ABSTRACT

Title of Document:	INVESTIGATING THE MOLECULAR MECHANISM OF RTE1 ACTIVATION OF THE ETHYLENE RECEPTOR ETR1 IN ARABIDOPSIS
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The plant hormone ethylene plays a vital role in regulating plant growth and development as well as plant defense to biotic and abiotic stresses during the entire life of the plant. In Arabidopsis, ethylene is perceived by a family of five receptors, one of which is ETR1. The Arabidopsis *REVERSION-TO-ETHYLENE SENSITIVITY1* (*RTE1*) gene is a positive regulator of *ETR1*. *RTE1* encodes a novel integral membrane protein that interacts with ETR1 at the Golgi apparatus and the endoplasmic reticulum (ER). Genetic evidence indicates that RTE1 is required for the formation of a functional ETR1 receptor, whereas the other ethylene receptors in Arabidopsis do not require RTE1. But the molecular mechanism by which RTE1 specifically activates ETR1 remains unknown. I took different approaches to gain insights into the molecular function of RTE1 and the basis for the specificity for activating ETR1.

In a library screen for RTE1-interacting proteins using the yeast split-ubiquitin assay, an ER-localized cytochrome *b*5 isoform (AtCb5-D) was identified. Cb5 is a small hemoprotein that functions in oxidation/reduction reactions. Mutants of three *AtCb5* isoforms show phenotypes in ethylene responses that are similar to those of the *rte1* mutant, suggesting the functional parallel between AtCb5 and RTE1 in ethylene signaling. Additional genetic analyses suggest that *AtCb5* might act in the same pathway as *RTE1* and that *AtCb5* is specific to *ETR1* like *RTE1*. Moreover, using a hemin-agarose affinity chromatography assay, I found that RTE1 homologs are able to bind heme in vitro, raising the possibility that RTE1 carries out redox with cytochrome *b*5s. I also found that the specificity for regulating ETR1 by RTE1 is largely due to a unique proline (P9) conserved only in ETR1 orthologs; introduction of P9 into the Arabidopsis ERS1 ethylene receptor was sufficient to convert ERS1 into an RTE1-dependent receptor. I propose that P9 may interfere with the proper folding of ETR1 EBD and formation of the ETR1 homodimer by affecting the conserved disulfide bond-forming cysteines (C4, C6) in the ETR1 homodimer. Taken together, our results suggest a model in which RTE1, together with cytochrome *b*5, promotes the active conformation of ETR1 through oxidative folding.

INVESTIGATING THE MOLECULAR MECHANISM OF RTE1 ACTIVATION OF THE ETHYLENE RECEPTOR ETR1 IN ARABIDOPSIS

By

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Dedication

This dissertation is dedicated to my parents for their unconditional love, endless support and encouragement. This dissertation is dedicated to my closest friend, confidant and husband Ruiqiang Chen. This dissertation is dedicated to my son Michael, who is the most important thing in the world to me.

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List of Abbreviations

5'-FOA, 5-fluoroorotic acid
ABA, Abscisic Acid
ABH, ABA Hypersensitive
ACC, 1-aminocyclopropane 1-carboxylic acid
ACS, ACC synthase
AVG, 1-aminoethoxyvinylglycine
AWE, Associate With ETR1
BLAST, Basic Local Alignment Search Tool
BiFC, Bimolecular Fluorescence Complementation Analysis
CaMV, Cauliflower Mosaic Virus
CAPS, Cleaved Amplified Polymorphic Sequence
Cb5, Cytochrome b5
CBP, mRNA Cap-binding Protein
CCS, Copper Chaperone for SOD1
cGMP, Cyclic Guanosine Monophosphate
CHX, Cation/H^+ Exchanger
Col, Columbia
CTR, Constitutive Triple Response
dCAPS, Derived Cleaved Amplified Polymorphic Sequence
EBD, Ethylene Binding Domain
EBF, EIN3-Binding F-box
EER, Enhanced-Ethylene Response
EIL, EIN3-Like
EIN, Ethylene Insensitive
ERF, Ethylene Response Factor
ERS, Ethylene Response Sensor
ETP, EIN2 TARGETING PROTEIN
ETR, Ethylene Resistant
GAF, cGMP-specific and Stimulated Phosphodiesterases, Anabaena Adenylate cyclases and E-coli Fhl1A
GFP, Green Fluorescent Protein
GID, Gibberelin acid Insensitive Dwarf
GR, Green Ripe
HA, Hemagglutinin
HK, Histidine Kinase
HRG, Heme Responsive Gene
JA, Jasmonic Acid
JAZ, Jasmonate Zim Domain
MAPK, Mitogen Activated Protein Kinase
MAPKKK, MAPKK Kinase
MS, Murashige and Skoog Medium
NR, Never Ripe
NRAMP, Natural Resistance-Associated Macrophage Protein
NS-LTP, Non-Specific Lipid Transfer Protein

PCR, Polymerase Chain Reaction
PPM, Parts Per Million
PPIase, Peptidylprolyl Isomerase
RBD, Ras-binding Domain
RAN, Responsive To Antagonist
RTE, Reversion To Ethylene Sensitivity
RTH, RTE1 Homolog
RT-PCR, Reverse Transcriptase PCR
SA, Salicylic Acid
SCF, Skp1-Cullin-F-box Ubiquitin Ligase
SLR, Slender Rice
SOD, Super Oxide Dismutase
TA, Tail-Anchored
TAP, Tandem Affinity Purification
TM, Transmembrane Domain
TPR, Tetratricopeptide Repeat
TRP, Tetratricopeptide Repeat Protein
Ub, Ubiquitin
UTR, Untranslated Region
WT, Wild Type
XRN, Exoribonuclease
YFP, Yellow Fluorescent Protein
ZIP, Zrt-, Irt-like Protein

