrogen), the *RTE1* promoter region was inserted upstream of the *GUS* reporter gene in the binary vector pBGWFS7 (Karimi et al., 2002).

To generate the *ETR1*-5xMyc construct, a 3.9Kb *Pst*I-*Bst*XI genomic DNA fragment containing the *ETR1* promoter region (3,167 bp upstream from the *ETR1* start codon including the native intron located in the 5'UTR) plus 733 bp of the *ETR1* coding sequence, was cloned into plasmid pBJ36 (Gleave, 1992) just upstream of the 3'UTR OCS terminator sequence. Just downstream of this *ETR1* fragment, we inserted a 1,593 bp *Bst*XI -*Bam*HI *ETR1* cDNA fragment (including the stop codon and 25 bp of the *ETR1* 3'UTR). Next, a fragment of approximately 400 bp containing the 3' end of the *ETR1* coding region was PCR-amplified, replacing the stop codon with *Stu*I-*Bam*HI restriction sites. After digesting the fragment with both *Afl*II (a natural internal site in the *ETR1* coding sequence) and *Bam*HI, the fragment was used to replace the *Afl*II-*Bam*HI fragment of the above construct (in which the *Bam*HI site was located just after the *ETR1* stop codon.) A *Stu*I-*Stu*I DNA fragment containing five copies of the Myc epitope (5xMyc) followed by a stop codon (from clone CD3-128; Arabidopsis Biological Resource Center, The Ohio State University) was then cloned in frame into the introduced *Stu*I site. The clone was verified by nucleotide sequencing, and then the entire composite gene including the OCS terminator was released with *Not*I and ligated into the *Not*I site of the binary vector pMLBart for stable plant transformation.

Fluorescent protein-tagged markers for organelle localization

The established fluorescent protein markers used in this study were: 1) GFP-HDEL (pVKH18En6-mGFPer) for the ER (Claude M. Saint-Jore, 2002), 2) ST-GFP (pVKH18En6-STtmd-GFP) for the Golgi apparatus (Claude M. Saint-Jore, 2002).

Histochemistry

GUS staining was carried out as previously described (Dong et al., 2001). Images of GUS-stained plants were obtained using a Nikon SMZ1000 dissecting microscope or a Nikon Eclipse E600 microscope using DIC.

For immunohistochemistry of ETR1-5xMyc, Arabidopsis seedlings were grown under white light and prepared essentially as described by (Friml et al., 2003). In brief, 5-day old light-grown seedlings were fixed in 4% paraformaldehyde in MTSB (50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$ (pH 7) adjusted with KOH) for 1 h. Samples were washed with MTSB/0.1% Triton (5-10 min) and with de-ionized water (5-10 min). Cell walls were digested with 1% Cellulase and 0.1% Maceroenzyme in MTSB for 30 min, and then samples were washed with MTSB/0.1% Triton (5-10 min). Incubation with 10% DMSO/3% NP-40 in MTSB for 1 h followed. After another washing in MTSB/0.1% Triton (5-10 min), seedlings were pre-incubated in 2% BSA/MTSB (1 h at 37°C) and incubated overnight (4°C) with the primary antibody, which was mouse monoclonal anti-c-Myc antibody (Invitrogen) at 1:200 dilution. After extensive washing with MTSB/0.1% Triton (8-10 min), the seedlings were incubated with 1:500 dilution of the appropriate secondary antibody in 3% BSA/MTSB (3 h at 37°C). The secondary antibody for co-localization with the GFP-tagged ER and Golgi markers was Alexa Fluor 633 goat anti-mouse IgG (H+L) (Invitrogen). For co-localization with RFP-RTE1, the secondary antibody was Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrogen). The samples were washed with

MTSB/0.1% Triton (5-10 min, then overnight) and transferred into VectaShield (Vector Company) mounting medium.

Fluorescence microscopy

Imaging of fluorescent proteins in protoplasts or seedling roots was conducted under a laser scanning confocal microscope (Zeiss LSM510). The excitation wavelengths (nm) for GFP and RFP were 488 and 543 respectively, and the emission filter wavelengths (nm) were 505-530 for GFP and 560-615 for RFP in the settings. Protoplasts were directly mounted on a glass slide in buffer solution (0.5M mannitol, 4mM MES (pH 5.7), 20mM KCl), and seedling root fragments were mounted in water for visualization of the fluorescent proteins. For immunohistochemistry imaging of ETR1-5xMyc using Alexa Fluor 633, the excitation and emission wavelengths (nm) were 633 and 650, respectively. For immunohistochemistry imaging of ETR1-5xMyc using Alexa Fluor 488, the same confocal microscopy settings were used as for GFP.

Membrane protein isolation, SDS-PAGE and western blotting

For isolation of Arabidopsis membranes, 8-day old etiolated seedlings were homogenized at 4°C in extraction buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 10 mM EDTA; and 20%[v/v] glycerol) containing plant culture tested protease inhibitors cocktail (Sigma). The homogenate was strained through Miracloth (Calbiochem-Novabiochem, San Diego) and centrifuged at 8,000*g* for 15 min. The supernatant was centrifuged at 100,000 *g* for 30 min, and the membrane pellet resuspended in 10 mM

Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA and 10% (v/v) glycerol with protease inhibitors. Immunoblot analysis was performed as described (Gamble et al., 2002). In brief, membrane proteins were treated with 100 mM DTT at 37°C for 1 h and then fractionated by SDS-PAGE on an 8% (w/v) polyacrylamide gel. After electrophoresis, proteins were electroblotted to a supported Nitrocellulose membrane (Bio-Rad). To detect ETR1-5xMyc, a 1:1000 dilution of the primary rabbit polyclonal anti-myc antibody (Sigma) was used, followed by a 1:5000 dilution of the goat anti-rabbit HRP secondary antibody (Chemical, Rockford, IL). For the ECA1 protein loading control, we used an anti-ECA1 antibody (Liang et al., 1997) kindly provided by Dr. Heven Sze. Immunodecorated proteins were visualized by enhanced chemiluminescence detection using the SuperSignal West Femto Maximum Sensitivity Kit (Pierce Chemical, Rockford, IL).

Chapter 3: Involvement of *RTE1* in conformational changes promoting ETR1 signaling

Introduction

In *Arabidopsis thaliana*, dark-grown seedlings display a specific ethylene response known as the triple-response phenotype, which consists of inhibition of hypocotyl and root elongation, radial swelling of the hypocotyl and exaggeration of the apical hook (Bleecker et al., 1988). The signaling pathway leading to the triple response and other ethylene responses has been dissected based on genetic screens for triple response mutants (Li and Guo, 2007).

Ethylene binding to the receptors (Schaller and Bleecker, 1995) requires a copper cofactor (Rodriguez et al., 1999) provided by *RAN1*, a homolog of the mammalian Menkes/Wilson P-type ATPase copper transporter (Hirayama et al., 1999; Woeste and Kieber, 2000). RAN1 appears to be required for the proper conformation and/or activity of all the ethylene receptors; reduced *RAN1* function alters the ligand specificity of the receptors (Hirayama et al., 1999) and a severe *ran1* loss-of-function confers a constitutive triple-response phenotype in dark-grown seedlings (Woeste and Kieber, 2000). The constitutive phenotype can be partially rescued by growing the seedlings on copper-supplemented medium (Woeste and Kieber, 2000).

As previously introduced, in the current model of ethylene receptor signaling, the receptors signal to repress ethylene responses in the absence of ethylene binding. Importantly, all known ethylene insensitive gain-of-function mutations lie within the hydrophobic transmembrane region comprising the ethylene-binding domain. For example, the *etr1-1* mutation substitutes a tyrosine for a cysteine residue (C65Y) that is required for binding of the copper cofactor (Rodriguez et al., 1999). In turn, the mutant ETR1-1 receptor cannot bind ethylene (Schaller and Bleecker, 1995) and the receptor is locked into a signaling conformation that cannot be turned off, resulting in ethylene insensitivity. In addition to mutations that disrupt ethylene binding, a class of gain-of-function mutations was identified that confers insensitivity without disrupting ethylene binding (Wang et al., 2006). Such alleles are thought to specifically prevent transmission of the ethylene signal to the signaling domains of the receptor (Wang et al., 2006). An example of such an allele is *etr1-2* (Hall et al., 1999). In addition to the missense mutations, the silver ion (I) has been shown to cause ethylene insensitivity for many years (Beyer, 1976). Silver is thought to cause ethylene insensitivity by taking the place of copper (I) in the receptor dimer and locking the receptor in the 'ON' conformation state (Binder et al., 2007). Silver has been shown to bind to the receptors in a yeast-based assay (Rodriguez et al., 1999) and this new conformation of the receptors can be reminiscent of a gain of function missense mutation in the hydrophobic domain of the EBD (Binder et al., 2007).

The negative regulator of ethylene responses, *RTE1*, was identified in *Arabidopsis* based on suppression of *etr1-2* (Resnick et al., 2006). Interestingly, the loss of *rte1* function is unable to suppress the *etr1-1* allele (Resnick et al., 2006).

etr1-1 is a strong allele giving complete ethylene insensitivity, whereas *etr1-2* gives a partial ethylene response (Hall et al., 1999). The *etr1-2* mutation results in a copper-independent conformational defect in the ETR1-2 receptor that is suppressed by the loss of *rte1* function. Similarly, the *etr1-1* mutation is thought to lock the ETR1-1 receptor into a copper-independent signaling conformation (Hirayama et al., 1999; Woeste and Kieber, 2000), yet the loss of *rte1* has no effect on *etr1-1*.

To gain insight into the basis for this difference, Dr. Resnick examined the ability of the severe loss-of-function mutation, *rte1-2*, to suppress additional dominant ethylene insensitive *etr1* alleles (Resnick, 2006). Existing *etr1* mutant transgenes that had been characterized in terms of the degree of ethylene insensitivity they confer in stably-transformed plants and the ability of the encoded proteins to bind ethylene in a yeast-based assay were used (Wang et al., 2006). Each transgene carries a missense mutation located in the ethylene-binding domain of ETR1. Wang et al. (2006) showed that complete disruption of ethylene binding confers strong ethylene insensitivity (as in the case of *etr1-1*), but that some strong ethylene-insensitive alleles encode receptors that can still bind ethylene to varying degrees. The alleles that confer insensitivity without disrupting ethylene binding (such as *etr1-2*) are thought to prevent the conformational change that occurs upon ethylene binding, and result in either strong or weak ethylene insensitivity (Wang et al., 2006).

Eleven missense mutations comprising both strong and weak alleles that are known to confer varying degrees of ethylene binding and signaling strengths (Table 3, Resnick et al., 2008) were tested. By including the previous data for the *etr1-2* and

Table 3. Summary of the ability of *rte1-2* to suppress a variety of dominant etr1 mutant alleles (Resnick et al., 2008)

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

[a]Data on percentage ethylene binding with respect to wild-type obtained by Wang *et al.* (2006).

[b]Values are the percentage hypocotyl length on 20 μM ACC with respect to that on no ACC in the wild-type background.

[c]Values are the percentage hypocotyl length in the *rte1-2* background with respect to that in the wild-type background on 20 μM ACC.

[d]Result provided by W. Wang (personal communication).

etr1-1 mutant alleles (Resnick et al., 2006), there were a total of seven suppressed and six non-suppressed *etr1* alleles. None of the suppressed lines were identical to the untransformed *rte1-2* mutant, but rather the lines showed variation in the extent of suppression. There is no clear pattern of features that distinguished the suppressed alleles from the non-suppressed alleles, such as ethylene binding ability, strength of signaling (*i.e.* degree of insensitivity) or the location of the mutation in the predicted protein sequence (Resnick et al., 2008). Ultimately, two different classes of dominant gain of function *etr1* alleles exist: *RTE1*-dependent and *RTE1*-independent. Since this work was performed using one homozygous line, my objective was to analyze additional independent lines in order to validate the interpretations. In Arabidopsis as well as other species, transgene insertions in the genome can lead to gene silencing and other uncontrolled effects on gene expression due to local position effects (Kooter et al., 1999). Therefore, characterizing more than one independent line is necessary to rule out the effects of insertion positions.

There has been speculation that *RTE1* and *GREEN-RIPE* might play a role in copper binding or transport (Barry and Giovannoni, 2006; Resnick et al., 2006), although copper treatment does not rescue *rte1* (Resnick et al., 2006). Recently, clear genetic evidence demonstrated *RTE1* to be independent of *RAN1* action and independent of copper supply/availability to the receptors. Moreover, *rte1* suppression of the etr1-2 protein is copper independent (Resnick et al., 2008).

One other possibility to address *RTE1* function is to look at its homologue, *RTH*. Examining the single null and *rte1 rth* double null may help to identify the role of the *RTE* family in Arabidopsis. *RTH* has been extensively characterized in

Arabidopsis and no relationship to the ethylene signaling pathway has been established (see Appendix B). I have performed phenotypic analysis of the *rth-1* null mutant and the double *rte1-3 rth-1* null mutant, but have not observed any obvious mutant phenotypes under a variety of different growth conditions (Appendix B).

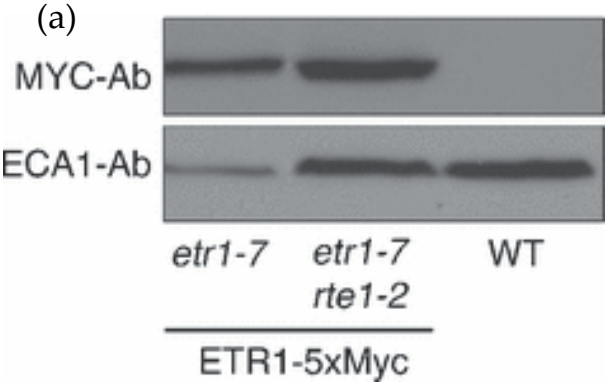
Examining the ETR1 protein, the only known target of any RTE, in the *rte1* null represents the best model to address the molecular function of *RTE1* and how it affects ETR1 signaling. Several hypotheses, such as *RTE1* having an affect on ETR1 protein stability, sub-cellular localization and protein conformation are credible possibilities. We plan to examine these hypotheses as to how *RTE1* affects the ETR1 receptor.

Results

RTE1 is not involved in ETR1 protein stability

To test the hypothesis of *RTE1* affecting ETR1 protein stability, we performed immunoblot experiments to detect ETR1-5xmyc in an *rte1* null background. As mentioned before, *rte1* only suppresses a subset of *etr1* alleles. *RTE1* is a known regulator of ETR1 signaling activity. *RTE1* plays a role in wild-type *ETR1* function since a loss-of-function of *RTE1* results in greatly reduced *ETR1* function (Resnick et al., 2006). To test whether the ETR1 protein is present in the *rte1-2* mutant , we stably-transformed the *rte1-2 etr1-7* double mutant with a transgene construct encoding ETR1 fused with a 5xMyc epitope at the carboxy-terminus. Expression of

the transgene was driven by the native *ETR1* promoter. This same construct is capable of rescuing the *etr1-7* null mutation (Dong et al., 2008). Using western blot analysis, we easily detected the ETR1-5xMyc fusion in the *etr1-7* mutant. Additionally, we find that ETR1-5xMYC is not altered in an *etr1 rte1* double null (Figure 3-1 a). Subsequently, we find ETR1-5xMYC and etr1-2(A102T)-5xMYC proteins in an *etr1 rte1* double null are not degraded (nor apparently reduced) when grown in the presence of ethylene (Figure 3-1 b). ETR1 and etr1-2, which are both *RTE1*-dependent alleles, show abundant protein levels in an *rte1* null that suggest


(a)
MYC-Ab
ECA1-Ab
etr1-7
etr1-7
rte1-2
WT
ETR1-5xMyc

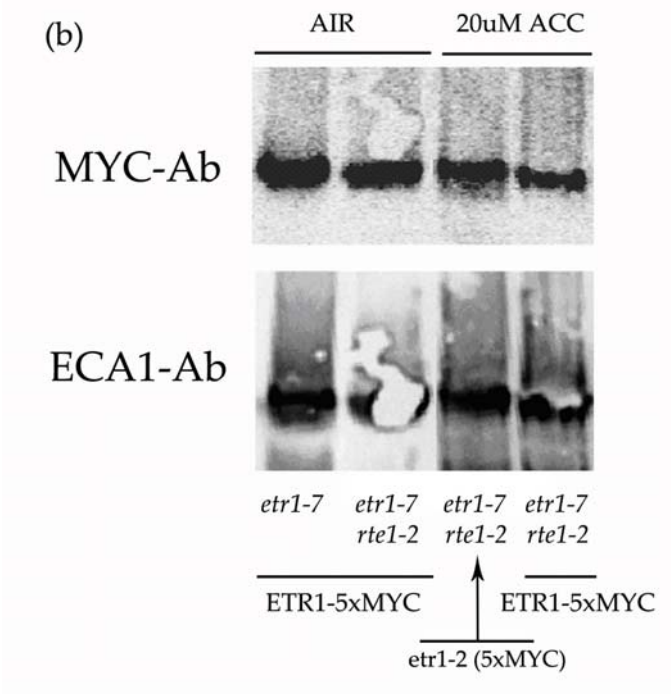

(b)
AIR
20uM ACC
MYC-Ab
ECA1-Ab
etr1-7
etr1-7
rte1-2
etr1-7
rte1-2
etr1-7
rte1-2
ETR1-5xMYC
ETR1-5xMYC
etr1-2 (5xMYC)

Figure 3-1. Presence of the ETR1 protein in the *rte1-2* loss-of-function mutant. Western blot showing the ETR1–5xMyc monomer isolated from the microsomal membrane fraction of Arabidopsis seedlings run on denaturing PAGE and detected by an anti-*c*-myc antibody (MYC-Ab). **(a)** The ETR1–5xMyc construct transformed into *etr1-7* and the *etr1-7 rte1-2* double mutant gives a band of approximately 80 kDa (left and center lanes) that is absent in the untransformed wild-type (WT, right lane). **(b)** The ETR1–5xMyc and etr1-2(A102T)-5xMYC constructs transformed into the *etr1-7 rte1-2* double mutant also give a band of approximately 80 kDa when treated with ACC. Seedlings exposed to ACC were dark-grown for four days in the presence of 20uM ACC. Seedlings with no treatment were light grown for 10 days. ECA1, an ER membrane protein detected by the ECA1-Ab antibody (Liang *et al.*, 1997), was used as a loading control.

other possible mechanisms for RTE1 to regulate ETR1 apart from protein turnover. This indicates that the *rte1-2* phenotype is not due to an absence of ETR1 protein but more likely due to a disruption of ETR1 signaling function.

RTE1 does not affect ETR1 sub-cellular localization

To test the hypothesis of RTE1 affecting ETR1 protein localization, we performed sub-cellular localization by immunohistochemistry of ETR1-5xMYC seedlings in wild-type and in *rte1* null background. One possibility we wanted to examine was if *RTE1* could be having an effect on ETR1 localization given that we found ETR1 to be localized to the ER and Golgi. ETR1 is distributed along the ER-Golgi membranes and therefore this distribution across these membranes could be important for ETR1 to signal correctly. Moreover, RAN1 is localized to the Golgi (Dunkley et al., 2006) and is believed to deliver the copper to ETR1 through some unknown mechanism. One possibility is that ETR1 makes its way to the Golgi receives the copper and is retrieved back to the ER. We set out to analyze if the distribution pattern of ETR1 would change in an *rte1* null background. Seedlings were grown under constant light as described in Dong et al. (2008). Given that the anti-Myc antibody detected an intact fusion protein, we proceeded with immunohistochemistry of root hair cells of plants that had been stably transformed with ETR1-5xMyc. We examined two different independent transgenic lines and observed no obvious difference in sub-cellular localization of ETR1 between wild-type and *rte1-2* plants (Figure 3-2). Further work is needed to eliminate the possibility

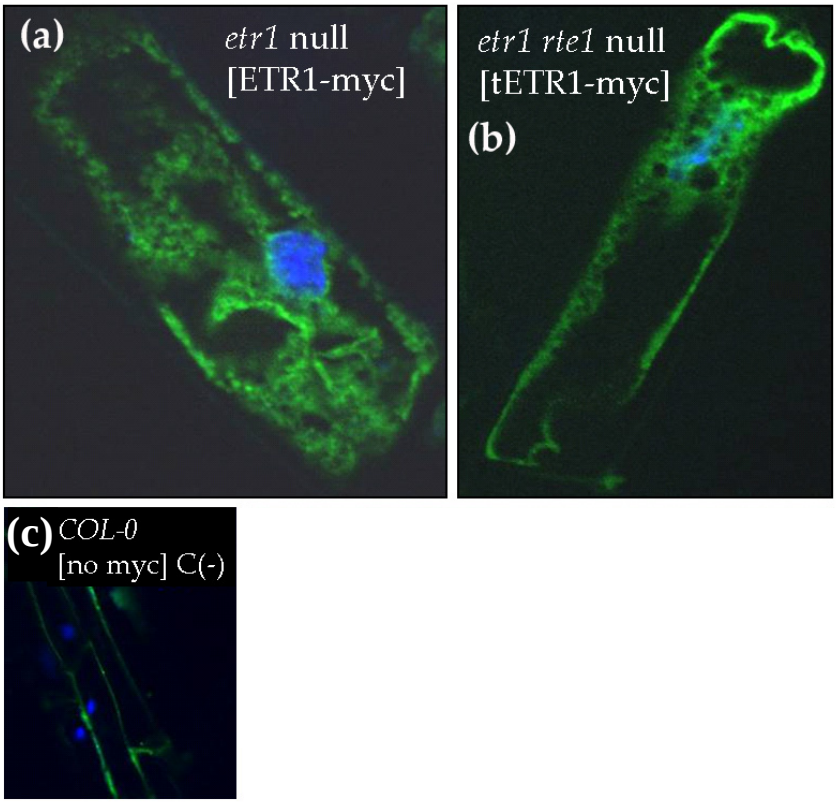


Figure 3-2: Similar subcellular localization patterns of ETR1-5xMYC in *etr1* null vs. *etr1 rte1* double null.

Representative root cells viewed by Apotome AxioVision fluorescence microscopy and stained with DAPI (a-c) to visualize nuclei.

(a) Root cell of a 5-day-old light-grown seedling expressing ETR1–5xMyc in an *etr1* null background, visualized by immunohistochemistry using an anti-c-myc antibody.

(b) Root hair cell of a 5-day-old light-grown seedling expressing ETR1–5xMyc in an *etr1 rte1* double null background, visualized by immunohistochemistry using an anti-c-myc antibody.

(c) Root hair cell of a 5-day-old light-grown wild type seedling untransformed as a negative control for auto fluorescence.

of a slight shift in the ER/Golgi distribution ratio of ETR1 when in an *rte1* null background.

The silver ion (I) causes the wild-type ETR1 receptor to become ethylene insensitive and *RTE1*-independent

rte1 loss of function mutants render ETR1 non-functional, therefore the *rte1* null has the same hypersensitivity as an *etr1* null to all doses of ethylene (Figure 1-4(b); Resnick et al., 2006). Moreover, in a high dose, with an equal ratio of silver to ethylene (100 μMolar), wild type and the single nulls *rte1* and *etr1* all have similar dark-grown phenotypes due to receptor redundancy (Resnick et al., 2006). Silver is thought to bind to the ethylene receptor and cause the receptors to be locked ON, resulting in constitutive signaling of the receptors that in turn represses the ethylene response (Rodriguez et al., 1999; Binder et al., 2007). To test the possible model that *RTE1* affects the ability of ETR1 to signal, we carried out the triple response assay with the addition of different concentrations of silver and ACC, which is readily converted to ethylene in plants by ACC oxidase, to the media. The rationale for this experiment was to look for differences in ethylene response between *etr1-7* and wild-type plants. We tested several doses of silver and ACC looking for a phenotypic difference between *etr1-7* null and wild-type plants. The experiment was designed this way due to redundancy of the receptors. In the ethylene signaling pathway, a majority of the receptors have to be shut off (binding of ethylene) in order for the ethylene response to occur. Ethylene and silver presumably compete with each other for the ethylene binding domain, where silver renders the receptors unfit to bind

ethylene (Binder et al., 2007). Similar to the ethylene dose response assay, we wanted to test if silver at low doses in comparison to ACC would give a different phenotype between wild type and an *etr1* null. The lack of ETR1 protein (a key receptor) lowers the signaling output potential considerably, rendering the plant more sensitive to ethylene. At a low ratio of silver to ACC the plants lacking ETR1 do not have enough signaling output to repress the responses. The assay was developed to visualize phenotypic differences between wild-type ETR1 and attenuated versions of *etr1* signaling (i.e. *etr1-7* or *rte1-3*) in the presence of silver. The concentration of 10uM silver and 100uM ACC is where wild-type is not be able to display any ethylene response but *etr1-7* null does show shortening of the hypocotyl due to the lack of the ETR1 receptor, rendering the seedling with less signaling output. Most interesting, at the same dose, *rte1* null seedlings behaved just like *WT*, which were long etiolated seedlings with no signs of any ethylene response (Figure 3-3). This experiment suggests that silver can rescue the effect that an *rte1-3* null has over ETR1 signaling. In addition, the double *etr1 rte1* null phenocopies the *etr1* null, placing *ETR1* downstream of *RTE1*, which had been predicted but never demonstrated (Figure 3-3). The evidence from these experiments supports the model that silver can restore ETR1 signaling in an *rte1* null and *RTE1* acts at or upstream of *ETR1*. These results indicate a role for *RTE1* in ETR1 signaling capacity and possibly in ETR1 protein conformation.

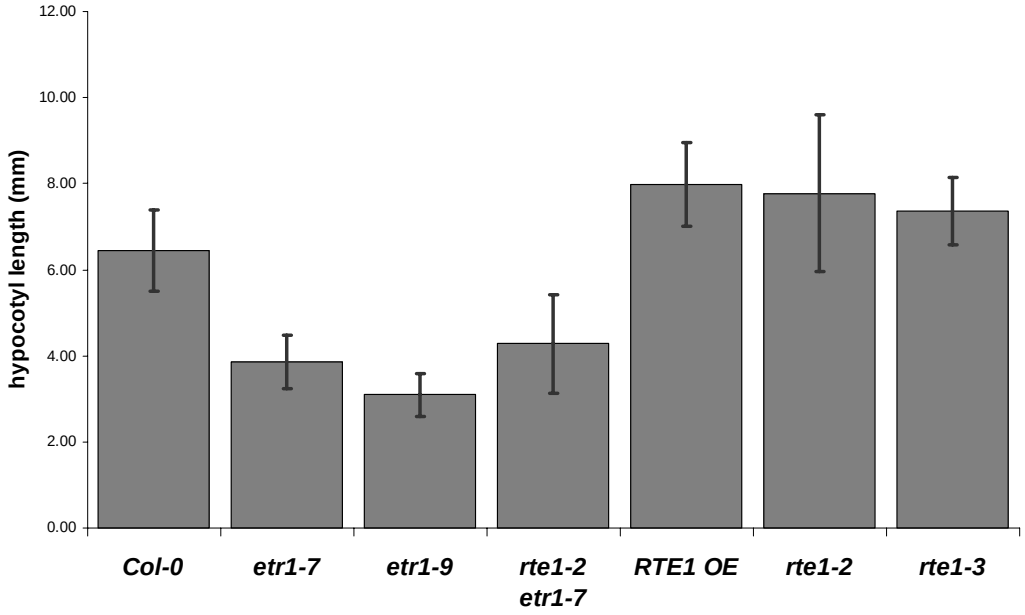


Figure 3-3. The silver ion can restore ETR1 signaling in an *rte1* null.

Treatment with the ethylene-response inhibitor silver nitrate ($AgNO_3$) at 10uM alleviates the ethylene response (100uM ACC) in wild type but not in an *etr1* null mutant (*etr1-7* and *etr1-9*). The *rte1* loss of function mutants (*rte1-2* and *rte1-3*) do not display an ethylene response. Seedlings were grown on 10uM silver nitrate plus 100uM ACC for four days in the dark. 10 – 15 seedlings were measured and mean ± standard deviations are shown.

Loss of *rte1* function suppresses a subset of dominant mutations in the ETR1 ethylene-binding domain; Additional independent transgenic lines confirm no insertion effect in the suppression analysis

In order to verify and validate the work on the *RTE1* dependence of certain *etr1* alleles to confer insensitivity initiated by Dr. Resnick in 2006 (PhD thesis), we set out to identify and measure additional homozygous lines of all the etr1 transgenes carrying dominant gain of function mutations. Previously, only one homozygous line was measured for the *RTE1* suppression analysis and further lines needed to be tested to rule out any insertional effect of the transgenes. Transgene insertion effects can result when a transgene inserted via *Agrobacterium tumefaciens* is inserted in a region of the Arabidopsis genome where transgene silencing occurs (Kooter et al., 1999). Transgene silencing may result from different mechanisms (i.e. epigenetic and/or siRNA regulation), which are just starting to be understood (Kooter et al., 1999). We measured additional lines of all the transgenes in the *rte1-2* or *Col-0* wild-type backgrounds. We measured (one or more) additional homozygous independent lines to rule out any insertion effect of the transgene on the phenotype scored. Table 4 shows that different independent lines from the ones used previously (Resnick, 2006) give the same results (Resnick et al., 2008). Thus, measurements of independent homozygous transgenic lines confirm the previous results that certain *ETR1* alleles are *RTE1*-dependent or independent. Hypocotyl lengths of dark-grown seedlings (Line2) per *etr1* transgene in wild-type vs. *rte1-2* backgrounds were measured. Therefore, we can be confident that the interpretations of the *RTE1*-dependent and *RTE1*-independent class of etr1 alleles are not an artifact of transgene insertion effect.

Table 4. *rte1-2* suppress additional independent lines of dominant etr1 mutant alleles.

Mutation / Background	Average Hypocotyl Length (mm) 0 μM ACC Line 1	20 μM ACC Line 1	20 μM ACC Line 2
WT (Col-0)*	10.91 +/- 0.14	4.03 +/- 0.12	n/a
rte1-2 *	7.74 +/- 0.09	2.47 +/- 0.07	n/a
D25A / WT	9.65 +/- 0.14	9.66 +/- 0.17	8.66 +/- 0.03
D25A / *rte1-2*	9.63 +/- 0.12	7.94 +/- 0.01	6.93 +/- 0.04
Y32A / WT	10.98 +/- 0.16	11.15 +/- 0.12	9.82 +/- 0.05
Y32A / *rte1-2*	10.61 +/- 0.18	4.72 +/- 0.09	5.46 +/- 0.03
E38A / WT	10.54 +/- 0.26	10.55 +/- 0.16	8.61 +/- 0.05
E38A / *rte1-2*	10.10 +/- 0.19	4.74 +/- 0.15	4.95 +/- 0.04
F58A / WT	10.40 +/- 0.17	9.01 +/- 0.26	8.64 +/- 0.07
F58A / *rte1-2*	10.46 +/- 0.13	4.14 +/- 0.13	4.54 +/- 0.02
F61A / WT	11.56 +/- 0.19	8.46 +/- 0.21	–
F61A / *rte1-2*	8.85 +/- 0.15	3.63 +/- 0.11	3.96 +/- 0.03
L64A / WT	10.54 +/- 0.24	7.39 +/- 0.11	7.52 +/- 0.04
L64A / *rte1-2*	9.34 +/- 0.15	3.32 +/- 0.08	2.95 +/- 0.02
etr1-1 (C65Y)*	12.53 +/- 0.19	12.15 +/- 0.12	n/a
etr1-1 rte1-2 *	11.50 +/- 0.25	11.34 +/- 0.17	n/a
T94M / WT	11.60 +/- 0.14	11.20 +/- 0.26	9.95 +/- 0.05
T94M / *rte1-2*	12.24 +/- 0.20	11.00 +/- 0.19	11.02 +/- 0.02
T101A / WT	10.68 +/- 0.16	10.58 +/- 0.17	7.13 +/- 0.03
T101A / *rte1-2*	11.18 +/- 0.20	10.02 +/- 0.09	8.85 +/- 0.03
etr1-2 (A102T)*	11.45 +/- 0.16	8.51 +/- 0.15	n/a
etr1-2 rte1-2 *	9.28 +/- 0.15	3.08 +/- 0.06	n/a
M104A / WT	11.83 +/- 0.17	8.79 +/- 0.13	–
M104A / *rte1-2*	9.58 +/- 0.30	3.97 +/- 0.13	3.10 +/- 0.04
L105A / WT	10.81 +/- 0.17	10.93 +/- 0.19	6.64 +/- 0.03
L105A / *rte1-2*	10.80 +/- 0.23	10.74 +/- 0.23	8.01 +/- 0.03
I108A / WT	10.00 +/- 0.13	9.77 +/- 0.22	9.13 +/- 0.03
I108A / *rte1-2*	10.78 +/- 0.23	7.79 +/- 0.11	–

Shown are the means +/- S.E. for 15-20 seedlings.
* genetic background carrying no mutant transgene

Discussion

Previous work has identified *RTE1* as a novel regulator of *ETR1*. Here, we carried out detailed genetic, biochemical and cell biological studies to analyze the dependence of *ETR1* on *RTE1*, including the placement of *RTE1* in the ethylene-signaling pathway in relation to *ETR1*. To date, the molecular function of *RTE1* still remains elusive and *Arabidopsis* is the only organism where a target of *RTE1* has been identified. Therefore, to gain insight into the role of *RTE1* in *ETR1* signaling, we have tested the ability of *rte1* loss of function mutants to alter or affect:

- ETR1 protein stability
- ETR1 sub-cellular localization
- ETR1 receptor signaling output

Similar to *ran1*, in which ETR1 is nonfunctional but present in the membrane fraction as revealed by western blot (Zhao et al., 2002), an *rte1* null does not affect protein abundance of ETR1, therefore suggesting that *rte1* results in a stable non-functional ETR1 protein. In addition, ETR1 sub-cellular localization studies showed no difference of ETR1 localization between *rte1* and *Col-0* wild-type backgrounds. All these results support a more subtle regulatory role of *RTE1* on ETR1, such that if *RTE1* was needed for correct localization or essential for protein stability, a difference in protein localization or abundance would have been revealed.

Wild-type ETR1 is dependent on *RTE1* to signal correctly. Therefore, the finding that silver can convert wild-type ETR1 to become *RTE1*-independent suggests that the silver ions (which are assumed to replace the copper cofactor) most likely have changed the conformation of the EBD (ethylene binding domain) in a way that

overrides the requirement of RTE1. This new EBD conformation may be reminiscent of an *etr1* dominant gain of function allele which is not suppressed by *rte1*. Moreover these silver and ethylene experiments were the first to show epistasis of *ETR1* to *RTE1*, placing *RTE1* upstream of *ETR1*. An alternative to this interpretation, although less likely, is that *rte1* may not fully abolish ETR1 signaling. Some ETR1 protein may still be able to signal in the absence of *RTE1*, therefore in the presence of silver, these ETR1 functional molecules may bind to silver and signal constitutively. Evidence for this model is the finding that the subfamily I null (*etr1 ers1* null) is more severe than the *rte1 ers1* double null (Resnick et al., 2006). This may suggest ETR1 is not completely shut off in an *rte1* null, although *rte1* and *etr1* single nulls have the same ethylene hypersensitivity. The rationale for this interpretation is the hypothesis that some ETR1 protein molecules may be functional in an *rte1* null.

rte1 loss of function mutants can suppress a variety of dominant ethylene-insensitive *etr1* mutations, which all lie within the ethylene-binding domain of ETR1 (defined as residues 1-128). Previous results revealed a class of mutations that requires *RTE1* in order to confer ethylene insensitivity, as well as a class that is independent of *RTE1* (Resnick et al., 2008). The underlying basis for these two classes remains unclear. A characteristic shared by all the *etr1* alleles tested is that they each presumably produce a unique structural defect within the ETR1 ethylene-binding domain that consequently inhibits, to varying degrees, the conformational transition required to turn ETR1 signaling off (Wang et al., 2006). Therefore, the *RTE1*-dependence of certain *etr1* alleles, including the wild-type *ETR1* allele, may be related to particular conformations of the ethylene-binding domain.