Chaper 1 : Introduction

Significance and history of ethylene research

Ethylene (C_2H_4) is a simple gaseous hydrocarbon that has profound effects upon plant growth and development (Abeles et al., 1992). Ethylene plays important roles throughout the life of the plant from the promotion of seed germination, root hair formation through promotion/inhibition of flowering, fruit ripening, leaf and petal abscission and organ senescence (Abeles et al., 1992). In addition, ethylene plays a dramatic part in plant defense responses. Plants, unlike animals, cannot evade biotic and abiotic stresses by moving away from external challenges such as flooding, drought, hypoxia, temperature changes, mechanostimuli, pathogen and insect attack and herbivory. Hormones play key roles in sensing these stresses and generating appropriate responses. Ethylene can be induced in response to numerous external and internal stimuli such as the stresses described above and the hormones cytokinin and auxin (Argueso et al., 2007), and triggers adaptive responses to protect the plant.

Because ethylene regulates so many physiological processes in plants, it is widely used in agriculture. For example, by controlling ethylene biosynthesis and sensitivity, we can manipulate the time of fruit ripening and flower fading, therefore reduce postharvest spoilage which results in big agricultural losses. In addition, some ethylene releasing compounds such as Ethephon are commercially used as a plant growth regulator in agriculture, such as to break seed dormancy, promote bulbs sprouting, synchronize flowering and fruit set in pineapple, degreen citrus, induce

fruit thinning or fruit drop, promote the female flower formation in cucumber, prevent self-pollination and increase yield and so on (Abeles et al., 1992). Because ethylene can impact so many physiological processes, which in turn impact crop yield, the importance of ethylene studies has long been recognized.

Ethylene has been utilized for agricultural purposes for over a thousand years; it is only recently that the mechanisms underlying ethylene biosynthesis and signaling have begun to be revealed. Ancient Egyptians knew wounding of figs can hasten fruit ripening (wounding induces the production of ethylene). Ancient Chinese would burn incense in closed room to promote ripening in pears (ethylene is generated as a byproduct of partial combustion of organic fuels). During the nineteenth century when the illuminating gas was used for lighting on the street, it was found that the plants near the pipelines were prone to have premature senescence and abscission. It was believed that some active components in coal gas injured the nearby plants. But at that time, it was not known the effects were due to ethylene. In 1901, a Russian scientist named Dimitry Neljubow firstly identified ethylene as the active molecule causing the effects. In 1934, R. Gane and others discovered that ethylene can be produced by plants and thus classified ethylene as a hormone because of its broad range of physiological effects on plants. Since then, ethylene was recognized as an endogenous regulator of plant growth and development, and the use of ethylene to manipulate the growth and development of agricultural crops started to be investigated.

Ethylene biosynthesis in plants was understood earlier than ethylene signaling. In 1979, the ethylene biosynthesis pathway was completed by Shang Fa Yang *et al*.

when they identified 1-aminocyclopropane-1 carboxylic acid (ACC) as an intermediate in the conversion of methionine to ethylene (Adams and Yang, 1979). The ethylene biosynthesis pathway is composed of three relatively simple steps (Kende, 1993). First, the amino acid L-methionine is adenylated to form *S*-adenosylmethionine (AdoMet) by AdoMet synthetase. Next, AdoMet is converted into 1-aminocyclopropane-1 carboxylic acid (ACC) by a large family of tightly regulated ACC synthase (ACS) genes. In the final step, ACC is converted into ethylene by ACC oxidase (ACO). The production of ACC is the rate-limiting step. Ethylene production is primarily controlled through the temporal and spatial regulation of the rate-limiting enzyme ACS at transcriptional and post-translational levels (Argueso et al., 2007). Ethylene biosynthesis is stimulated by many factors, including developmental stages, biotic and abiotic stresses and other plant hormones.

In the past two decades, outstanding progress had been made concerning the identification of key components in the ethylene signaling pathway. This largely relied on genetic screens in the model system *Arabidopsis thaliana*. A simple, highly ethylene-specific and readily distinguishable phenotype, the triple response, has greatly facilitated the isolation of mutants that have defects in ethylene signaling. The triple response is a striking morphology that etiolated seedlings exhibit in the dark when treated with ethylene, which consists of the shortening and thickening of the hypocotyl, the reduced root elongation and the formation of exaggerated apical hook (Guzman and Ecker, 1990) (**Figure 1-1**). The identified triple response mutants largely fall into 3 categories: 1) ethylene insensitive mutants which fail to display a triple response under high ethylene doses; 2) constitutive ethylene response mutants

Figure 1-1 The Arabidopsis ethylene triple response phenotype

Wild-type Arabidopsis seedlings were grown in dark for 4 days in the absence (left) or presence (right) of ethylene gas. The etiolated seedling exhibits 'triple response' in response to ethylene. The features of ethylene triple response include the shortening and thickening of the hypocotyl, the reduced root elongation and the formation of exaggerated apical hook.

which exhibit a triple response in the absence of ethylene; 3) ethylene hypersensitive mutants which show a triple response at a very low dose of ethylene whereas the wild type doesn't. Besides the above standard triple response mutant screens, there are other mutant screen approaches leading to the identification of new components which cannot be discovered through standard triple response screen. For example, Hirayama et al. isolated *response to antagonist* (*ran*) mutant through screening for the mutants that displayed ethylene responsive phenotype upon exposure to an antagonist of ethylene action (Hirayama et al., 1999). Another example is that, by screening for the suppressors of *etr1-2* ethylene insensitivity, a novel ethylene receptor ETR1 regulator *REVERSION-TO-ETHYLENE SENSITIVITY1* (*RTE1*) was discovered (Resnick et al., 2006). Some representative triple response mutants are given in **Table I**. Epistatic analysis of these mutants and cloning of the genes that the mutations affect have resulted in a linear but probably incomplete pathway from the ethylene receptors at ER membrane to transcription of ethylene-response genes in nucleus (**Figure 1-2**). Homologs of Arabidopsis ethylene receptors, CTR1, and transcription factors involved in activating ethylene response genes have been identified in other higher plant species such as rice, tomato, tobacco (Adams-Phillips et al., 2004; Goff et al., 2002; Leclercq et al., 2002; Tieman and Klee, 1999; Tieman et al., 2000; Xie et al., 2002; Yu et al., 2002; Zhang et al., 2001). Some identified homologs in other plant species can functionally complement the mutation of the corresponding Arabidopsis homolog (Adams-Phillips et al., 2004; Leclercq et al., 2002). The existence of these homologs suggests that the ethylene signaling pathway is conserved in higher plants. Although the ethylene signaling pathway has been largely

Table I Representative ethylene triple response mutants in Arabidopsis

Mutant	Mutation	Phenotype	Gain- or loss-of-function	Identity	Reference
etr1-1	Missense C65Y	Ethylene insensitive	Gain-of-function	Ethylene receptor	(Bleecker et al., 1988; Chang et al., 1993)
etr1-2	Missense A102T	Ethylene insensitive	Gain-of-function	Ethylene receptor	(Bleecker et al., 1988; Chang et al., 1993)
etr1-7	Nonsense W74stop	Ethylene hypersensitive	Loss-of-function	Ethylene receptor	(Hua and Meyerowitz, 1998)
etr2-1	Missense P66L	Ethylene insensitive	Gain-of-function	Ethylene receptor	(Sakai et al., 1998)
ers1-10	Missense R320C	Ethylene insensitive	Gain-of-function	Ethylene receptor	(Alonso et al., 2003)
ctr1-1	Missense D1342E	Constitutive ethylene response	Loss-of-function	Raf-like kinase	(Kieber et al., 1993)
ctr1-8	Missense G354E	Constitutive ethylene response	Loss-of-function	Raf-like kinase	(Huang et al., 2003)

Mutant	Mutation	Phenotype	Gain- or loss-of-function	Identity	Reference
ein2-5	**Frameshift**	**Ethylene insensitive**	**Loss-of-function**	**Nramp-like protein**	**(Alonso et al., 1999)**
ein3-1	**Nonsense W215stop**	**Ethylene insensitive**	**Loss-of-function**	**Transcription factor**	**(Chao et al., 1997)**
ran1-3	**Missense G759R**	**Constitutive ethylene response**	**Loss-of-function**	**Copper transporter**	**(Woeste and Kieber, 2000)**
eto2-1	**Frameshift**	**Constitutive ethylene response**	**Gain-of-function**	**ACC synthase 5**	**(Kieber et al., 1993; Vogel et al., 1998)**
ein5-1	**Frameshift**	**Ethylene insensitive**	**Loss-of-function**	**5'-3' exoribonuclease**	**(Olmedo et al., 2006)**
rte1-1	**Missense C161Y**	**Ethylene hypersensitive**	**Loss-of-function**	**Novel protein**	**(Resnick et al., 2006)**
rte1-2	**Frameshift**	**Ethylene hypersensitive**	**Loss-of-function**	**Novel protein**	**(Resnick et al., 2006)**
rte1-3	**Nonsense W57stop**	**Ethylene hypersensitive**	**Loss-of-function**	**Novel protein**	**(Resnick et al., 2006)**