Accordingly, from all our results, we propose that RTE1 has an effect on the conformation of the ethylene-binding domain that results in the promotion or stabilization of the ETR1 signaling "ON" state. This is based on several lines of reasoning. As proposed by Wang et al. (2006), a conformational shift in the ETR1 ethylene-binding domain upon ethylene binding is responsible for transmission of the signal to the transmitter (signaling) domain in order to shut signaling off. Consistent with the model of RTE1 affecting the amino terminal of ETR1, Zhou et al. (2007) showed that loss of the *RTE1* over-expression phenotype in the *etr1-7* null mutant is rescued by co-expressing a truncated form of ETR1 consisting of residues 1-349 (Zhou et al., 2007). (ETR1 (1-349) confers signaling in *etr1-7* through interactions with the ERS1 ethylene receptor (Xie et al., 2006). Thus, it appears that RTE1 acts through the amino-terminal portion of ETR1.

It was proposed that the ETR1 receptor normally exists in several different states, including the on/off states and a transitional state in between the on/off states, as shown in Figure 3-4. *RTE1* could promote or stabilize the ETR1 signaling "ON" state at either of two transitions. One involves the formation of functional ETR1 ("A" in Figure 3-4), while the other inhibits the transition from the signaling "ON" state to the signaling "OFF" state ("B" in Figure 3-4). We propose that in the wild-type situation, *RTE1* helps to create the ETR1 signaling "ON" state from an undefined ground state that is phenotypically equivalent to the *etr1* null (Figure 3-4); ethylene binding turns ETR1 signaling "OFF", which is a state that is known to be phenotypically different from the *etr1* null. We favor the model in which *RTE1* acts at "A" in Figure 3-4, based on the finding that the *rte1* loss-of-function results in an

etr1 null-like phenotype (ethylene hypersensitivity), although we cannot rule out that this null-like phenotype includes non-ethylene-related effects, since the *rte1-2* and *rte1-3* mutants do not phenocopy the *etr1* null mutant in every respect (Resnick et al., 2006; Zhou et al., 2007). Involvement of *RTE1* in ETR1 biogenesis would be consistent with the ability of *rte1-2* to suppress the Y32A mutant receptor. The *RTE1*-dependent Y32A mutant binds a negligible amount of ethylene, and so would rarely achieve the ethylene-bound, signaling "off" state in the absence of *RTE1*. Similarly, the *etr1-2* mutation in the *ran1-3* mutant background would be unable to bind ethylene in the absence of copper, yet is still suppressed by *rte1-2* in the presence of *ran1-3*. Presumably, the *RTE1*-independent *etr1* alleles are capable of bypassing the requirement for *RTE1* in step "A' of Figure 3-4 in order to reach the signaling "ON" state. Nevertheless, *RTE1* may act at both points (A) and (B) in Figure 3-4, promoting the ETR1 "ON" state by altering the equilibrium between the various states.

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

(Dong et al., 2008), but it is currently unclear whether the two proteins physically interact. The basis for the apparent specificity of *RTE1* for *ETR1* versus the other ethylene receptor family members in *Arabidopsis* is also not yet understood.

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

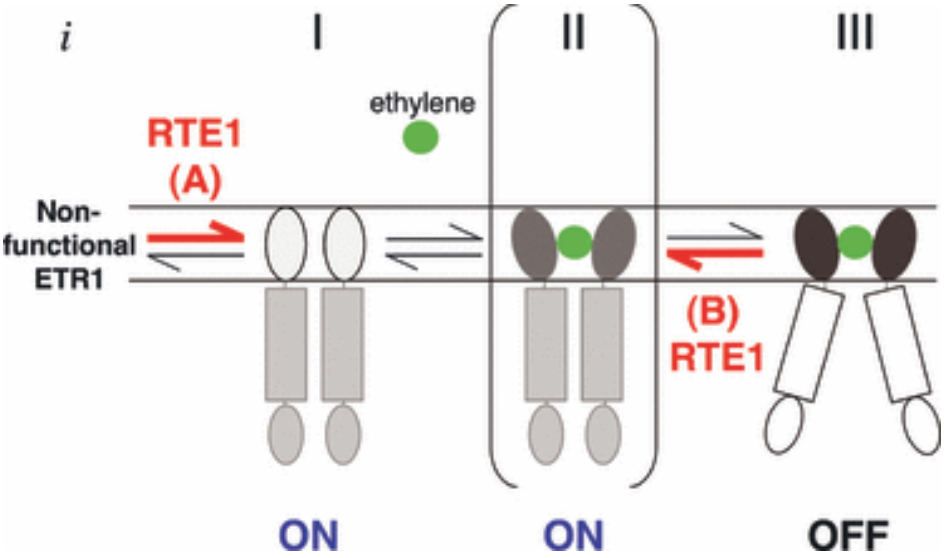


Figure 3-4. Model for the promotion of ETR1 signaling by RTE1.

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

Experimental Procedures

Construction of ETR1 (A102T)-5xMYC

The ETR1-5xMYC wild-type construct (see Chapter 2) was used as a template for mutagenesis. To engineer mutations into *ETR1, in vitro* site-directed mutagenesis was carried out using Stratagene's QuikChange XLII mutagenesis kit (www.stratagene.com). All mutations were verified by nucleotide sequencing. To create the ETR1 A102T (etr1-2 allele) amino acid substitution the following primers were used:

5'-GTTGTCTCGTGTGCTACTACGTTGATGCTTGTTCATATTAT-3' sense;

5'-ATAATATGAACAAGCATCAACGTAGTAGCACACGAGACAAC-3' antisense.

Western blotting

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

Immunohistochemistry

For immunohistochemistry of ETR1-5xMyc, Arabidopsis seedlings were grown under white light and prepared essentially as described by (Friml et al., 2003; Dong et al., 2008).

Fluorescence microscopy

Imaging of fluorescent proteins (secondary GFP-labeled antibodies) in seedling roots was conducted under an Apotome AxioVision microscope as described in (Melani et al., 2008).

Chapter 4: ETR1-specific signaling mechanism as revealed by RTE1.

Introduction

The five ethylene receptors in *Arabidopsis* fall into 2 subfamilies, subfamily I (*ETR1* and *ERS1*) which contains a conserved carboxy terminal histidine kinase domain and a hydrophobic region consisting of three transmembrane domains. In contrast, subfamily II (*ERS2, ETR2,* and *EIN4)* has a degenerate histidine kinase domain and a hydrophobic region consisting of four transmembrane domains. Elusive remains the exact mechanism by which the ethylene receptors (of particular interest ETR1) signal to CTR1, the next downstream component which is known. In all plant species the ethylene receptors have a highly conserved amino acid sequence, in particular the amino terminal region consisting of the ethylene binding domain, GAF domain, and Coiled coil domain (Figure 4-1). Previous work done by several ethylene groups have shown that ethylene insensitive dominant gain of function mutations like the original missense mutation isolated by Dr. Caren Chang in 1993, *etr1-1*, which encodes a tyrosine at amino acid 65 replacing a cysteine (C65Y), can be transferred to other receptors (Wilkinson et al., 1997; Sakai et al., 1998; Terajima et al., 2001) or other species (Wilkinson et al., 1997) and still confer ethylene insensitivity. These results suggest a conserved ethylene signaling mechanism of the receptors present in all receptors and across different species.


LeETR2 ----------MDCNCFDP--LLPADELLMKYQY 21
NtETR1 ----------MDCNCFDP--QWPADELLMKYQY 21
MdETR1 ----------MLACNCIEP--QWPADELLMKYQY 22
AtETR1 ----------MEVCNCIEP--QWPADELLMKYQY 22
MaERS1 ----------MDGCDCIEP--QWPADELLIKYQY 22
OsERS1 ----------MDGCDCIEP--LWPTDELLIKYQY 22
PhETR1 ----------MEGCDCIEP--QWPADELLVKYQY 22
LeNR ----------MESCDCIEA--LLPTGDLLVKYQY 22
NtERS1 ----------MMESCDCIEA--LLPNDDLLVKYQY 23
AtERS1 ----------MESCDCFET--HVNQDDLLVKYQY 22
AtERS2 ------MLKTLLVQWLVFFFFFLIGSVVTAAEDDGSLSLCNCDDE-DSLFSYETILNSQK 53
AtETR2 ------MVKEIASWLLILSMVVFVSPVLAIN--GGGYPRCNCEDEGNSFWSTENILETQR 52
AtEIN4 ------MLRSLGLGLLLFALLALVS-------GDNDYVSCNCDDE--GFLSVHTILECQR 45
ZmETR2 MVVGTALLRGVSSAWILLFLSSLLLSPSAASVDFGHCGGCDDADD-GALSSTYNILQCQK 59
LeETR2 ISDFFIAVAYFSIPIELVYFVQKSAVFP-YRWVLVQFGAFIVLCGATHLINLWT-STPHT 79
NtETR1 ISDFFIAVAYFSIPIELVYFVQKSAVFP-YRWVLVQFGAFIVLCGATHLINLWT-STAHT 79
MdETR1 ISDFFIALAYFSIPLELIYFVKKSAVFP-YRWVLVQFGAFIVLCGATHLINLWT-FSIHS 80
AtETR1 ISDFFIAIAYFSIPLELIYFVKKSAVFP-YRWVLVQFGAFIVLCGATHLINLWT-FTTHS 80
MaERS1 ISDFFIALAYFSIPLELIYFVKKSSFFP-YRWVLIQFGAFIVLCGATHLINLWT-FALHS 80
OsERS1 ISDFFIALAYFSIPLELIYFVKKSSFFP-YRWVLIQFGAFIVLCGATHLINLWT-FTTHT 80
PhETR1 ISDFFIALAYFSIPLELIYFVKKSSFFP-YRWVLIQFGAFIVLCGATHLINLWT-FTMHS 80
LeNR LSDFFIAVAYFSIPLELIYFVHKSACFP-YRWVLMQFGAFIVLCGATHFISLWT-FFMHS 80
NtERS1 LSDFFIAFAYFSIPLELIYFVHKSACFP-YRWVLMQFGAFIVLCGATHFISLWT-FFMHS 81
AtERS1 ISDALIALAYFSIPLELIYFVQKSAFFP-YKWVLMQFGAFIILCGATHFINLWM-FFMHS 80
AtERS2 VGDFLIAIAYFSIPIELVYFVSRTNVPSPYNWVVCEFIAFIVLCGMTHLLAGFT-YGPHW 112
AtETR2 VSDFLIAVAYFSIPIELLYFVSCSNVP--FKWVLFEFIAFIVLCGMTHLLHGWT-YSAHP 109
AtEIN4 VSDLLIAIAYFSIPLELLYFISFSNVP--FKWVLVQFIAFIVLCGMTHLLNAWTYYGPHS 103
ZmETR2 VSDFLIAAAYFSIPLELLYFATCSDLFP-LKWIVLQFGAFIVLCGLTHLITVFT-YEPHS 117
LeETR2 RTVAMVMTTAKFSTAAVSCATAVMLVHIIPDLLSVKTRELFLKNKAAELDREMGLIRTQE 139
NtETR1 RTLAIVMTTAKVLTAVVSCATALMLVHIIPDLLSVKTRELFLKNKAAELDREMGLIRTQE 139
MdETR1 RTVAMVMTTAKVLTAVVSCATALMLVHIIPDLLSVKTRELFLKNKAAELDREMGLIRTQE 140
AtETR1 RTVALVMTTAKVLTAVVSCATALMLVHIIPDLLSVKTRELFLKNKAAELDREMGLIRTQE 140
MaERS1 RTLAIVMTVAKVSTAVVSCATALMLVHIIPDLLSVKTRELFLKNKAEELDREMGLIRTQE 140
OsERS1 KTVAMVMTVAKVSTAVVSCATALMLVHIIPDLLSVKTRELFLKNKAEQLDREMGLIRTQE 140
PhETR1 RTLAIVMTVAKVSTAVVSCATALMLVHIIPDLLSVKTRELFLRNKAEELDKEMGLIRTQE 140
LeNR KTVAVVMTISKMLTAAVSCITALMLVHIIPDLLSVKTRELFLKTRAEELDKEMGLIIRQE 140
NtERS1 KTVAVVMTIAKILTAAVSCITALMLVHIIPDLLSVKTRELFLKARAEELDKEMGLIIRQE 141
AtERS1 KAVAIVMTIAKVSCAVVSCATALMLVHIIPDLLSVKNRELFLKKKADELDREMGLILTQE 140
AtERS2 PWVMTAVTVFKMLTGIVSFLTALSLVTLLPLLLKAKVREFMLSKKTRELDREVGIIMKQT 172
AtETR2 FRLMMAFTVFKMLTALVSCATAITLITLIPLLLKVKVREFMLKKKAHELGREVGLILIKK 169
AtEIN4 FQLMLWLTIFKFLTALVSCATAITLLTLIPLLLKWKVRELYLKQNVLELNEEVGLMKRQK 163
ZmETR2 FHLVLALTVAKFLTALVSFATAITLLTLIPQLLRVKVRENFLMNKARELDREVGRMKRKE 177
LeETR2 ETGRYVRMLTHEIRSTLDRHTILKTTLVELGRALQLEECALWMPTRTGVELQLSYTLHHQ 199
NtETR1 ETGRYVRMLTHEIRSTLDRHTILKTTLVELGRTLALEECALWMPTPPGLELQLSYTLRHQ 199
MdETR1 ETGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLALEECALWMPTRTGLELQLSYTLRQQ 200
AtETR1 ETGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLALEECALWMPTRTGLELQLSYTLRHQ 200
MaERS1 ETEGNVRMLTHEIRSTLDRDTVLKTTLVELGRTLDLKDCMLWMPSHSGTSLQLSHTLHHQ 200
OsERS1 ETGRHVRMLTHEIRSTLDRHTILKTTLVELGGTLGLEECALWMPSRSGSSLQLSHTLRHQ 200
PhETR1 ETGRHVRMLTHEIRSTLDRHTILRTTLVELGRTLDLAECALWMPSRTGLNLQLSHTLNNQ 200
LeNR ETGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLDLAECALWMPCQGGLTLQLSHNLNNL 200
NtERS1 ETGRHVRMLTHEIRSTLDRHTILKTTLVELGRTLDLAECALWMPCOGGLNLOLSHNLNNL 201
AtERS1 ETGRHVRMLTHGIRRTLDRHTILRTTLVELGKTLCLEECALWMPSQSGLYLQLSHTLSHK 200
AtERS2 ETSLHVRMLTTKIRTSLDRHTILYTTLVELSKTLGLKNCAVWIPNEIKTEMNLTHELRPR 232
AtETR2 ETGFHVRMLTQEIRKSLDRHTILYTTLVELSKTLGLQNCAVWMPNDGGTEMDLTHELRGR 229
AtEIN4 EMSVQVRMLTREIRKSLDKHMILRTTLVELSKILDLQNSAVWMPNENRTEMHLTHELRAN 223
ZmETR2 EASWHVRMLTQEIRKSLDRHTILYTTMVELSKALELQNCAVWMPDETRSTMILTHQLRER 237