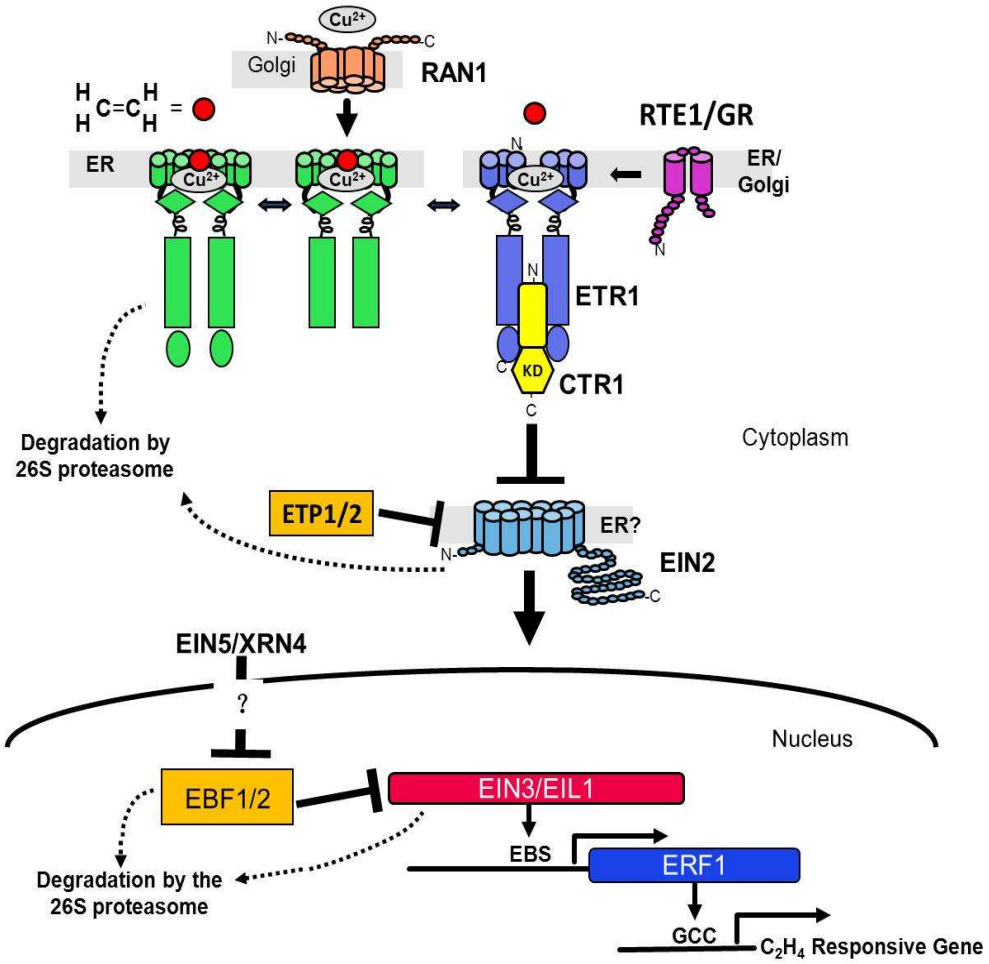


Figure 1-2 Current model of the ethylene-signaling pathway in Arabidopsis

The figure is modified from (Kendrick and Chang, 2008). The ethylene molecule is perceived at the endomembrane by a family of receptors that form disulfide linked homodimers. Ethylene binding requires copper cofactor provided by RAN1 in the Golgi membrane. The ETR1 receptor depends on a novel regulator RTE1 for proper function. In the absence of ethylene, ethylene receptors repress downstream ethylene responses through activation of CTR1, another negative regulator of ethylene responses. Ethylene binding turned off receptor signaling, causing the inactivation of

CTR1, and thereby allowing downstream signaling to proceed through EIN2, which is a key positive regulator of ethylene response. EIN2 is negatively regulated in the absence of ethylene by two F-box proteins ETP1/2 through the 26S proteasome-dependent degradation pathway. Downstream of EIN2 are transcription factors EIN3/EIL, which activate a transcriptional cascade in nucleus. Like EIN2, EIN3 and EIL1 are targeted for degradation by two F-box proteins, EBF1/2 in the absence of ethylene. Ethylene stabilizes EIN3 by eliminating EBFs at both mRNA and protein levels. EBFs mRNA degradation is mediated by an EIN5/XRN4 exoribonuclease and EBFs proteins are degraded through the 26S proteasome-mediated pathway in response to ethylene.

established, there are still many unanswered questions regarding to the mechanisms underlying ethylene signaling and its regulation. The future directions will be uncovering unknown new components, revealing how the components are regulated, elucidating the biochemical mechanisms by which the ethylene signal is transduced, unraveling the crosstalk of ethylene's with other signaling pathways, etc.

The ethylene receptor family

Ethylene gas is readily permeable through the cell and in Arabidopsis is perceived by a family of five receptors (ETR1, ERS1, EIN4, ETR2 and ERS2) (Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998), which are negative regulators of ethylene response (**Figure 1-3**). In the absence of ethylene, the ethylene receptor is in an active state, repressing downstream ethylene responses. In the presence of ethylene, the receptors are turned off, allowing ethylene responses to occur. The five receptors have a high degree of sequence similarity and functional redundancy. A loss-of-function mutant of any one receptor gene gives a wild-type phenotype except the *etr1* null mutant, which displays hypersensitivity to ethylene (Cancel and Larsen, 2002). However, multiple loss-of-function receptor mutants show a constitutive ethylene response (Hua and Meyerowitz, 1998; Qu et al., 2007). On the other hand, dominant gain-of-function receptor mutants exhibit ethylene insensitivity (e.g. *etr1-1* and *etr1-2*). Based on their structure and function similarity, the ethylene receptors are divided into two subfamilies. In Arabidopsis, subfamily I receptors include ETR1 and ERS1 and subfamily II receptors consist of ETR2, ERS2 and EIN4. In Arabidopsis, subfamily I ethylene receptors are believed to play a

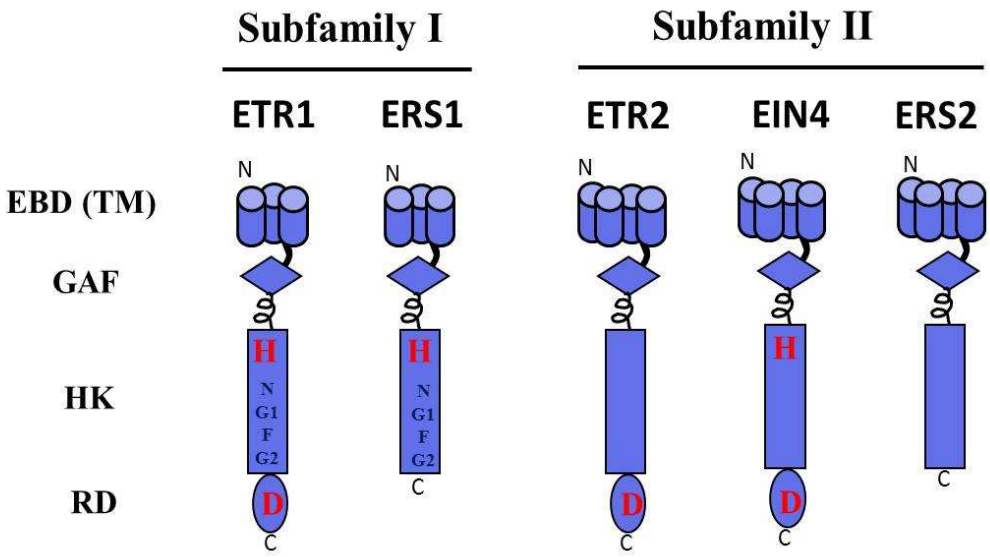


Figure 1-3 Basic structure of the Arabidopsis ethylene receptor family

Arabidopsis has a family of five ethylene receptors which are classified into two subfamilies based on their structure: subfamily I (ETR1 and ETRS1) and subfamily II (ETR2, EIN4, ERS2). Subfamily I and II receptors have 3 and 4 integral transmembrane (TM) segments at N-terminus, respectively, harboring the ethylene binding domain (EBD). Following the EBD is a GAF domain, a histidine kinase (HK) domain, and a receiver domain (RD), which is absent in ERS1/2. The conserved histidine residue that is the site of in histidine autophosphorylation in the histidine protein kinase family is indicated by a red 'H' and the conserved aspartate residue that characterizes the two-component receiver domain is shown by a red 'D'. Only subfamily I receptors possess all of the conserved motifs (N, G1, F and G2) that are required for histidine kinase activity.