LeETR2 ----------NPVGFTVPIQLPVINQVFSANCAVKISPNSAVAR-LRPTR-KYIPGEVV 246
NtETR1 ----------NPIGFTVPIQLPVINQVFGTNRAVKISPNSPVAR-LRPAG-KYMPGEVV 246
MdETR1 ----------NPVGYTVPIHLPVINQVFSSNRAVKISANSPVAK-LRQLAGRHIPGEVV 248
AtETR1 ----------HPVEYTVPIQLPVINQVFGTSRAVKISPNSPVAR-LRPVSGKYMLGEVV 248
MaERS1 ----------IPTGSAVPINLPVVNQIFSSNHAMIIPHTCRLAR-IWPLSVRHVPPEVA 248
OsERS1 ----------ITVGSTVSINLPVVNQVFSSNRAIIIPHTSPLAR-IRPLAGRYVPPEVA 248
PhETR1 ----------IPVGSVVSINLPVVNQVFNSSRAVRIPHTCQLAR-FQPHTGRYVPPEVV 248
LeNR ----------IPLGSTVPINLPIINEIFSSPEAIQIPHTNPLAR-MRNTVGRYIPPEVV 248
NtERS1 ----------LPLGSTVPINLPIINEIFSSPGAIQIPHTNALAR-MRNTAGRYIPPEVV 249
AtERS1 ----------IQVGSSVPINLPIINELFNSAQAMHIPHSCPLAK-IGPPVGRYSPPEVV 248
AtERS2 IDDENENEHFGGYAGFSIPISESDVVRIKRSEEVNMLSPGSVLASVTSRG----KSGPTV 288
AtETR2 ----------GGYGGCSVSMEDLDVVRIRESDEVNVLSVDSSIARASGGGGDVSEIGAVA 279
AtEIN4 ----------PMRSFRVIPINDPDVVQVRETKVVTILRKNSVLAVESSGCGGSEEFGPVA 273
ZmETR2 --------DIMDPQKHSIPIDDPDVQEIKATKDAKVLGPDSALGVSSRSK---HEAGPVA 286
:.: : .: : . : :. ..
LeETR2 AVRVPLLHLSNFQTNDWPELSPKSYALMVLMLPSNSARQWHVHELELVDVVADQVAVALS 306
NtETR1 AVRVPLLHLSNFQINDWPELSTKRYALMVLMLPSGSARQWHVHELELVEVVADQVAVALS 306
MdETR1 AVRVPLLHLSNFQINDWPELSTKRYALMVLMLPSDSARQWHVHELELVEVVADQVAVALS 308
AtETR1 AVRVPLLHLSNFQINDWPELSTKRYALMVLMLPSDSARQWHVHELELVEVVADQVAVALS 308
MaERS1 AVRVPLLHLSNFQISDWSELSTKSYAVMVLILPSDSGRKWHVHELELVEVVADQVAVALS 308
OsERS1 AVRVPLLHLSNFQINDWPELSAKSYAIMVLMLPSDSARKWHVHELELVEVVADQVAVALS 308
PhETR1 AVRVPLLHLSNFQINDWPELSAKNFAVMVLMLPSDSARKWHVYELELVEVVADQVAVALS 308
LeNR AVRVPLLHLSNFTN-DWAELSTRSYAVMVLVLPMNGLRKWREHELELVQVVADQVAVALS 307
NtERS1 AVRVPLLHLSNFHINDWPELSARSYAVMVLILPMNGLRKWRDHELELVQVVADQVAVALS 309
AtERS1 SVRVPLLHLSNFQGSDWSDLSGKGYAIMVLILPTDGARKWRDHELELVENVADQVAVALS 308
AtERS2 GIRVPMLRVCNFKGGTPEAI-HMCYAILVCVLPLRQPQAWTYQELEIVKVVADQVAVAIS 347
AtETR2 AIRMPMLRVSDFNG-------ELSYAILVCVLPGGTPRDWTYQEIEIVKVVADQVTVALD 332
AtEIN4 AIRMPMLHGLNFKGGTPEFV-DTPYAIMVLVLPSANSRVWTDKEIEIAEVVADQVAVAIS 332
ZmETR2 AIRMPMLRVSNFKGGTPEVM-QTSYAILVLVLPNDGSLGWGRRELEIVEVVADQVAVALS 345
.:*:*:*: :* :**: :** * *:*:.. *****:**:.
LeETR2 HAAILEESMRARDLLIEQNVALDLARREAETAVRARNDFLG
NtETR1 HAAILEESMRARDLLMEQNVALDLARREAEMAVRARNDFLA
MdETR1 HAAILEESMRARDLLMEQNIALDLARREAETAIRARNDFLA
AtETR1 HAAILEESMRARDLLMEQNVALDLARREAETAIRARNDFLA
MaERS1 HAAILAESMRARDLLTEQNVALYLARQEAELAIRARNDFLA
OsERS1 HAAILEESMRARDLLMEQNVALDLARREAEMAIRARNDFLA
PhETR1 HAAILEESMRARDQLMDQNVALDLARREAEMAIRARNDFLA
LeNR HAAILEDSMRAHDQLMEQNIALDVARQEAEMAIRARNDFLA
NtERS1 HAAILEESMRAHDQLMEQNIALDVARQEAEMAIHARNDFLA
AtERS1 HAAILEESMHARDQLMEQNFALDKARQEAEMAVHARNDFLA
AtERS2 HAVILEESQLMREKLAEQNRALQVARENALRANQAKAAFEQ
AtETR2 HAAVLEESQLMREKLAEQNRALQMAKRDALRASQARNAFQK
AtEIN4 HASVLEESQLMREKLGIQNRALLRAKQNAMMASQARNTCQK
ZmETR2 HAALLEESQLMREKLAEQHRDLLQAKDEAMRAGDARNSFQT
** :* :* :: * *: * *: :* * *:

Figure 4-1. Ethylene receptor sequence similarity of the amino terminal region across plant species.

Amino acid sequence alignment of the EBD-GAF-CC domains from fourteen plant ethylene receptors; Approximately amino acids 1 to 349 (ETR1) are shown. EBD sequence is boxed in dotted red line; GAF in solid blue line; Coiled coil in dotted green line. "*****" denotes conserved residues, "**:**" denotes conserved substitutions, and "**.**" denotes semi-conserved substitutions; aligned using ClustalW (Larkin et al., 2007). Below are the gene names, the Genebank accession numbers, and from what species they belong to:

At-ETR1 NP_176808.3 [Arabidopsis thaliana]; At-ERS1 NP_181626.1 [Arabidopsis thaliana]; At-ERS2 NP_171927.1 [Arabidopsis thaliana]; At-ETR2 NP_188956.1 [Arabidopsis thaliana]; At-EIN4 NP_187108.1 [Arabidopsis thaliana]; Le-ETR2 AAC02214.1 [Lycopersicon esculentum]; Le-NR AAC49124.1 [Lycopersicon esculentum]; Ma-ERS1 AAF08300.1 [Musa acuminata]; Md-ETR1 AAC31123.1 [Malus x domestica]; Nt-ETR1 AAB97160.1 [Nicotiana tabacum]; Nt-ERS1 AAB96765.2 [Nicotiana tabacum]; Os-ERS1 AAB72193.1 [Oryza sativa]; Ph-ETR1 AAD04949.1 [Phalaenopsis sp. 'KCbutterfly']; Zm-ETR2 NP_001104852.1 [Zea mays].

All ethylene receptors contain an Ethylene Binding Domain (EBD), a GAF domain (GAF), a Coiled Coil domain (CC), a Histidine Kinase domain (HK), and in some receptors a carboxy terminal Receiver Domain (RD) (Chang and Stadler, 2001). As mentioned earlier, the EBD is a hydrophobic region with either three or four transmembrane motifs that serve to create the pocket where ethylene binds. The GAF domain was originally described as a noncatalytic cGMP-binding domain conserved in cyclic nucleotide phosphodiesterases (Charbonneau et al., 1990). Later, this domain was recognized in histidine kinases and other proteins. The CC domain is a small motif mainly involved in the interface of protein-protein interactions. The 3D shape of a particular coiled-coil domain determines its oligomerization state, rigidity and ability to function as a molecular recognition system (Burkhard et al., 2001). The histidine kinase activity does not seem to play a role in the ethylene signaling pathway (Wang et al., 2003) but the entire HK domain is required for wild-type signaling of ETR1 (Qu and Schaller, 2004). Lastly, the receiver domain does not seem to be involved at all in the ethylene signaling pathway (Chen et al., 2005). Although no obvious phenotypes have been observed with receptors lacking the receiver domain, Binder et al. (2004), demonstrated a likely function of these receiver domains in ethylene-dependent recovery of growth rates. Dr. Binder showed that *ers1* and *ers2* nulls, which lack their RD have no defect in ethylene recovery growth rates while *etr1*, *ein4*, and *etr2* single nulls significantly prolong the time for recovery of growth rate after ethylene was removed (Binder et al., 2004).

In the current model of ETR1 signaling; *RTE1* is required for the necessary conformational change(s) in order for ETR1 to signal and repress the ethylene

responses in the absence of ethylene binding (Resnick et al., 2008). When ethylene is bound, transmission of the signal occurs via a conformational change in the receptors, turning signaling off and allowing responses to proceed (Wang et al., 2006). This model is supported by dominant gain-of-function mutations in the receptor genes. Importantly, all known gain-of-function mutations lie within the hydrophobic transmembrane region comprising the ethylene-binding domain (EBD). Elegant work done by our laboratory classified these dominant gain-of-function mutations as either *RTE1*-dependent or independent. The *RTE1*-dependent *etr1* alleles, i.e., those *etr1* alleles that require *RTE1* to signal, can not be predicted based on their ethylene binding capacity, amino acid position in the EBD, nor signaling strength (Resnick et al., 2008). The lack of suppression correlation is intriguing and suggests a model in which only particular ETR1 EBD conformation(s) require *RTE1*. We are interested in ETR1's requirement for *RTE1* and whether *RTE1* solely acts on ETR1. Moreover, given that only certain conformations of the ETR1 EBD require *RTE1*, we will also address if the EBD of another receptor can properly function in place of the ETR1 EBD. Therefore, we will address two fundamental questions: Is (and why) ETR1 unique in its signaling mechanism regulation? Does ETR1 uniquely require *RTE1* to properly function?.

Results

RTE1-dependent alleles of _etr1_ do not confer ethylene insensitivity when carried by other ethylene receptor genes

We set out to examine whether the loss of *rte1* function could suppress dominant mutations carried by a different member of the ethylene receptor gene family. Previously we had found that *rte1* did not suppress existing gain-of-function alleles in the four other *Arabidopsis* ethylene receptor genes, but we could not exclude the possibility that this was a function of the particular dominant mutant alleles that had been tested (Resnick et al., 2006), especially since previous results revealed that only certain alleles of *etr1* are suppressed by *rte1* (Resnick et al., 2008). Having been identified those particular alleles, we could re-examine the question of receptor specificity using known suppressible mutations. We focused on the ethylene receptor ERS1, because among the *Arabidopsis* ethylene receptors, ERS1 has the most closely related ethylene-binding domain (amino acids 1 to 128) to that of ETR1 (75% identity, 83% similarity and Figure 4-1). We tested four dominant missense mutations (encoding Y32A, E38A, F58A, and A102T mutations) that had been found to be dependent on *RTE1* for ethylene insensitivity and one (encoding C65Y) that was *RTE1* independent, as shown in Table 3 (Resnick et al., 2008) and Table 4. All of the substitutions (except for A102T) act as strong alleles within the *etr1* transgene (Table 3) and reside in highly conserved regions within the three predicted transmembrane domains of the ethylene-binding domain (Figure 4-2 a).

The five mutations were each introduced into the corresponding conserved position of the *ERS1* coding sequence by *in vitro* site-directed mutagenesis. In order to control for possible differences in expression between *ETR1* and *ERS1*, two

separate *ers1* transgene (T*ers1*) constructs were created for each mutation, one driven by the native *ERS1* promoter and the other driven by the native *ETR1* promoter. Each of the ten constructs was stably transformed into wild-type and *rte1-2* null mutant plants, and six transformed lines were analyzed for each construct. As expected, all of the T*ers1* (C65Y) lines showed strong ethylene insensitivity (Figure 4-2 b; Table 5), which was not suppressed by *rte1-2* (data not shown). Contrary to expectations, none of the T*ers1* lines carrying Y32A, E38A, F58A and A102T exhibited ethylene insensitivity, even in the wild-type background (Figure 4-2; Table 5). This was surprising, because dominant mutations have been known to be transferred between different ethylene receptor isoforms, even across species, such as tomato *Never-ripe* and *Arabidopsis etr1-2* (Wilkinson et al., 1995; Sakai et al., 1998). The fact that these four mutations in the *etr1* gene had all required *RTE1* for insensitivity suggested a correlation between *RTE1*-dependence and the inability to confer insensitivity when carried by another ethylene receptor gene. To test this using another ethylene receptor gene, we introduced the A102T (*etr1-2*) mutation into *EIN4* driven by the CaMV 35S promoter. Like *ETR1*, the *EIN4* ethylene receptor gene encodes a C-terminal receiver domain (which the ERS1 receptor lacks). The results with *EIN4* were consistent with those of *ERS1*; wild-type plants transformed with the mutant *EIN4* transgene failed to display ethylene insensitivity (Figure 4-2; Table 5). Taken together, these results suggested that *ETR1* missense mutations that require *RTE1* to confer ethylene insensitivity do not cause ethylene insensitivity when present in the corresponding conserved positions within other ethylene receptors. These findings are consistent with a model, in which RTE1 acts specifically on ETR1 to maintain or

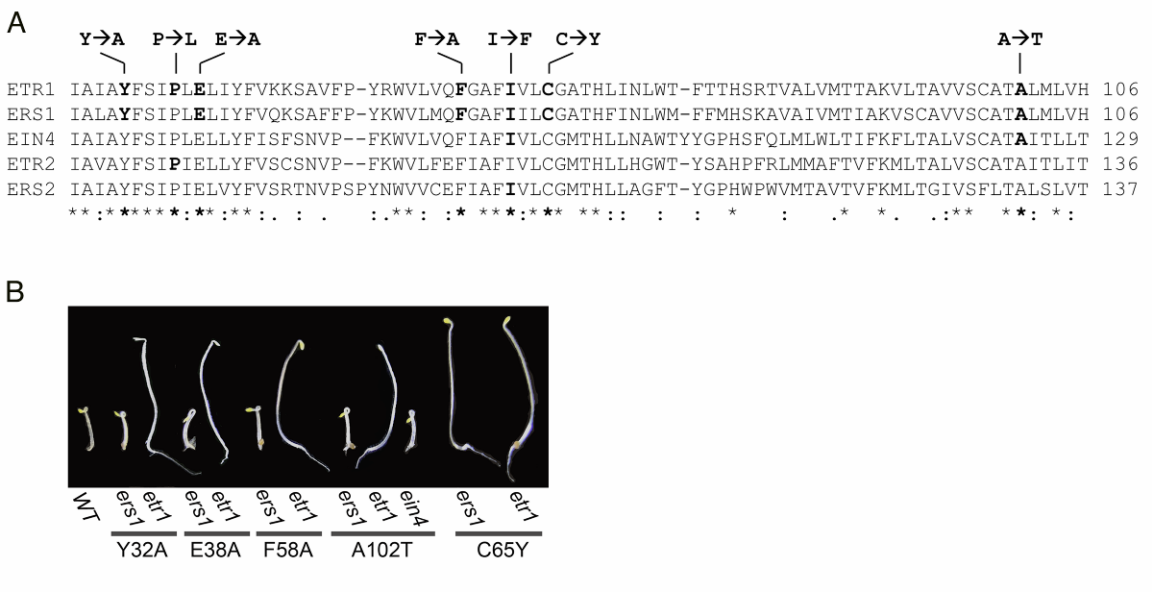


Figure 4-2. *RTE1*-Dependent *etr1* mutations do not confer ethylene insensitivity when carried by *ers1 and ein4* transgenes.

(a) Sequence alignment of the ethylene-binding region of the five Arabidopsis ethylene receptors showing the amino acid substitutions that were tested in Table 5. Amino acids that were substituted are shown in boldface within the sequences, with the corresponding substitutions indicated above the alignment. "*" denotes conserved residues, ":" denotes conserved substitutions, and "." denotes semi-conserved substitutions aligned using ClustalW (Larkin et al., 2007).

(b) Effect of various amino acid substitutions carried by the indicated receptor transgenes. *etr1*, *ers1*, or *ein4* transgenes carrying the specified amino acid substitutions were transformed into wild-type plants. Shown are representative dark-grown four-day old seedlings in the presence of 10 μM ACC.

Table 5. Dominant ethylene-insensitive *etr1* mutations that are suppressed by *rte1* do not confer insensitivity when carried by *ERS1* or *EIN4*

Ethylene receptor transgene	Amino acid substitution	Ethylene insensitivity conferred in WT?	Equivalent *etr1* mutation suppressed by *rte1-2*? *
T*ers1*	C65Y	YES	NO
	Y32A	NO	YES
	E38A	NO	YES
	F58A	NO	YES
	A102T	NO	YES
T*ein4*	A125T	NO	YES

* Data based on Table 3, Resnick et al., 2008.

stabilize active conformations of certain ETR1 mutant forms, but does not act on the other ethylene receptors, despite the high sequence conservation among the receptors.

In support of this model, we also showed that ethylene-insensitive mutations in the four other ethylene receptor genes could not be suppressed by *rte1* when carried by the *etr1* gene. We tested two different amino acid substitutions, I62F and P36L. The I62F substitution lies in an identical conserved position in four existing mutant alleles of four different ethylene receptor genes, all of which confer strong ethylene insensitivity: *etr1-4* (Chang et al., 1993), *ers1-1* (Hua et al., 1995), *ein4-1* (Hua et al., 1998) and *ers2-2* (Hua et al., 1998). It was shown previously that *ein4-1* and *ers2-2* are not suppressed by *rte1-2* (Resnick et al., 2006), and when we constructed the *ers1-1 rte1-2* double mutant, we found that *ers1-1* is not suppressed by *rte1-2* (Resnick et al., 2006). We then tested whether the *rte1-3* null suppresses *etr1-4* by creating an *etr1* transgene encoding the corresponding I62F substitution, followed by transformation into the wild type and *rte1-3*. Consistent with the model, the T*etr1* (I62F) transgene conferred ethylene insensitivity, which failed to be suppressed by *rte1-3* (Table 6). The second mutation that we analyzed, encoding the P36L substitution**,** is identical to that encoded in the corresponding position by *etr2-1* (Sakai et al., 1998). As above, we created an *etr1* transgene encoding P36L, and then transformed it into the wild type to test for ethylene insensitivity and into *rte1-3* to test for suppression. As with T*etr1* (I62F), the T*etr1* (P36L) transgene conferred ethylene insensitivity, which was not suppressed by *rte1-3* (Table 6).

Table 6. Dominant ethylene-insensitive receptor mutations in each of the four other ethylene receptor genes are not suppressed by *rte1* when introduced into *ETR1*.

Amino acid substitution	Dominant insensitive mutation	Suppressed by *rte1*? (X%)[1]	Equivalent *etr1* mutation	Equivalent *etr1* mutation suppressed by *rte1*? (X%)[3]
I to F	*ers1-1* (I62F)	NO (84%)	T*etr1* (I62F)	NO (94%)
	ein4-1 (I84F)	NO (96%)[2]		
	ers2-2 (I94F)	NO (101%)[2]		
P to L	*etr2-1* (P66L)	NO (98%)[2]	T*etr1* (P36L)	NO (106%)

[1, 3] X is % hypocotyl length in the *rte1-3* background relative to that in the wild-type background on 20 μM ACC

[2] Data from Resnick *et al.* (2006)

The EBDs of subfamily I members are not interchangeable

We next set out to explore if the ethylene binding domains of ERS1 and ETR1 were interchangeable and if the chimeric translational fusion protein could have signaling capacity (functional) in the absence of RTE1. We used a chimeric protein fusion construct with the ERS1 EBD (amino acids 1- 128) containing a dominant missense mutation (E38A or C65Y) fused to the rest of ETR1 (GAF, CC, HK, RD) labeled 'RTTT E38A' or 'RTTT C65Y'. The chimeric transgene was cloned in the pPZP221 plasmid and driven by the *ETR1* native promoter and also carried the 3'UTR of *ETR1* as a terminator sequence. The EBD of ETR1 and ERS1 share 75% identity and 83% similarity. Previous results showed that lack of *RTE1* does not affect ERS1 wild-type protein and that normal ERS1 signaling is independent of *RTE1* (Resnick et al., 2006). Moreover, we showed that the ERS1 protein carrying a C65Y missense mutation confers ethylene insensitivity independently of *RTE1*. Interchanging the ethylene binding domains of ERS1 and ETR1 will allow us to test the precise domain(s) of ETR1 that confer its dependency on RTE1.

Previous work from Dr. Chi-Kuang Wen's laboratory (Chinese Acadamy of Sciences, Shangai, China) had shown that an ETR1 truncated protein (consisting of the EBD-GAF-CC; amino acids 1-349) was sufficient for RTE1 to act on, either directly or indirectly. This conclusion came from well-designed genetic experiments where an *RTE1* over-expression plant was able to confer ethylene insensitivity when the only ETR1 protein available was an ETR1 truncated protein (amino acids 1-349) (Zhou et al., 2007).

Performing an experiment with the ERS1 EBD fused to the ETR1 GAF-CC-HK-RD will allow us to answer two different and interesting questions. First, are the EBD of ERS1 and ETR1 interchangeable, i.e. will the chimeric protein function (signal)? Second, will this chimera require *RTE1* for its signaling?

To examine the signaling by the chimeric protein RTTT, we assayed if RTTT could give insensitivity when carrying different gain of function missense mutations. We created two different missense mutations in the EBD of the RTTT chimera. First, we made an *RTE1*-independent mutation, C65Y, that when in ETR1 is known to give insensitivity even when heterologously expressed across different species (Wilkinson et al., 1997). Secondly, we introduced an alanine at amino acid 38 in place of a glutamate (E38A), a strong allele which is *RTE1*-dependent. Unexpectedly, preliminary results show both chimeric transgenes carrying either missense mutation did not confer ethylene insensitivity at low doses of ACC (ethylene). These assays were carried out in a heterozygous population and six to eight independent lines were examined (data not shown). This was very surprising given that the EBD domains of ERS1 and ETR1 are so well conserved and the *RTE1*-independent mutation, C65Y, confers insensitivity when carried by either ETR1 or ERS1 full length proteins. Nonetheless, the transgenic plants carrying either the RTTT E38A or the RTTT C65Y chimera transgenes were capable of restoring/alleviating the *etr1 etr2 ein4* phenotype back to a phenotype similar to the double *etr2 ein4* phenotype (Figure 4-3).

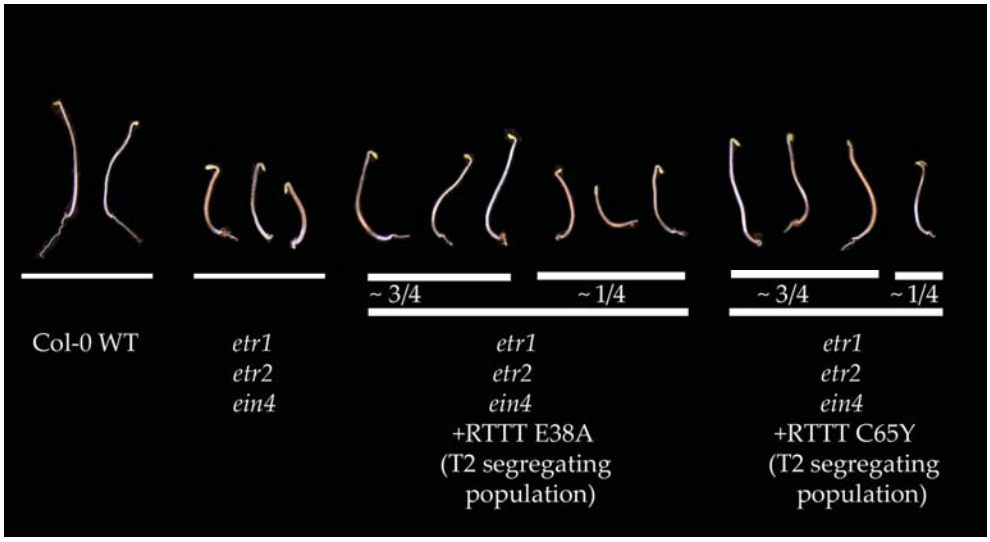


Figure 4-3. The chimera RTTT E38A and RTTT C65Y both alleviate ethylene responses.

Both RTTT E38A and C65Y alleviate the ethylene response in the triple receptor null mutant *etr1 etr2 ein4.* Representative seedlings are shown. The seedlings were grown on 1X Murashige and Skoog medium in air at 20 °C for four days in the dark and pictures were taken immediately after opening the plates.

Therefore, this suggests that the chimera transgenes are capable of signaling similar to an 'ETR1 wild-type' protein. Moreover, Dr. Wuyi Wang (of the late Dr. Tony Bleecker's lab, University of Wisconsin, Madison) had done a similar experiment with a wild-type version of the chimera ERS1 EBD (amino acids 1- 128) fused to the rest of ETR1 (GAF, CC, HK, RD) transgene and observed that his wild-type RTTT transgene rescues an *etr1* null (Dr. Wang personal communication).

This suggests the possibility that while the RTTT chimeric protein fusion was enough to signal in the place of an *etr1* null, it could not fully transmit the conformational change(s) from the ERS1 EBD and transmit a constitutive signaling to the rest of the ETR1 protein, mainly the GAF-CC domain. This result was also surprising given that the amino terminal region of all ethylene receptors, even across species, share a high degree of similarity (see Figure 4-1). In particular, the C65Y mutation that confers strong *RTE1*-independent ethylene insensitivity in ERS1 or ETR1 but not in the RTTT chimera suggests ETR1 and ERS1 may have their own unique conformations of the EBD, which are not in fact interchangeable. These preliminary results will be repeated and the triple response assay will be performed with homozygous lines as soon as I obtain them.

Discussion

Interestingly, we find that not all dominant ethylene-insensitive alleles of *etr1* are transferable to other ethylene receptor genes. We discovered that dominant *etr1* mutations that require *RTE1* to confer ethylene insensitivity are silent when

transferred to the identical conserved positions in other ethylene receptor genes. This was unexpected, given the strong sequence conservation among the ethylene receptors particularly within the ethylene-binding domain. Furthermore, dominant missense mutations that confer ethylene insensitivity have been previously transferred to the corresponding positions in other ethylene receptor isoforms, even across species. Therefore, a general assumption has been that dominant mutations identified in one ethylene receptor gene can be introduced into a different ethylene receptor gene (of the same or different species) to create a dominant mutant transgene capable of conferring ethylene insensitivity to wild-type plants. In fact, this approach was employed to demonstrate that the *Arabidopsis ERS1* and *ERS2* genes encode functional ethylene receptors in the absence of endogenous mutant alleles isolated for these genes: the *etr1-4* (I62F) mutation was introduced to an *ERS1* transgene (Hua et al., 1995) and the *etr1-4* (I62F) and *etr2-1* (P66L) mutations were each introduced to an *ERS2* transgene (Hua et al., 1998). Similarly, the ethylene-insensitive mutation of tomato *Never-ripe* (*Nr*) was introduced into the tobacco *Nt-ERS1* ethylene receptor gene to confer ethylene insensitivity to tobacco (Terajima et al., 2001). In another example, the H69A mutation, which blocks ethylene binding in *Arabidopsis ETR1* (Hall et al., 1999), was engineered into melon *Cm-ETR1* and found to confer ethylene insensitivity in *Nemesia strumosa* (Cui et al., 2004).

Our findings demonstrate that *RTE1* is highly specific for *ETR1* and complements previous data showing that ethylene insensitivity conferred by ectopic over-expression of *RTE1* is dependent upon *ETR1* and is largely independent of the other *Arabidopsis* ethylene receptor genes (Zhou et al., 2007; Resnick et al., 2006;

Resnick and Chang, unpublished data). Moreover, the ability of *RTE1*-dependent mutations to confer ethylene insensitivity in *ETR1*, yet have no effect in *ERS1* and *EIN4*, distinguishes *ETR1* from the other ethylene receptors. The *RTE1*-dependent *etr1* mutations presumably lead to an altered ETR1 ethylene-binding domain conformation that confers ethylene insensitivity, yet the same mutations in other ethylene receptors either do not result in the same altered conformation and/or they lack the action of *RTE1* to bring about ethylene insensitivity. Further (preliminary) evidence using 2,5-norbornadiene (NBD) supports the model of RTE1 having an effect in the ethylene-binding domain of ETR1. A dose response using the ethylene antagonist NBD showed partial insensitivity at high doses in the *rte1* null when compared to wild type and *etr1* null (see Appendix A). This finding suggest/indicates some kind of conformational defect in the EBD of ETR1 in an *rte1* plant that makes the plant less susceptible to high doses of NBD. It is unclear why *RTE1* affects *ETR1* but lacks this role for *ERS1* (and probably for the other three ethylene receptors). Interestingly, our results indicate that it is unlikely that a gene similar to *RTE1* acts on *ERS1*, because if that were the case, then the ethylene insensitivity of mutant *etr1* alleles would have given insensitivity when transferred to *ers1*. In other words, the lack of ethylene insensitivity from the *ers1* and *ein4* mutant transgenes suggests that *ERS1* and *EIN4* signaling is not only independent of *RTE1*, but independent of an RTE1-like protein. The *RTE1* mechanism that promotes *ETR1* signaling therefore appears to be unique to *ETR1*.

As for the ETR1 signaling mechanism, we find that the RTTT chimera carrying a strong *RTE1*-independent or dependent missense mutation (C65Y or

E38A) can not confer insensitivity yet still rescues an *etr1* null phenotype. This experiment may suggest two different signaling conformations of the EBD in ETR1 vs. ERS1 and/or the need for the EBD and the GAF-CC domain to work as one unit. On one hand, the RTTT C65Y chimera protein retains some 'wild type' signaling, yet on the other hand, it can not transmit the constitutive signaling from the missense mutation (C65Y) in the ERS1 EBD. A possible interpretation of these results is that wild-type signaling is due in large part to recruitment of CTR1 to the membrane through CTR1 interaction with the ETR1 HK domain (Clark et al., 1998). This recruitment of CTR1 to the membrane may be enough to repress some ethylene responses when grown on air. Schaller's group showed that in the *etr1 etr2 ein4* triple receptor null, CTR1 is mainly found in the cytosol (Gao et al., 2003). Therefore, an RTTT chimera independently of any missense mutations, may recruit some CTR1 to the membrane. An interesting fact remains that RTTT can signal, we assume that RTTT can recruit CTR1 to the membrane where CTR1 is functioning to some extent properly. Nevertheless, RTTT does not have the capacity to transmit the conformational change(s) in its EBD as a result of a C65Y mutation to the GAF CC domain and therefore can not constitutively activate CTR1. This result suggests that the EBD-GAF-CC act as one unit and the EBD can not be swapped, even in between subfamily I members. Interestingly, this raises the question of whether ETR1 overall has different conformational properties in the amino terminal region that are different from ERS1. Further work utilizing more swapping experiments of different domains may result in a better understanding if ETR1 has a specific signaling mechanism and/or conformation(s) different from the other four receptors.

It remains very interesting that the RTTT C65Y protein, which presumably cannot bind ethylene, has an ETR1-like 'wild-type' signaling capability yet is able to presumably turn 'OFF' its receptor signaling in the presence of ethylene. The RTTT C65Y does not confer insensitivity. This suggests that the ERS1 C65Y EBD in the presence of ethylene is able to shut off signaling in RTTT, yet the same ERS1 C65Y EBD cannot confer constitutive signaling to the rest of the RTTT protein. This contradiction does not fit in the receptor signaling model. One possibility (explanation) is that the RTTT chimera has a very weak signaling capacity (recruits some CTR1 to the membrane) and therefore can only be detected in a triple receptor null sensitized background. For that reason, a comprehensive dose response with very low doses of ethylene may show that the alleviation of the triple receptor null by RTTT is not capable of shutting 'OFF' its signaling.

To further address *RTE1's* effect on ETR1 signaling, I have started transforming plants with an RTTT wild-type chimera (no mutations). Examining the phenotypes of RTTT WT in *rte1* vs. *etr1* nulls in 0.5uM ACC will uncover if RTTT WT chimera requires RTE1 to function. This experiment will narrow the location of were RTE1 affects or genetically interacts with ETR1. Whether RTE1 directly or indirectly interacts with ETR1 remains unsolved. Previous results from other groups support the possibility of ETR1 to form a large protein complex that may include RTE1. Analysis by a gel-filtration assay showed that the ETR1 receptor is part of a stable 670 KDa complex (Schaller, personal communication; (Gao et al., 2004). Moreover, exciting preliminary results from Dr. Chunhai Dong show weak ETR1 – RTE1 protein interaction when assayed transiently through a bimolecular

fluorescence complementation (BiFC) test in tobacco leaf cells. In summary, more work is needed to confirm if RTE1 acts on the EBD or the GAF-coiled coil domain(s) through a direct physical interaction or if other proteins are required. Understanding where RTE1 acts on ETR1 and whether it's a direct protein – protein interaction will help further identify new targets of the other RTE family members in other species.

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

Experimental Procedures

Plant strains and growth conditions

The *Arabidopsis thaliana* wild-type ecotype Columbia (Col-0) was used throughout this work. For all seedling analyses, seeds were sown on Murashige and Skoog (MS) medium containing 0.8% agar. Following three-day stratification at 4^{o}C, seeds were incubated at 20^{o}C for 4 days in either complete darkness or 24-hour light. Plants were grown in soil under a 16-hour light / 8-hour dark cycle in controlled environment chambers under fluorescent lights. For the triple-response assay, seedlings were germinated on medium containing ACC (Sigma Aldrich) at the stated

concentrations. To measure hypocotyl lengths, seedlings were digitally photographed and measurements were made using IMAGEJ software (http://rsb.info.nih.gov/jj/).

Transgenic constructs and plant transformation

Construct were transformed by the floral dip method (Clough and Bent, 1999) using *Agrobacterium tumefaciens* strain GV3101. Transformants were selected using the herbicide Gentamycin (pPZP221) at 90mg/L on ½ MS or Kanamycin (pCambia1380) at 50mg/L on 1X MS.

To engineer mutations into *ERS1*, *EIN4* and *ETR1*, *in vitro* site-directed mutagenesis was carried out using Stratagene's QuikChange mutagenesis kit (www.stratagene.com). For *ERS1*, missense mutations were introduced into the genomic *ERS1* sequence driven by the native *ERS1* promoter in the binary vector pCAMBIA1380 (kindly provided by G. Eric Schaller, Dartmouth College) and into c-*ERS1* (the *ERS1* coding sequence in the binary vector pPZP221 driven by the *ETR1* native promoter and carrying the *ETR1* 3' UTR) (Wang et al, 2003). For *EIN4*, a missense mutation was created in the *EIN4* cDNA, which was then cloned as a *Bam*HI fragment into the plasmid pART7 (Gleave et al., 1992), between the CaMV 35S promoter and 3' OCS terminator sequence. The composite gene was then released with *Not*I and ligated into the *Not*I site of binary vector pMLBart (Gleave et al., 1992), which is a derivative of pART27 containing the *bar* gene. For *ETR1*, missense mutations were introduced into c-*ETR1* (the *ETR1* coding sequence in pPZP221 driven by the *ETR1* native promoter and carrying the *ETR1* 3' UTR) (Wang

et al, 2003). All mutations were verified by nucleotide sequencing. To create the ERS1 amino acid substitutions the following primers were used:

Tyrosine at amino acid 32 to alanine (Y32A):

5'-tgcgttgattgctcttgcagccttctcaatcccactcgag-3' and

5'-ctcgagtgggattgagaaggctgcaagagcaatcaacgca-3'.

Glutamic acid at amino acid 38 to alanine (E38A):

5'-cttctcaatcccactcgcgcttatctatttcgtgc-3' and

5'-gcacgaaatagataagcgcgagtgggattgagaag-3'.

Phenylalanine at amino acid 58 to alanine (F58A):

5'-tacaaatgggtgcttatgcaggctggagcctttatcattctctg-3' and

5'-cagagaatgataaaggctccagcctgcataagcacccatttgta-3'.

Alanine at amino acid 102 to threonine (A102T):

5'-ttgtgtcgtgtgctaccacgttgatgttggttcat-3' and

5'-atgaaccaacatcaacgtggtagcacacgacacaa-3'.

Cysteine at amino acid 65 to tyrosine (C65Y):

5'-gtttggagcctttatcattctctatggagctacgca-3' and

5'-tgcgtagctccatagagaatgataaaggctccaaac-3'.

To create the ETR1 amino acid substitution I62F:

primers 5'-ggtacttgttcagtttggtgctttttcgttctttgtggagcaac-3' and

5'-gttgctccacaaagaacgaaaaaagcaccaaactgaacaagtacc-3' were used to incorporate a phenylalanine to an isoleucine at amino acid 62 in the c-ETR1 pPZP211 vector (Bleecker).