bigger role in ethylene signaling, since the loss of both subfamily I receptors mutant has a more severe phenotype than loss of other receptor combination mutants (Qu et al., 2007).

Unlike many other receptor proteins, which are localized in the plasma membrane (PM) or nucleus, the ethylene receptors predominantly reside at the endoplasmic reticulum (ER) membrane (Chen et al., 2002; Dong et al., 2008; Ma et al., 2006; Zhong et al., 2008). However, there is still possibility of low levels or transient receptors localization to other membrane systems. For example, Arabidopsis ETR1 was observed at the Golgi apparatus by immunohistochemistry (Dong et al., 2008). Tobacco NTHK1, a subfamily II ethylene receptor was reported to be localized to the plasma membrane in protoplasts (Xie et al., 2003).

Ethylene receptors are composed of an N-terminal transmembrane domain containing the ethylene-binding site, followed by a soluble domain that has weak similarity to GAF domains (c**G**MP-regulated mammalian phosphodiesterases, cyanobacterial **a**denyl cyclases, and a **f**ormate-hydrogen lyase transcriptional activator), a predicted coiled-coil, and a C-terminal signaling output domain that has similarity to the bacterial two component histidine protein kinase family (**Figure 1-1**). The N-terminal transmembrane domain has three (subfamily I receptors) or four (subfamily II receptors) membrane spanning segments and a short amino terminal fragment in the lumen. The ethylene binding domain (EBD) is the most conserved portion of the ethylene receptors.

The receptors forms disulfide-linked homo-dimers at two conserved Cysteines (Cys4 and Cys6 in ETR1), which are in the lumen (Schaller et al., 1995). Each

receptor dimer encompasses one ethylene molecule binding pocket at the transmembrane domain. Ethylene binding is mediated by a molecule of copper that is coordinated by two conserved amino acids (Cys65 and His69 in ETR1) within the membrane (Rodriguez et al., 1999; Schaller and Bleecker, 1995). Alterations of these copper binding ligands are sufficient to eliminate ethylene binding (Rodriguez et al., 1999). Copper is provided by RAN1, a P-type ATPase copper transporter homolog in the Golgi membrane (Hirayama et al., 1999; Woeste and Kieber, 2000). *ran1* loss-of-function mutants result in a constitutive ethylene response phenotype, suggesting all the ethylene receptors are in an inactive state in the absence of copper. The mechanism by which how ethylene binding shuts off the ethylene receptors has not been elucidated yet. However, recent studies favor the following model: ethylene binding causes a conformational change in the EBD domain, which is presumably propagated to the cytoplasmic transmitter domain to affect a change in signaling status (Wang et al., 2006). Interestingly, almost all known gain-of-function dominant mutations encode an amino acid substitution in the EBD domain and confer ethylene insensitivity regardless of ethylene binding ability. Some of them abolish ethylene binding, and therefore are insensitive to ethylene. For example, the *etr1-1* mutation causes the Cys65Tyr substitution and disrupts the copper binding that is essential for ethylene binding. As a result, the mutant ETR1-1 receptor is probably locked to a signaling state that cannot be turned off. However, other gain-of-function mutations can confer ethylene insensitivity without disrupting ethylene binding, suggesting that they are crucial for transmitting conformational changes to turn off receptor signaling. An example is the *etr1-2* mutation, which encodes an Ala102Thr conversion.

Following the EBD is a GAF domain, which is found in phytochromes and cGMP-specific phosphodiesterases where it is known to bind small molecules (Charbonneau et al., 1990). The function of the GAF domain in ethylene signaling is currently unknown. But recent studies suggest that one of the GAF domain functions may be to mediate non-covalent heteromeric interactions among ethylene receptors (Gao et al., 2008). Yeast two hybrid analysis demonstrated that the GAF domains of ETR1 and ETR2 are sufficient for their association (Gao et al., 2008). Higher order interactions between ethylene receptors not only explain why single ethylene receptor mutation can confer insensitivity despite the presence of redundant wild-type family members, but also provide a mechanism as to why plants can rapidly respond to minute ethylene amounts: the ethylene signal could be amplified by associated ethylene receptors.

The Carboxyl-terminal cytoplasmic portion of the ethylene receptors is the signal output domain. It has the striking features of the bacterial two-component systems which are used by bacteria to sense and respond to the environmental stimulus. The two components refer to a sensor component and a response regulator component (Stock et al., 2000; WurglerMurphy and Saito, 1997). In the two component system, the signal is perceived by the amino-terminal signal input domain in a sensor protein and transmitted to the carboxyl-terminal histidine protein kinase (HPK) domain of the sensor protein where histidine kinase auto-phosphorylates on a conserved histidine. This phosphate is then transferred to a conserved aspartate residue in the receiver domain of the response regulator. The receiver domain sometimes is covalently joined to a histidine kinase instead of existing as a separate

response regulator protein. The ethylene receptor signal output domain has a HPK-like domain and a covalently attached response regulator-like receiver domain, which are only found in ETR1, ETR2 and EIN4. Although subfamily I receptors contain the conserved histidine protein kinase motifs necessary for the histidine kinase activity and ETR1 was shown to display histidine kinase activity in vitro (Gamble et al., 1998), the histidine kinase activity seems not to play a substantial role in ethylene receptor signaling (Gamble et al., 2002; Moussatche and Klee, 2004; Wang et al., 2003). The role of histidine kinase activity in ethylene receptor ETR1 has not been understood yet, but it could be involved in the interaction of ethylene signaling with other signaling pathways which also use the two-component system such as cytokinin and osmosensing (Inoue et al., 2001; Urao et al., 1999). The biochemical mechanism by which ethylene receptors transduce the ethylene signal to the downstream component CTR1 is still unknown. If phosphorylation is not involved in the ethylene receptor signaling, the allosteric regulation could be one of possible ethylene receptor signaling mechanisms. Supporting evidence is that CTR1 is associated with ER membrane in an ethylene receptor dependent manner in Arabidopsis and physically interacts with the signal output domain of ETR1, ERS1 and ETR2 receptors in vitro (Cancel and Larsen, 2002; Clark et al., 1998; Gao et al., 2003).

Homologs of ethylene receptors identified in other species such as rice, tobacco, tomato may have similar structure and function as those in Arabidopsis (Goff et al., 2002; Tieman and Klee, 1999; Tieman et al., 2000; Xie et al., 2002; Yu et al., 2002; Zhang et al., 2001). Tomato contains 6 ethylene receptors: LeETR1-LeETR6. The first three belong to subfamily I and receptors 4-6 constitute subfamily II (Klee and

Tieman, 2002). LeETR3 also called Never ripe (Nr) is the only one that lacks the receiver domain. In tomato, subfamily II receptors (LeETR4 and LeETR6) play a larger role in ethylene signaling (Kevany et al., 2007; Klee, 2004). Introducing an *etr1-1* equivalent mutation into *LeETR4* and *LeETR6* cause strong ethylene insensitivity in Arabidopsis, suggesting Tomato ethylene receptors may be functionally similar to Arabidopsis ethylene receptors (Tieman and Klee, 1999). Interestingly, proteins with EBD, GAF and HPK domain were also found in cyanobacteria *Synechocystis,* raising the possibility that the ethylene receptor in plants is derived from the chloroplast, a cyanobacterial symbiont (Bleecker, 1999; Mount and Chang, 2002; Rodriguez et al., 1999).

Recently it was found that some ethylene receptors can be regulated by protein degradation. Arabidopsis ETR2 is targeted for degradation by a proteasome-dependent pathway in response to ethylene (Chen et al., 2007). Similarly, in tomato, ethylene induces LeETR4 and LeETR6 to be rapidly degraded probably through the 26S proteasome-dependent pathway (Kevany et al., 2007). The decrease in certain ethylene receptors induced by ethylene could be a mechanism by which plants propagate the ethylene signal, increase sensitization to ethylene and modulate ethylene responses at specific developmental stages and tissues.

Downstream components of the ethylene signaling pathway

The receptors repress ethylene responses through activation of CTR1, a Raf-like mitogen activated kinase kinase kinase (MAPKKK) (Kieber et al., 1993). *ctr1* loss-of-function mutants exhibit constitutive ethylene responses, suggesting it is a negative

regulator of the ethylene response (Kieber et al., 1993). CTR1 is composed of an N-terminal regulatory domain and a C-terminal serine/threonine kinase domain. The N-terminal domain of CTR1 is able to directly interact with the cytoplasmic part of the ethylene receptors, therefore bringing CTR1 to ER membrane (Clark et al., 1998; Gao et al., 2003). Mutations that eliminate the CTR1 kinase activity such as *ctr1-1* and mutations that disrupt the interaction of CTR1 with ethylene receptors such as *ctr1-8* result in a constitutive ethylene response, indicating that both the kinase activity and the localization to ER through association with ethylene receptors are required for CTR1 to suppress ethylene responses (Huang et al., 2003). The biochemical mechanism by which how CTR1 is regulated by the ethylene receptor has not been established yet. The current model is based on the protein kinase Raf. The CTR1 C-terminal Ser/Thr kinase activity may be autoinhibited by the N-terminal regulatory domain. In the absence of ethylene, the N-terminal domain of CTR1 associates with ethylene receptor, therefore, the C-terminal kinase activity is not inhibited and can repress downstream ethylene response. Ethylene binding might induce a presumed conformational change of the N-terminal domain of CTR1, which autoinhibit the C-terminal kinase activity, relieving the repress of downstream ethylene response. Another important unsolved question is whether there is a MAPK module in ethylene signaling pathway since CTR1 is a putative MAPKKK. Moreover, the *ctr*1 loss-of-function mutant still can respond to ethylene and has a less severe phenotype than a quadruple loss-of-function ethylene receptor mutant (Hua and Meyerowitz, 1998). So other CTR1-like proteins are thought to exist. However, the identity of the alternate pathway bypassing CTR1 is unknown.

The next component downstream of CTR1 is a homolog of the Nramp membrane protein, EIN2, a positive regulator of the pathway (Alonso et al., 1999). EIN2 is a large integral membrane protein with the significant sequence similarity to the Nramp family of metal ion transporters at N terminus and a cytoplasmic portion with unknown function at C terminus. However, EIN2 is not shown to bind or transport any metal ion (Thomine et al., 2000). EIN2 is believed to play a central role in ethylene signaling because *ein2* loss-of-function mutation results in complete ethylene insensitivity. Ethylene response strength seems to be proportional to the EIN2 protein level which is regulated through the degradation by a 26S proteasome-dependent pathway mediated by SCF complex containing two F-box proteins ETP1 and ETP2 (Qiao et al., 2009). The basis of EIN2 action in ethylene signaling remains unknown. However, the latest mass spectrometry-based proteomic data revealed for the first time that EIN2 may be phosphorylated in the absence of ethylene and dephosphorylated upon ethylene treatment, suggesting a possible mechanism that the activity and/or stability of EIN2 could be modulated by differential phosphorylation (Chen et al., 2011b).