To construct the RTTT transgene with the missense mutations, we cloned the ERS1 EBD (amino acids 1 to 128) into the pGEM-T vector using a forward primer starting at the *Sac*I (5'-tgtggaattgtgagcggata-3') site just upstream of the ETR1 promoter in the c-ERS1 pPZP221 construct and a reverse primer with an engineered *Sac*I restriction site at nucleotide 384 (amino acid 128) (5'-atcgagctcatcagctttcttc-3'). The PCR was performed with the c-ERS1 E38A and C65Y as the corresponding DNA templates. The PCR fragment and the c-ETR1 construct were cut with the restriction enzyme *Sac*I; the corresponding ~11 Kb partial ETR1-vector and the 1.92 Kb c-ERS1 EBD PCR fragment were cloned by ligation with the T4 Ligase (New England Biolabs).

The wild-type RTTT constructs have been kindly provided by Wuyi Wang (unpublished results). The RTTT wild-type clone from Wang had been cloned by the same method as the RTTT missense mutation constructs I cloned earlier.

The plasmids pPZP221 and pCAMBIA 1380 containing individual *etr1* or *ers1* transgenes were transformed into plants by the floral dip method (Clough and Bent, 1998). Transformed T_1 individuals were selected on MS plates containing 100mg/L of Kanamycin for vector pPZP211, 90mg/L of Gentamycin for pPZP221, and 200mg/L of Hygromycin for pCAMBIA 1380 constructs.

Chapter 5: Conclusions and perspectives

Conclusions

In summary, I have addressed several key and important questions on both *RTE1* action and ETR1 signaling. The work done over the past six years has been very challenging and exciting. I started this project to examine more closely *RTE1's* molecular function and how it plays a role in ETR1 signaling and why its function is specific to ETR1. A related goal was to advance our knowledge of *RTE1's* molecular function in Arabidopsis, and thereby provide a framework to further understand its role in other organisms. Conducting research in a unique system where a prokaryotic-like receptor (ETR1) signals to a eukaryotic-specific downstream component (CTR1) made understanding the ETR1 signaling mechanism more fascinating. After extensive research, *RTE1's* molecular function is still unanswered but several new insights have been made. Below are some of the findings on *RTE1* and how ETR1 appears to be unique in the way it requires *RTE1* and possibly signals to downstream components.

1) Localization of ETR1 at the Golgi/ER presents an interesting modification to our current understanding of ethylene receptor signaling, but is consistent with the overall model of ethylene signaling. Due to the solubility of ethylene in aqueous and lipid environments, ethylene should be readily perceived by receptors residing at either organelle.

2) Subcellular co-localization of RTE1 and ETR1 supports the possibility that RTE1 promotes ETR1 signaling through physical interaction with ETR1.

3) The *rte1* null does not affect protein abundance of ETR1, therefore suggesting that *rte1* null plants render an abundant non-functional ETR1 protein. In addition, ETR1 sub-cellular localization studies showed no difference of ETR1 localization between *rte1* and *Col-0* wild-type backgrounds. All these results support a more subtle regulatory role for *RTE1*, because if *RTE1* were needed for correct ETR1 localization or essential for ETR1 protein stability, a difference in protein localization or abundance would have been revealed.

4) Wild-type ETR1 is dependent on *RTE1* to signal correctly. Therefore, the finding that silver can convert wild-type ETR1 to become *RTE1*-independent suggest that the silver ions (which are assumed to replace the copper cofactor) most likely have changed the conformation of the EBD (ethylene binding domain) in a way that overrides the requirement of RTE1. This new EBD conformation caused by silver may be reminiscent of an *etr1* dominant gain of function allele which is not suppressed by *rte1*. Previous results revealed a class of mutations that requires *RTE1* in order to confer ethylene insensitivity, as well as a class that is independent of *RTE1*. The underlying basis for these two classes remains unclear. A characteristic shared by all the *etr1* alleles tested is that they each presumably produce a unique structural defect within the ETR1 ethylene-binding domain that consequently inhibits, to varying degrees, the conformational transition required to turn ETR1 signaling off. Therefore, the *RTE1*-dependence of certain *etr1* alleles, including the wild-type *ETR1* allele, may be related to particular conformations of the ethylene-binding domain. Accordingly, from all our results, we propose that *RTE1* has an effect on the

conformation of the ethylene-binding domain that results in the promotion or stabilization of the ETR1 signaling "ON" state.

5) Interestingly, we find that not all dominant ethylene-insensitive alleles of *etr1* are transferable to other ethylene receptor genes. We discovered that dominant *etr1* mutations that require *RTE1* to confer ethylene insensitivity are silent when transferred to the identical conserved positions in other ethylene receptor genes. Our findings demonstrate that *RTE1* is highly specific for *ETR1* and may suggest differences between ETR1 and ERS1 signaling.

6) The lack of ethylene insensitivity from the *ers1* and *ein4* mutant transgenes suggests that *ERS1* and *EIN4* signaling is not only independent of *RTE1*, but independent of an RTE1-like protein. The *RTE1* mechanism that promotes *ETR1* signaling therefore appears to be unique to *ETR1*.

7) The finding that the RTTT chimera carrying a strong *RTE1*-independent missense mutation (C65Y) can not confer insensitivity yet still rescues an *etr1* null suggests a conformational difference between ETR1 and ERS1. We therefore propose that the entire amino terminal region (EBD-GAF-CC) probably acts together as one unit and that differences in conformation of the EBD exist between ETR1 and ERS1.

8) *RTH*, the Arabidopsis *RTE1* homologue, does not seem to be involved in the ethylene signaling pathway. Characterization of the *rth-1* null and the *rth-1 rte1-3* double null indicates no distinct phenotype when treated with ethylene (See Appendix B).

9) Finally, our findings demonstrate that ETR1 is unique in its signaling mechanism/conformation(s) as evident through the requirement of *RTE1*, since the

other ethylene receptors do not require RTE1 nor any RTE1-like proteins. Moreover, we find that the ethylene binding domain (EBD) of subfamily I receptors are not interchangeable. We suggest that the EBD-GAF-CC act as one unit in ethylene signaling. In addition, the findings in this thesis suggest a difference in the conformations/signaling of the ethylene receptors, especially in between subfamily I (ETR1 vs. ERS1).

Significance

The two goals of this thesis were the following: To further advance our understandings of *RTE1* molecular function and to further comprehend the relationship ('genetic' interaction) between *RTE1* and *ETR1*. Prior to this work all of our understanding of *RTE1* came from elegant genetics in plants. The power of genetics in Arabidopsis is a great tool to study and characterize identify genes of interest. Yet as with any tool, genetics has its limitations, especially when trying to assign a molecular function to a novel gene. The power of genetics to identify genes with phenotypes has set the framework of the ethylene signaling pathway. The new challenge is to identify and examine the molecular function of these new components, such as *RTE1*, in this pathway.

This thesis provides a better understanding of *RTE1* at the cellular and molecular levels resulting from the cellular and biochemical analyses carried out here. Prior to this work, several hypotheses were formulated on *RTE1's* molecular function based on genetic data. For example, it was hypothesized that *RTE1* may play a role in protein stability, subcellular localization, or the signaling output of ETR1. The work

in this thesis provides compelling evidence that *RTE1* does not affect the protein stability or the subcellular localization of its target, ETR1. Furthermore, our work supports that hypothesis that *RTE1* acts on the EBD of ETR1 probably by affecting the lipid bilayer endomembrane. Although this latter model needs to be further tested, we now have narrowed the possible functions of *RTE1*. Of a broader significance, we also have advanced our understanding of the cellular function of *RTE1* in general. *RTE1* likely has a conserved cellular process that has yet to be fully elucidated, but the work on both *RTE1* (and *RTH*) has brought us many steps closer to revealing that cellular process.

In addition, we find ETR1 most likely has a unique RTE1-dependent conformation(s) that are not present in other ethylene receptors. This model supports previous genetic evidence that ETR1 is a key player in the ethylene signaling pathway. This hypothesis refines the receptor signaling model and proposes a more complex mechanism of ethylene receptor signaling between family members. Many different experiments have placed ETR1, and in many cases ERS1, as key components of receptor signaling, yet the findings in this thesis are the first to demonstrate differences in signaling capabilities between them and to distinguish ETR1 from the rest of the receptors.

Finally, the examination of the function of a novel gene conserved in animals and plants, *RTE1*, provides a great starting platform that will eventually lead to the understanding of the role of other *RTE* family members in other organisms. The findings in this thesis may suggest a role for RTE1 in the lipid bilayer environment that affects the conformation of the ETR1 EBD, currently being tested (see below).

What is the molecular function of RTE1? Some speculation and a new hypothesis

Does RTE1 affect the endomembrane (lipid bilayer) environment?

The ETR1 conformational changes proposed by Wang (Wang et al., 2006) fit with the idea of the two-component signaling mechanism of the receptors flowing their information from an amino-terminal sensory domain to the carboxy-terminal transmitter or signal output domain(s) (N-to-C flow) (Aravind et al., 2003).

RTE1 only affects certain etr1 alleles (including the wild-type allele) in which there seems to be no correlation of suppression with ETR1 signaling strength, location of the missense mutation, nor ethylene binding. In addition, preliminary work from Jianhong Chang (a graduate student in our laboratory) has identified two proteins involved in membrane/lipid maintenance that physically interact with RTE1 in the yeast split ubiquitin assay (Johnsson and Varshavsky, 1994).

We propose that *rte1* shapes/changes the lipid bilayer membrane in a way that renders the wild-type ETR1 non-functional. This non-functional ETR1 EBD may in turn be unable to make the necessary conformational changes to the GAF-CC domain(s) and as a result ETR1 has no signaling capacity. A hypothesis would be that some *etr1* alleles (*RTE1*-independent) result in an unwavering configuration of the EBD that is not affected by the changes in the lipid bilayer environment. These *etr1* alleles which are *RTE1*-independent result in a conformation which shifts the GAF-CC domain to an active conformation. RTE1 specificity to ETR1 could be the result

of a unique EBD conformation of the ETR1 EBD. We hypothesize that ETR1 takes up different conformation(s) than its subfamily I counterpart, ERS1, therefore ERS1 may have a different conformation and/or affinity to signal to its GAF-CC. Moreover, the chimera RTTT carrying a C65Y mutation could not confer insensitivity indicating the ERS1 EBD is not accurately communicating with the GAF-CC domain of ETR1. Both ETR1 and ERS1 when carrying a C65Y mutation confer strong ethylene insensitivity that is *RTE1*-independent. In order to better interpret these results, more work on how RTTT signals is needed. Does RTTT give the same ethylene response at all doses as wild-type? Understanding and quantifying RTTT vs. wild-type receptor signaling is crucial to making the correct interpretations.

Animals, including humans, do not have proteins that contain a sequence similar to the ethylene binding domain and no proteins known to bind ethylene. Therefore the target of the human RTE homolog is probably some other transmembrane protein unrelated to the ethylene receptors. It is not clear, however, how *RTE1* is specific to ETR1. An interesting model is where ETR1 signals or changes its conformation to 'ON/OFF' signaling in a way that makes it unique when compared to the other receptors, in particular the ERS1 receptor. In this model where ETR1 takes up unique configurations, specifically at the EBD, and requires RTE1 to 'create' the corresponding lipid bilayer environment is the hypothesis that best fits all our data.

Future experiments to test working models

Searching for the (molecular) truth about *RTE1*

So after many years of hard work and enjoying a great learning experience, there are new questions to be answered and some old ones. More importantly one main question remains, what is the molecular function of *RTE1*. As mentioned above, we propose that *RTE1* is in some way affecting the endomembrane environment which in turn affects ETR1. Future strategies to address this question and provide greater insight are discussed.

One main point that may shed some light into *RTE1's* molecular function is to identify its molecular partners. In order to find these partners, several approaches could be taken. First, Jianhong Chang, graduate student in the lab, is currently screening a cDNA library made from dark grown seedlings in the yeast split ubiquitin protein interaction assay to uncover RTE1 protein partners. This approach gave some preliminary evidence that RTE1 interacts with two lipid binding-like proteins. A second approach also initiated in the lab is to discover RTE1 protein partners using a proteomic strategy. In this strategy, the use of a tandem affinity purification (TAP) system and mass spectrometry (MS) will be used. TAP-RTE1 proteins will be purified from plants and the protein partners will be identified by MS. Both of these strategies have the potential to discover proteins with known molecular functions that interact with RTE1.

On the other hand, other cell biology/biochemical experiments may lead to a better understanding of RTE1 function. Photobleaching time experiments of ETR1-GFP proteins in wild-type vs. *rte1* null could probe membrane fluidity differences in an *rte1* mutant. Another assay worth pursuing is to analyze the lipid content in an *rte1* null seedling. The difficulty with this assay is that there are not many good markers of lipids and RTE1 probably would only affect the lipid bilayer of the ER and Golgi. A final assay worth mentioning is to examine ETR1 protein conformation changes by analyzing the ETR1-CTR1 protein-protein interaction in the *rte1* null background. ETR1 and CTR1 have been shown to interact in a yeast two hybrid assay (Clark et al., 1998) as well as *in planta* via BiFC (Kendrick and Chang, unpublished). The rationale of the experiment would be to look at the known ETR1 and CTR1 physical interaction via BiFC in wild type and in the *rte1* null plants. If the results show that CTR1 can no longer bind to ETR1 in an *rte1* null, then this will directly suggest conformational changes in ETR1 that do not allow its interaction with CTR1.

Testing the molecular structure/function of ethylene receptor signaling

In the quest to identify RTE1's function, we came across an unanticipated finding. We identified that the ethylene binding domains of the subfamily I, ERS1 and ETR1, differ from each other and it is likely that the EBD and GAF-CC domains act as one unit. Moreover we propose that the EBDs can not be interchanged. Two new hypotheses arise from these interpretations that need to be addressed. First, we propose that the differences between ETR1 and ERS1 are in the specificity of RTE1 to ETR1, as can be interpreted from the lack of insensitivity in ERS1 when carrying

mutations that are *RTE1*-dependent in ETR1. Second, other differences, apart from *RTE1*, may exist between the two EBD since a strong *RTE1*-independent mutation (C65Y) also failed to give insensitivity when carried by the RTTT chimeric transgene. Future strategies to address these hypotheses/models are discussed. One of the first experiments is to address the chimeric fusion protein RTTT signaling. It is unclear if the RTTT C65Y or E38A chimeric proteins are slightly (very weak) insensitive or not. Performing an ethylene dose response with all RTTT chimeric proteins in the *Col-0* wild type background and the triple receptor null background should aid in determining what type of signaling RTTT C65Y, RTTT E38A, and wild-type RTTT have. However, as mentioned earlier, the lack of strong insensitivity from RTTT C65Y implies that the EBD of ERS1 does not fully communicate with the GAF-CC domain of ETR1. We propose that the EBDs are different among these receptors. How are they different? Both ERS1 full length and ETR1 full length when carrying a C65Y mutation confer strong insensitivity. Consequently, we propose that any difference in the EBD of ETR1 (vs. ERS1) has a compensatory mutation in the GAF-CC domain to create the correct protein conformation. To address this model, *in vitro* site directed mutagenesis of regions of the ERS1 EBD in the RTTT chimera will be changed to the corresponding ETR1 residues. This strategy will enable us to pinpoint which amino acids are required for the C65Y RTTT and/or E38A RTTT chimera to become fully insensitive. Only a few regions of two to four amino acids per region are divergent in the EBD of ETR1 vs. ERS1. This makes the experiment reasonably feasible. Once we locate the important amino acid region(s) we can test whether this new chimeric protein is able to confer insensitivity in an *rte1* null. These

sets of experiments will add a great deal of knowledge in how we think the receptors function as well as how and where RTE1 plays a role in it.

Finally, one experiment would in fact give us a better understanding of what makes an *etr1* dominant gain-of-function allele *RTE1*-dependent or independent. The experiment would be to obtain the crystal structure of the ETR1 receptor. Analyzing the 3D structure and where each amino acid resides within the structure, especially in the EBD, may shed some light into what makes an *etr1* allele insensitive and may also provide clues into why some of these alleles require *RTE1*. Examining the structure of the ETR1 receptor and to further characterize what conformation changes occur in its ethylene binding domain where there is a missense mutation will surely advance our understanding of receptor signaling and in its requirement for *RTE1*.

Appendix A

Preliminary results with 2,5-norbornadiene may support a conformational defect of the ETR1 EBD in the *rte1* null mutant

2,5-norbornadiene (NBD) is an ethylene-like molecule which at elevated doses elicits an ethylene response (Larsen and Chang, 2001). Nevertheless, the cyclic olefin 2,5-norbornadiene (C_7H_8) is an ethylene antagonist which at low doses (~200ppm) inhibits the action of ethylene by competing for the receptors without eliciting a response (Sisler, 1991; Larsen and Chang, 2001). NBD at very high doses (~200000ppm) can shut off the receptors enough to give shortening of hypocotyl, therefore suggesting that maybe the effectiveness of NBD to force the conformational changes required at the EBD to shut off signaling are very weak (Matoo and Suttle, 1991; Larsen and Chang, 2001). An alternative explanation is that such high doses of NBD elicit stress responses from the plant which are not directly related to ethylene receptor signaling.

rte1 and *etr1* have the same ethylene hypersensitivity at all doses when assayed for the triple response phenotype (Resnick et al., 2006; Zhou et al., 2007). To examine more closely the ETR1 EBD, we decided to test if *rte1* had the same hypersensitivity as *etr1* to NBD. Our results demonstrate that *rte1* has a different hypersensitivity dose curve than *etr1* (Figure A-1). The difference in sensitivity to NBD may be indicative of a change in the EBD of ETR1 in an *rte1*. *rte1* seedlings may still have a subtle and weak ETR1 signaling capability as is supported by the double null *ers1 rte1* which does not mimic a subfamily I null *etr1 ers1* phenotype

(Resnick et al., 2006). That very weak signaling of ETR1 in an *rte1* null is not detectable when in the presence of a strong inhibitor of receptor signaling such as ethylene. In the presence of NBD which competes for the receptors but is very inefficient in turning off the signaling, this small undetectable difference may be observed. Moreover, what is very interesting is the fact that at high doses of NBD, *rte1* seems to be responding less to NBD than wild type (when compared to 0ul/L of NBD). Can the EBD of ETR1 in an *rte1* not shut off completely in the presence of NBD? This in turn may explain the results in which *rte1* responds less to NBD than wild type or *etr1*. In any case these experiments are preliminary and further work needs to be done for a more clear interpretation.

The finding that *rte1* has different dose response to NBD in comparison to an *etr1* null or wild-type suggest/indicates some kind of conformational defect in the EBD of ETR1 which renders the ETR1 receptor in an *rte1* plant less susceptible high doses of NBD. This finding was unexpected since previous results showed the same ethylene hypersensitivity in *rte1* and *etr1* single nulls. This finding may support the model were RTE1 affects the EBD of the ETR1 receptor. Further examination of the *rte1* null seedlings under other different ethylene-like molecules may shed some light into the function of RTE1 on ETR1 EBD.

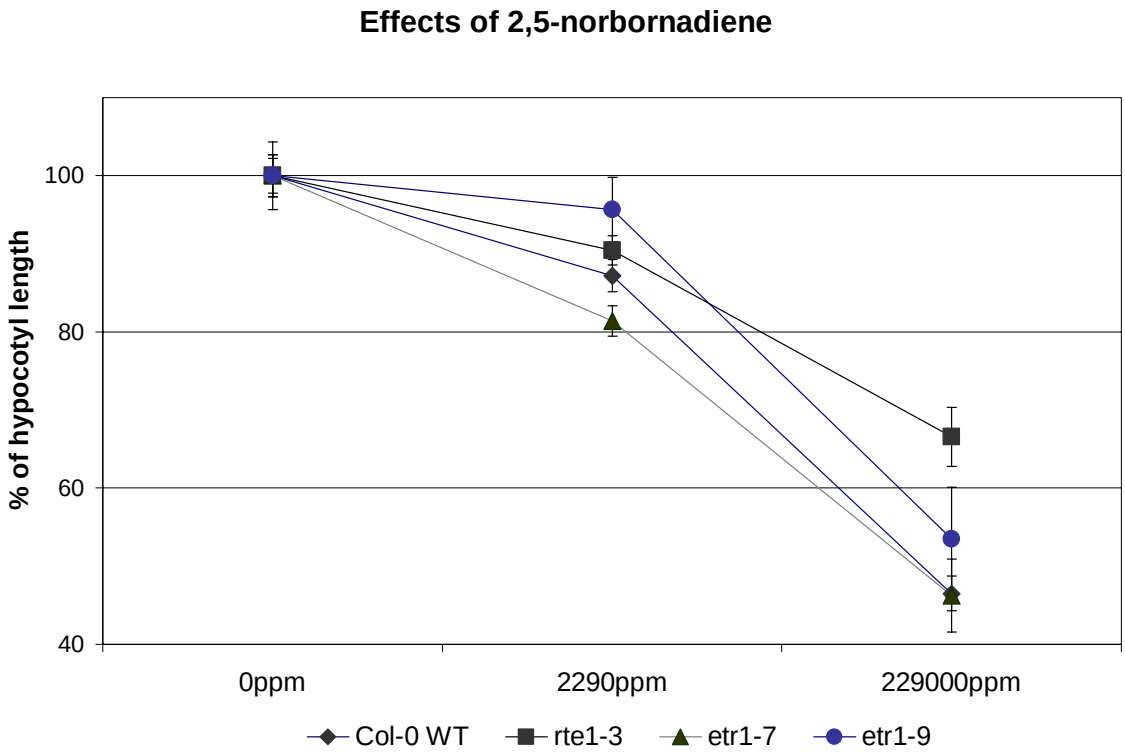


Figure A-1. Slight insensitivity of *rte1* to 2,5-norbornadiene (NBD) could support RTE1's role in affecting the ETR1 EBD.

Preliminary results from an NBD dose-response assay show the *rte1* mutant displays less sensitivity to NBD than Col-0 WT and the *etr1* null. For the graph, relative hypocotyl length compared to 0ppm for each genotype is ploted. The mean ± standard error converted to % is shown; n≥10 seedlings.

rte1-3 is a true null and RTE1 is membrane bound

To further investigate the nature of *rte1-3* mutation, we decided to examine if the *rte1-3* plant made an RTE1 protein. We used an existing amino terminal targeted antibody (For details, see Resnick 2006; Thesis). After many attempts to identify the correct antibody bleed and the right conditions, we were moderately successful in obtaining clear results. As shown in Figure A-2, we could establish that *rte1-3* is a true null and does not synthesize any RTE1 protein. We also showed that RTE1 appears to be correctly synthesized in yeast. Furthermore, we show that RTE1 is present in the membrane fraction (100000xg pellet) which supports its prediction of being an integral transmembrane protein.

ETR1 – RTE1: protein - protein interaction assays:

Split Ubiquitin experiments (yeast)

To test a possible protein - protein interaction between RTE1 and ETR1, we decided to use an assay where it would be feasible to work with two full length membrane proteins. In the more traditional 'yeast two hybrid' system (Fields and Song, 1989), the activating domain protein must travel to the nucleus to activate transcription, therefore the use of two transmembrane proteins creates a situation where the shuttling to the nucleus is improbable. We decided to pursue the interaction via the Split Ubiquitin system (Johnsson and Varshavsky, 1994). After an extensive list of probable combinations of RTE1 and ETR1 as prey or bait and in several

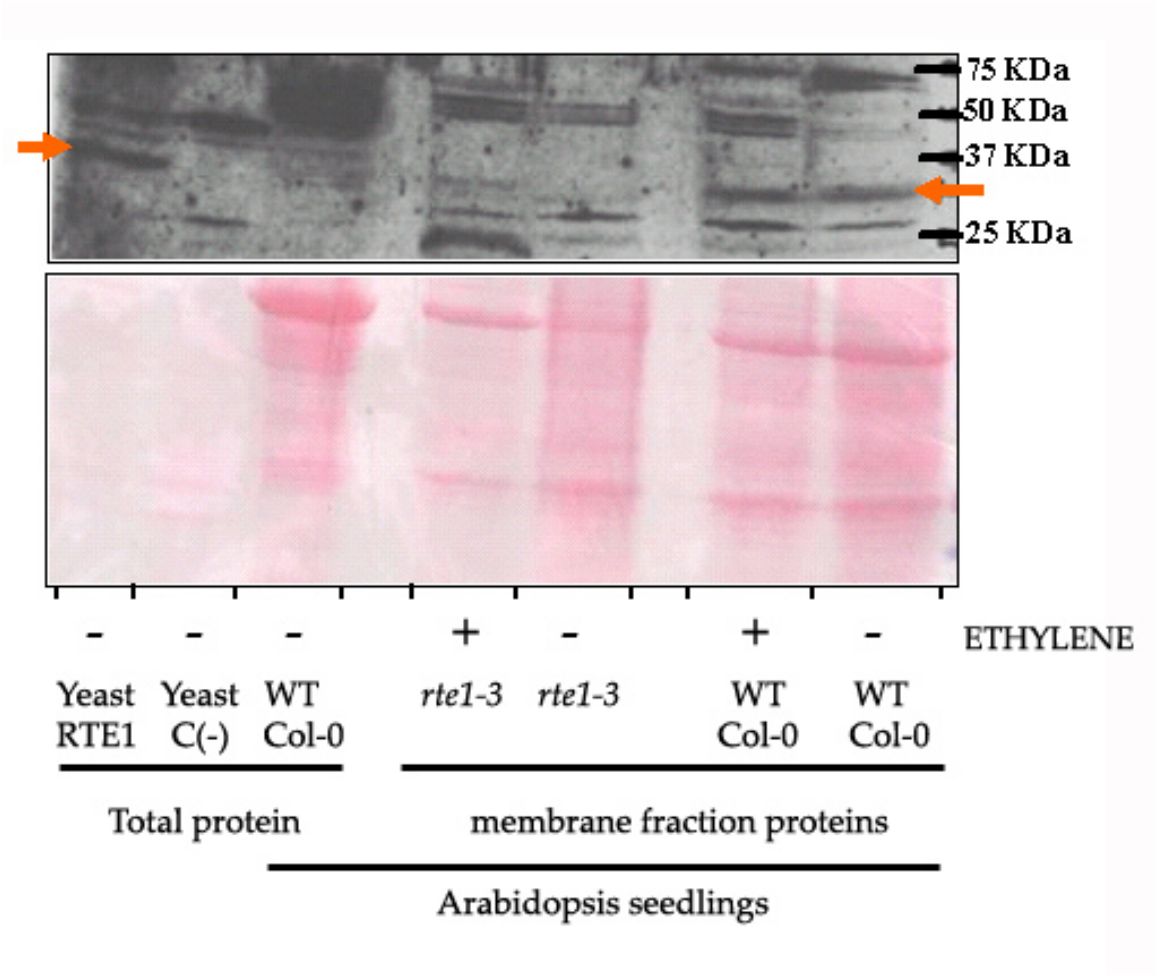


Figure A-2. Presence of the RTE1 protein in WT but absent in the *rte1-3* mutant. Western blot showing the RTE1 isolated from the microsomal membrane fraction of Arabidopsis seedlings run on denaturing 16% PAGE, prior treatment with BME for 1 hour at 37C. The proteins were detected by an RTE1 antibody (bleed #5, 1:500 dilution). RTE1 gives a band of approximately 28 KDa that is absent in the *rte1-3* background, red arrow. In the Col-0 WT total protein extract, RTE1 could not be detected. A Ponceau S stain of the blot is shown to show a semi quantitative measure of protein loading per lane.

conditions varing the amount of bait protein being made, we found that RTE1 and ETR1 do not consistently interact in this system. We tried all these conditions in two different split ubiquitin systems; a 5-fluoroorotic acid (5FOA) selection strategy (Johnsson, 2002) and a HIS/LacZ selection strategy (Obrdlik et al., 2004). In both cases we found no repeatable interaction between *Arabidopsis* RTE1 and ETR1 proteins in yeast.

Co-IP using an *HA-RTE1* and *ETR1-5xMYC* constructs

A better alternative to the yeast based method of testing protein-protein interaction is to perform co-immunoprecipitation (Co-IP) experiments. Performing Co-IP experiments with two transmembrane proteins present a greater challenge, on the other hand Co-IP experiments performed *in planta* provide the native environment where the targeted proteins are synthesized and localized. To be successful in co-immunoprecipitation, a good reliable set of antibodies is essential. Previously we had constructed an ETR1-5xMYC transgene, which we showed was functional and could be easily and cleanly detected by western blot (Dong et al., 2008 and Figure 2-2). The RTE1 antibody, as shown in Figure A-2, gives too many background bands and therefore is not suited for co-immunoprecipitation. Consequently, we constructed an epitope tagged version of *RTE1*. We cloned *RTE1* with an amino terminal hemagglutinin epitope (HA) tag driven by the constitutive CaMV 35S-driven promoter in the vector pEarleyGate201 (p35S-HA-RTE1) (Keith W. Earley et al., 2006). We are currently in the process of isolating independent homozygous *Arabidopsis* lines of HA-RTE1 and HA-RTE1 crossed with ETR1-

5xMYC. Once we obtain these lines and test for HA-RTE1 functionality, we will begin to perform Co-IP experiments. This work will be done in collaboration with Dr. Dong of our laboratory and Dr. Inhwan Hwang at the Center for Plant Intracellular Trafficking, Pohang University of Science and Technology, Korea.

Appendix B

Characterization and study of *RTH*

RTE1 has one homologue in *Arabidopsis,* named *RTH* (RTE1-HOMOLOG) whose amino acid sequence shows 69% similarity (Figure 1-5, ClustalW2 (Larkin et al., 2007). The *RTE* gene family is conserved in animals, plants and lower eukaryotes (except fungi) which suggests a conserved function. There is one conserved domain, Domain of Unknown Function 778 (DUF778), which encompasses the whole *RTE1* family. The molecular/biochemical function of *RTE* is completely unknown in all organisms. Our current understanding of *RTE1* function comes entirely from studies in plants (Barry and Giovannoni, 2006; Resnick et al., 2006; Zhou et al., 2007; Dong et al., 2008; Resnick et al., 2008).

RTH has a similar profile as *RTE1* in the plant tissues but with lower expression intensity. Moreover, both *RTE1* and *RTH* are up regulated in roots of seedlings where there has been nitrogen deprivation (Table 2) (eFP browser: http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi and NASC array browser http://affy.arabidopsis.info/narrays.com). The fact that there is only one *RTE1* homolog in *Arabidopsis* (*RTH*) made perusing the role of *RTE* function in *Arabidopsis* manageable. We believed that the use of reverse genetics for *RTH* and the double null *rte1 rth* would broaden our understanding of the biological role that *RTE* plays in Arabidopsis and eventually in all other eukaryote organisms.

I was fortunate enough to receive a mutant plant with a frame shift mutation that gave an early stop codon at amino acid 108 of 231, this mutant was named *rth-1*

(Q108stop) (Table 1). The *rth-1* mutant *Arabidopsis* plant came from the Tilling Project, a large scale point mutation project in *Arabidopsis thaliana* (Till et al., 2003).

Unfortunately, after much extensive reverse genetic type of experimentation for phenotypic differences in *rth-1* and *rte1-3 rth-1* double null plants, we found no clear and repeatable assay were we observed a hypersensitive or hyposensitive phenotypes. Nonetheless, the double null *rte1-3 rth-1* was very similar to the single null *rte1-3*, in particular in its hypersensitivity to ethylene (Figure B-1). As can be seen in Figure B-1, no phenotypic differences between *rth-1* and *WT* exist when treated with ethylene and assayed for the triple response.

rth-1 is a true null and RTH is membrane bound

The *rth-1* mutant creates an early stop codon at amino acid 108. We were interested in confirming if *rth-1* is a true null allele. To examine *rth-1*, we obtained a specific polyclonal RTH antibody from Alpha Diagnostics Intl. Inc. (www.4adi.com). The antibody was ordered by Dr. Resnick at the same time the RTE1 antibody order was placed. The antibody was created to target the amino terminal region of RTH aa10-HRMMIGLSDPMKID(C)-aa23. After many attempts to identify the correct antibody bleed and the right conditions, we were successful in obtaining clear results. As shown in Figure B-2, *Col-0* wild-type and *rte1-3* have the correct band of approximately 26KDa and the same band is missing from the *rth-1* lane. This result also indicates that the RTH antibody does not cross react with the RTE1 protein.

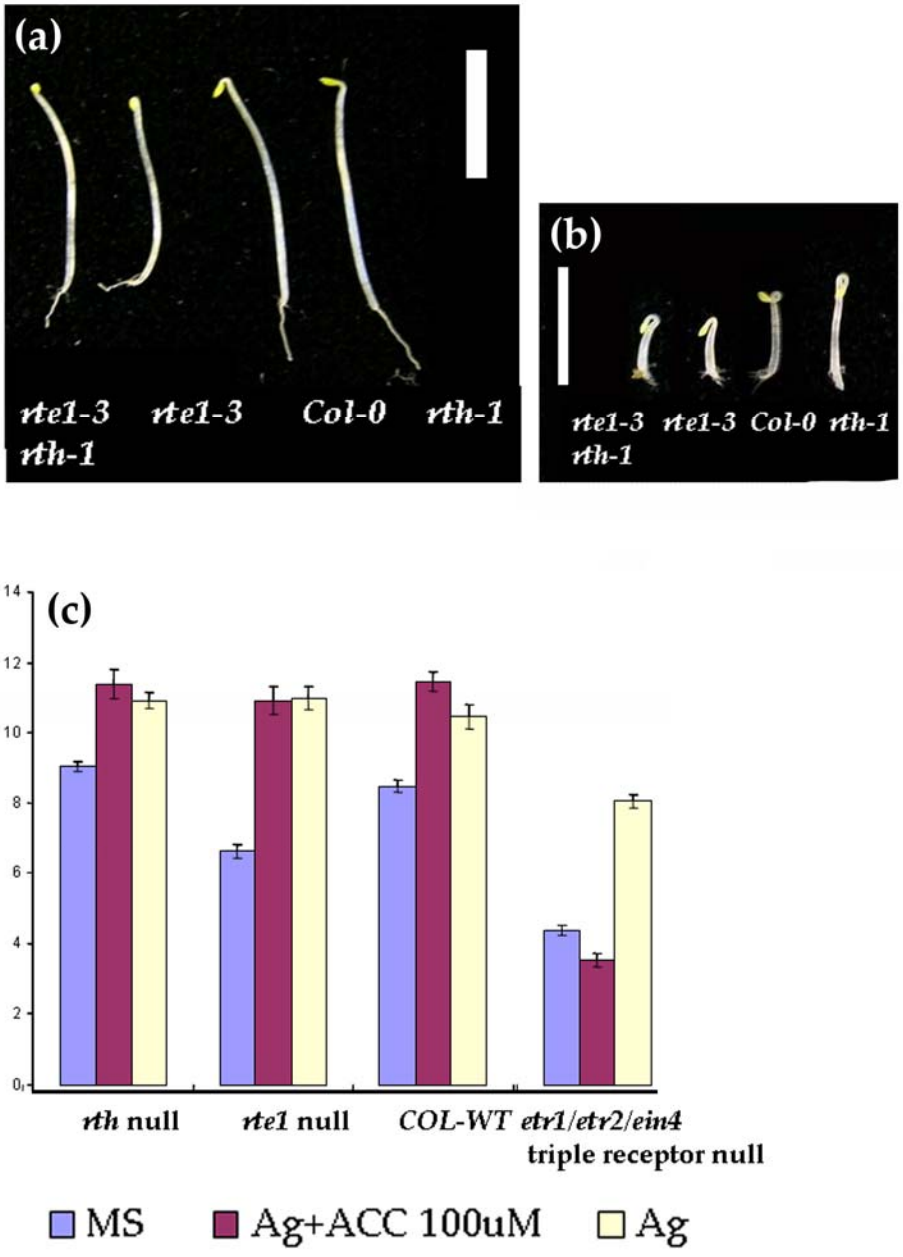

(a)
rte1-3
rte1-3
Col-0
rth-1
rth-1
(b)
rte1-3
rte1-3
Col-0
rth-1
rth-1
(c)
mm
14
12
10
8
6
4
2
0
rth null
rte1 null
COL-WT
etr1/etr2/ein4
triple receptor null
MS
Ag+ACC 100uM
Ag

Figure B-1: Similar phenotypes between WT and *rth-1* null in dark grown assays.