Based on genetic analysis, EIN3 and its homologs EIN3-like (EIL) proteins are the known components downstream of EIN2, which are another positive regulator of ethylene signaling pathway (Chao et al., 1997). *EIN3* encodes a transcription factor that activates the transcription cascades in nucleus. Like EIN2, EIN3 and EIL1 are also negatively regulated by two F-box proteins, EBF1 and EBF2 which in the absence of ethylene, ubiquinate EIN3 and EIL1 constitutively, targeting them for degradation (Guo and Ecker, 2003; Potuschak et al., 2003). Ethylene stabilizes EIN3

by eliminating EBFs at both mRNA and protein levels. EBF1 and EBF2 mRNAs are targeted for degradation by a 5'-3' exoribonuclease called EIN5 or XRN4 (Olmedo et al., 2006; Potuschak et al., 2006). Lately, a new finding revealed that EBF1 and EBF2 proteins are degraded through the 26S proteasome-mediated pathway in response to ethylene (An et al., 2010).

EIN3 directly binds to the promoter element of another transcription factor ERF1 and activates it (Solano et al., 1998). ERF1 binds to the GCC-box which is present in the promoters of many ethylene- and pathogen-induced genes such as *basic chitinase* and *plant defensin* (*PDF1.2*) (Ohmetakagi and Shinshi, 1995; Solano et al., 1998). ERF1 is a shared downstream component in both ethylene and jasmonate (JA) signaling pathways which synergistically regulate the defense responses to pathogen (Lorenzo et al., 2003). Recent studies revealed that EIN3/EIL is a key integration node between ethylene and several other signaling pathways such as JA and iron acquisition. JA positively regulates the transcriptional activity of EIN3/EIL1 by removing the repressor JA-Zim domain (JAZ) proteins (Zhu et al., 2011). The basic helix-loop-helix (bHLH) transcription factor FER-LIKE FE DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT), which induces the expression of iron acquisition genes and plays a key role in iron deficient responses, is stabilized through physical interaction with EIN3 (Lingam et al., 2011).

RTE1, a novel regulator of the ethylene receptor ETR1

In the past several years, the Chang lab looked for additional components in the ethylene signaling pathway by using various approaches. Analysis of the functions of

the new players might provide us with more clues for understanding the ethylene signaling pathway. A positive regulator of *ETR1*, *REVERSION-TO-ETHYLENE SENSITIVITY1* (*RTE1*), was identified through screening for *etr1-2* ethylene insensitivity suppressors (Resnick et al., 2006). Loss of *RTE1* function suppresses *etr1-2* ethylene insensitivity, probably due to a non-functional ETR1-2 receptor in the absence of RTE1. Moreover, the *rte1* loss-of-function mutant phenotype resembles the *etr1-7* null mutant, and the *etr1-7 rte1-2* double mutant is indistinguishable from the *etr1-7* single null mutant (Resnick et al., 2006). These data suggest that *RTE1* is required for both wild-type ETR1 and ETR1-2 function and that RTE1 and ETR1 are in the same pathway. When *RTE1* is overexpressed, seedlings show a weak ethylene insensitive phenotype in an ETR1 ethylene binding domain (EBD) dependent manner (Resnick et al., 2006; Zhou et al., 2007). Similarly, a mutation causing ectopic expression of *Green Ripe* (*GR*), the tomato RTE1 homolog, results in a non-ripening phenotype in tomato due to the reduced ethylene responsiveness in fruit (Barry and Giovannoni, 2006). These results indicate that *RTE1* negatively regulates ethylene response through positively regulating the ethylene receptor ETR1 in both Arabidopsis and Tomato (**Figure 1-4**).

Further genetic studies on RTE1 suggested that RTE1 is only required for ETR1 receptor function, not other ethylene receptors (Resnick et al., 2006). More surprisingly, RTE1 is not required for all dominant *etr1* ethylene insensitive mutations for conferring ethylene insensitivity (e.g. *etr1-1*) (Resnick et al., 2008; Resnick et al., 2006). Although the underlying basis for RTE1-dependent and RTE1-independent alleles remains unknown, all *etr1* mutant alleles are thought to yield