(a) Representative dark-grown four-day old seedlings in air showing similar phenotypes between *Col-0* wild type and *rth-1*. The double null *rte1-3 rth-1* resembles the *rte1-3* phenotype.

(b) Representative dark-grown four-day old seedlings on 20uM ACC showing similar phenotypes between *Col-0* wild type and *rth-1*. The double null *rte1-3 rth-1* resembles the *rte1-3* null phenotype.

(c) Treatment with the ethylene-response inhibitor $AgNO_3$ (10uM) alleviates the ethylene response (100uM ACC) in wild type but not in an ethylene sensitized triple receptor null background.The *rth-1* shows the same phenotypes on air, ethylene, and silver plus ethylene as WT. For each treatment, the mean ± SE is shown for ≥10 seedlings per genotype.

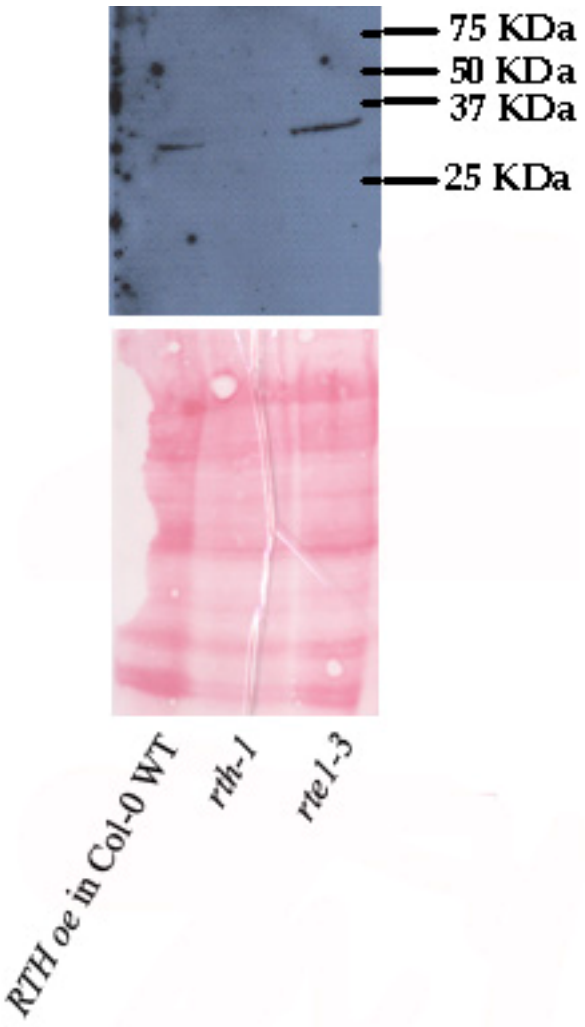


Figure B-2. **Presence of the RTH protein in WT and *rte1-3,***

but absent in the *rth-1* mutant.

Western blot showing RTH isolated from the total fraction of Arabidopsis seedlings run on denaturing 16% PAGE, prior treatment with BME for 1 hour at 37C. The proteins were detected by an RTH antibody (affinity purified, 1:1000 dilution). RTH gives a band of approximately 27 KDa that is absent in the *rth-1* background. A Ponceau S stain of the blot is shown to show a semi quantitative measure of protein loading per lane.

Reverse genetics on the *rth-1* null and *rth-1 rte1-3* double null

We pursued several conditions based on the expression data I could find on several online repositories of microarrays and massively parallel signature sequencing (MPSS), and other expression data (Table 2; http://mpss.udel.edu/at (Meyers et al., 2004). The negative results listed in Table 7 show the conditions used and phenotypes scored. In addition, we crossed *rth-1* to many ethylene insensitive and hypersensitive mutants to look for any suppression or exaggeration of ethylene response phenotypes, respectively (Table 8). After many different crosses to different receptor mutants, *RTH* has no effect on the ethylene pathway that could be observed. In performing all these different conditions and assaying for distinct phenotypes, I came across phenotypes (in particular for the glucose assay) where one backcrossed line behaved differently than another subsequent backcrossed line. This phenomenon affected my interpretations and resulted in phenotypes which did not correlate with the genotype of *rth-1*. I tested for co-segregation of the glucose hypersensitive phenotype with the *rth-1* genotype and found them not to correlate. Therefore, I have further backcrossed the *rth-1* line, I have performed three backcrosses with *rth-1* to Col-0 wild-type.

***pRTH::GUS* expression patterns in different developmental stages**

To further examine *RTH* expression pattern *in planta*, we set out to construct a *GUS* expression reporter system driven by the *RTH* promoter. To examine *RTH* gene expression, we fused the *RTH* promoter region (consisting of a 2.9 kb genomic DNA fragment just upstream of the *RTH* translation start codon and including the 5' UTR

Table 7. List of Assays performed on *rth-1* and *rth-1 rte1-3* mutants

Condition ; Assay	*rth-1* cross18.1.7	*rte1-3*	*rth-1 rte1-3* cross28.
ABA ; germination	wt	wt	wt
Cytokinin +/- ethylene; Triple response (DARK)	wt	wt	wt
GA; seedling growth	wt	wt	wt
Blue and Far Red Light; hypocotyl length	wt	shorter in far red	shorter in far red
Low and High Light Intensity with 1xMS, 1/10 xMS, soil; seedling and adult stages	wt	wt	wt
Cold 4°C and Heat Stress 28°C; seedling growth	wt	wt	wt
Auxin; seedling growth	wt	wt	wt
Ethylene;	wt	hypersensitive	hypersensitive
Mannitol; seedling growth	wt	wt	wt
NaCl; seedling growth	wt	wt	wt
Normal 16h light at 20 C; seedling growth and adult	wt	wt	wt
Glucose; seedling growth/germination	wt/ inconclusive	wt	wt
Mannose; seedling growth/germination	wt/ inconclusive	wt	wt
2-deoxy-glucose; seedling growth	wt/ inconclusive	wt	wt

Table 8. Crosses performed with *rth-1*; neither suppression nor enhanced ethylene sensitivity was detected with any of the double mutants.

Crosses: *rth-1 x* …	ethylene phenotype
rth-1 rte1-3	similar to *rte1-3*
rth-1 etr1-9	similar to *rth-1;* longer hypocotyl than *etr1-9* single
rth-1 etr1-2	*etr1-2*
rth-1 ers1-10	*ers1-10*
rth-1 ers1-1	*ers1-1*
rth-1 etr1-1	*etr1-1*
rth-1 etr2-1	*etr2-1*
rth-1 ers1-3	*ers1-3*
rth-1 ein2-1	*ein2-1* (preliminary)

of *RTH*) with the β-glucuronidase (*GUS*) reporter gene and transformed the resulting construct into wild-type Col-0 *Arabidopsis* plants by *Agrobacterium* infiltration (Clough and Bent, 1998). The expression pattern of *RTH* was observed by staining for *GUS* activity in the transgenic lines. As shown in Figure B-3, *RTH* has a very similar expression pattern as *RTE1* and is interestingly highly expressed in all tissues examined were there is active cell replication and elongation. For example, there is high *RTH* expression in new developing rosettes leaves, in new developing stems when plants are bolting, and in the root meristem of growing root tips.

Preliminary RTH subcellular localization; CaMV35Spromoter-YFP-RTH appears to be localized to the endomembrane compartment.

Preliminary results utilizing a CaMV-35Sdriven-YFP-RTH construct in tobacco cells suggest RTH may reside in the endomembrane compartment. The *RTH* gene with its stop codon was cloned into the vector pEarleyGate-104 (Keith W. Earley et al., 2006). Most of this work was done by Kevin Meng under my supervision and help. Dr. Chunhai Dong helped Mr. Meng with the microscopy and tobacco infiltration. As can be seen in Figure B-4, RTH appears to show a pattern that's similar to the Golgi marker pattern (when compared to a Golgi GFP marker, picture not shown). More experiments need to be performed such as localizing RTH in Arabidopsis under its native promoter to accurately determine its subcellular localization. If RTH is localized at the endomembrane compartment (mainly Golgi), this would place RTH and RTE1 in the same subcellular compartment.

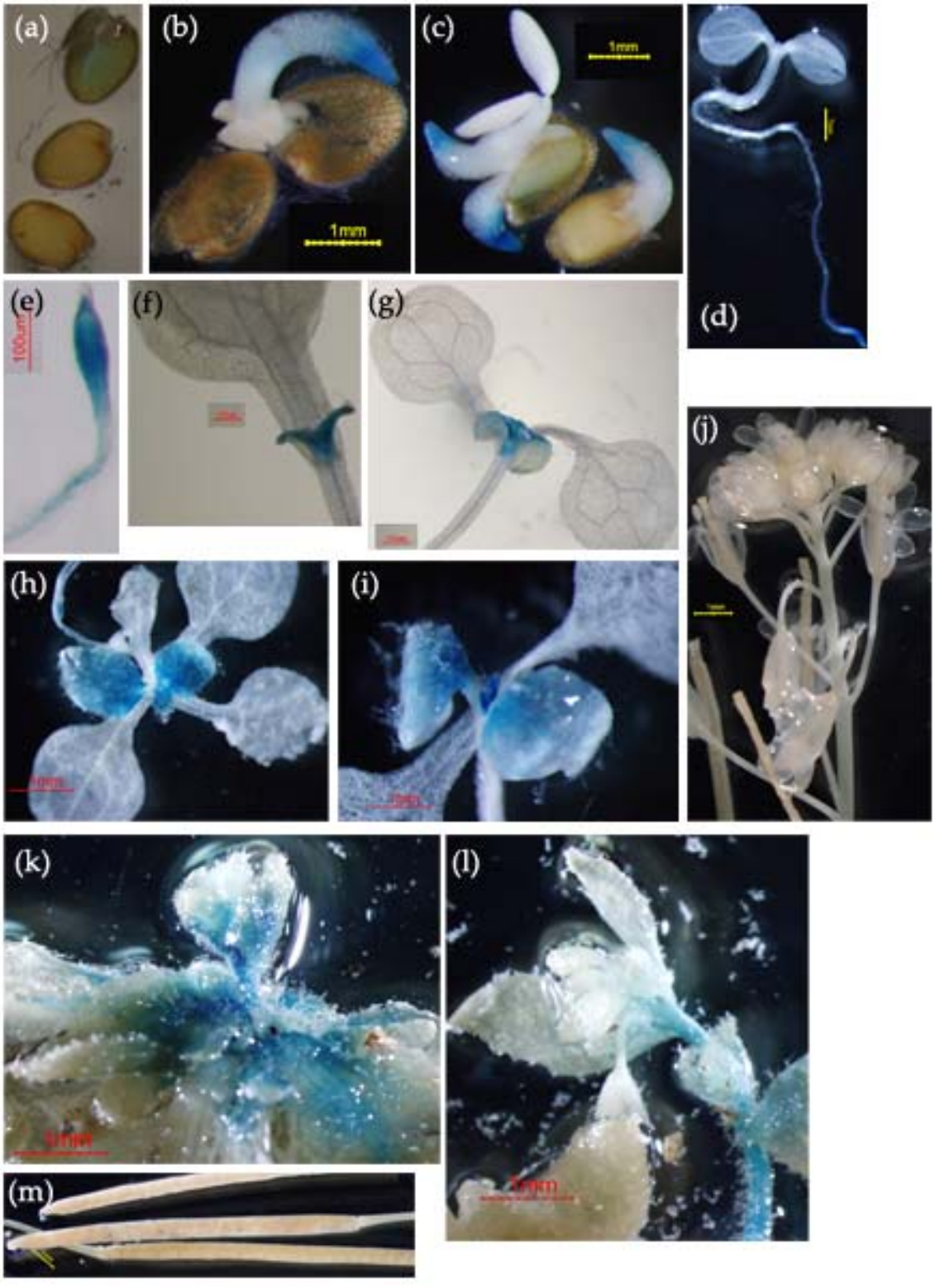

(a)
(b)
1mm
(c)
1mm
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)

Figure B-3. $RTH_{promoter}$::GUS gene expression patterns.

Representative GUS expression is seen in the following wild-type tissues. **(a)** Seeds. **(b-c)** 1-day-old light-grown seedlings. **(d)** 3-day-old light-grown seedling. Scale bar 1mm. **(e-g)** 7-day-old light-grown seedlings; **(e)** is root tip. Scale bar 100um. **(h-i)** 9-day-old light-grown seedlings. Scale bar 1mm. **(j)** Adult stage inflorescence from primary stem. Scale bar 1mm. **(k-l)** Early bolting stage (aprox. 3 weeks). Scale bar 1mm. **(m)** Fully developed siliques from primary stem. Scale bar 1mm.

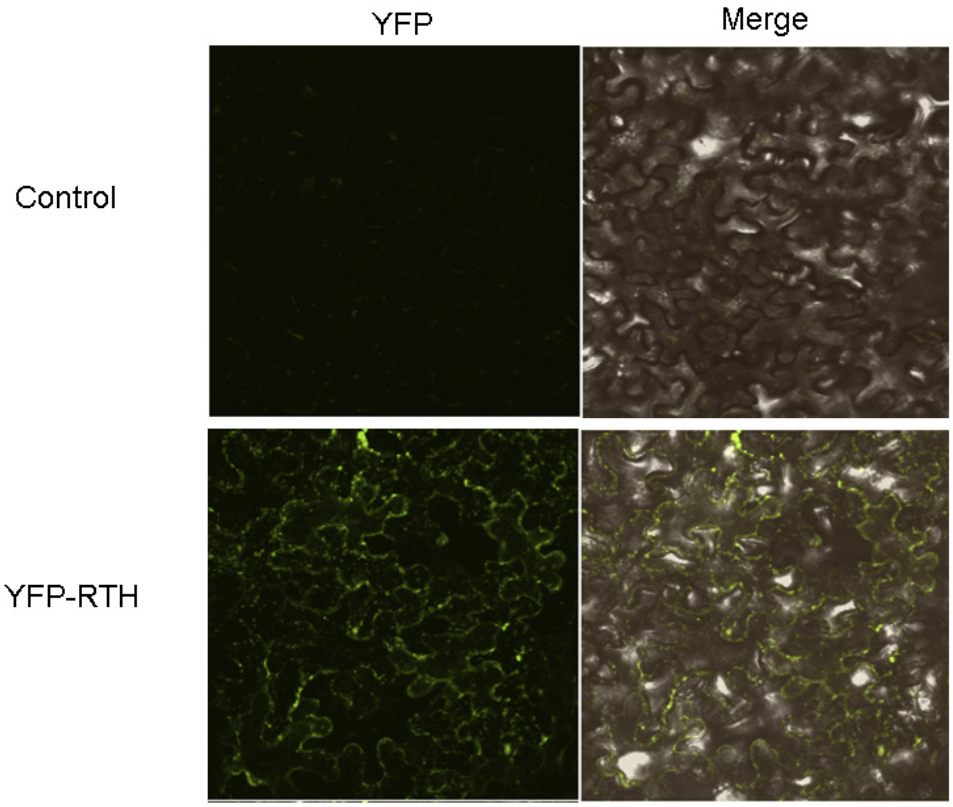


Figure B-4. Preliminary subcellular localization of RTH.

Preliminary results may suggest RTH is localized to the endomembrane. Pictures were taken by Dr. Dong and Mr. Kevin Meng. Pictures of YFP-RTH from tobacco leaves were taken after 3 or 4 days after infiltration by Agrobacterium carrying the YFP-RTH plasmid.

Constructs (clones) in plants that need to be further examined:

- *pRTH-RFP-RTH* (Gateway 3 piece cloning), Have several independent lines; T2 seeds have been collected.
- Artificial miRNA targeting of *RTH* (construct bought from OpenBiosystems Co. AMR4844-99731007 (openbiosystems.com). *RTH* miRNA is cloned in plasmid pAmiR (6.4 Kb). I have 3 independent lines; T2 seeds collected from each line.
- Second allele of *RTH*. A T-DNA insertion (not confirmed) presumably inserted immediately after the first ATG of the *RTH* gene. The seed stock ET9854 was obtained from Joe Simorowski (Lab Manager), Martienssen Lab at Cold Spring Harbor Laboratory. The Landsberg erecta ET9854 is a line from an enhancer trap line developed by the Martienssen Lab (http://genetrap.cshl.edu).
- *pETR1::ETR1*(1-349aa)*::ETR1 3'UTR* (Gateway 3 piece cloning). In the process of transforming *pETR1::ETR1*(1-349aa)-*ETR1 3'UTR* containing a E38A mutation and a C65Y mutation.

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