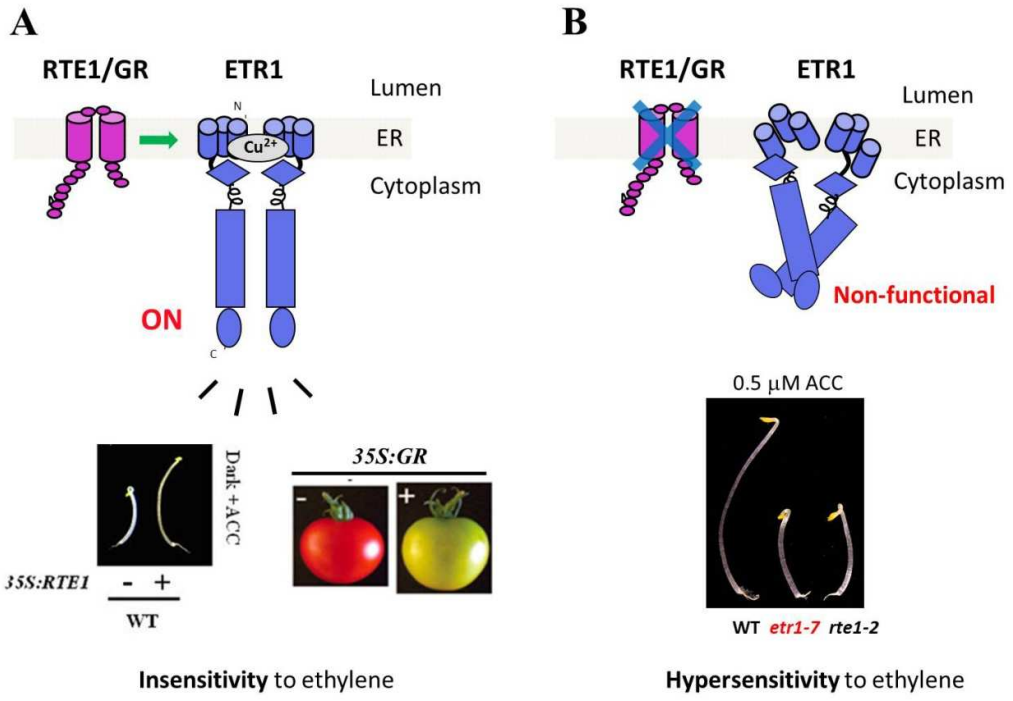


Figure 1-4 RTE1 positively regulates the ethylene receptor ETR1

(A) The wild-type ETR1 receptor requires RTE1 for signaling. When RTE1 (GR in tomato) is overexpressed, it promotes ETR1 signaling, which strongly represses ethylene response. Thus ethylene insensitivity phenotypes are observed. For example, dark-grown Arabidopsis seedlings lose the triple response in the presence of ethylene, which is provided by the ethylene precursor ACC, and in tomato, fruit ripening is inhibited. (B) When RTE1 is eliminated, ETR1 is presumed to be non-functional. The lack of ETR1 renders plants hypersensitive to ethylene since the receptor signaling strength is much reduced. On the low dose of ethylene provided by the precursor ACC, the wild type does not display the triple response, but *rte1-2* and *etr1-7* null mutants do display the triple response. Arabidopsis dark-grown seedlings and tomato fruit images are from (Resnick et al., 2006) and (Barry and Giovannoni, 2006) respectively.

structural defects within the ETR1 ethylene binding domain (EBD), which cause, to varying degrees, an inability to switch the signaling output domain off (Wang et al., 2006). Thus, the *RTE1*-dependence of certain *etr1* alleles, including wild-type ETR1, suggests that RTE1 may affect ETR1 receptor signaling through subtle modification of the ethylene binding domain conformation. This is also supported by in vivo association of RTE1 and ETR1 and subcellular co-localization of RTE1 and ETR1 in both ER and Golgi (Dong et al., 2010; Dong et al., 2008). In addition, it was demonstrated that *RTE1* does not affect ETR1 protein stability and sub-cellular localization (Rivarola and Chang, unpublished). RTE1 does not have any detectable effect on ethylene binding, nor does RTE1 itself bind ethylene (Michiels and Chang, unpublished).

After map-based cloning of the *rte1* mutation in Arabidopsis, the *RTE1* gene was found to encode a novel integral membrane protein conserved in plants, animals and some protists (Resnick et al., 2006). But the biochemical function of RTE1 in any of these organisms is unknown. There are no known functional motifs found in the RTE1 protein or in the orthologs in other species. The only known RTE1 action is promoting ETR1 signaling in Arabidopsis. Interestingly, animals also carry one copy of RTE but do not have ethylene receptor homologs. It is speculated that RTE1 may have some general function that is not limited to ethylene. Elucidating the molecular function of RTE1 will not only enhance our understanding of the ethylene signaling pathway in plants, but will help to uncover the conserved function of RTE in other organisms.

Based on the previous study focusing on RTE1 and ETR1, I continued to investigate the molecular function of RTE1 and the basis of RTE1 specificity for the ETR1 receptor to help understanding how ETR1 is regulated. The extensive genetic tools provided by the ethylene-signaling pathway in Arabidopsis may help to elucidate the conserved function of RTE1 in other organisms. In this thesis, firstly, I further provided supporting evidence for the previously proposed hypothesis that RTE1 may regulate ETR1 receptor signaling through affecting the ethylene binding domain conformation of ETR1 (**Chapter 2**). Secondly, I isolated and characterized an RTE1-interacting protein, cytochrome *b*5, which may provide an important clue for understanding the molecular function of RTE1 (**Chapter 3**). Thirdly, I demonstrated that RTE proteins may bind heme, suggesting that RTE proteins may be able to carry out redox (**Chapter 4**). Lastly, I investigated the underlying mechanism by which ETR1 is distinct from other Arabidopsis ethylene receptors in terms of RTE dependence, and a unique proline residue conserved only in ETR1 orthologs is involved in the specificity of RTE1 for ETR1 (**Chapter 5**). All the above novel findings lead us to a hypothesis that RTE1, together with cytochrome *b*5, may promote the active conformation of the ETR1 receptor through oxidative protein folding.

Chaper 2 : RTE1 may be involved in regulating the ETR1 EBD conformation

Introduction

Previous genetic analyses implicated that *RTE1* is a positive regulator of the ETR1 receptor and that ETR1 is largely non-functional in the absence of RTE1 (Resnick et al., 2006). The *etr1-7* null mutant displays the ethylene hypersensitivity at low dose of ethylene, probably because the lack of the ETR1 receptor, which is responsible for the majority of ethylene signaling, reduces the receptor signaling output considerably, rendering plants more sensitive to ethylene. The *rte1* mutant also has an enhanced ethylene-response phenotype that largely phenocopies the *etr1-7* null mutant (Resnick et al., 2006). Therefore, the ETR1 receptor may be inactive in the absence of *RTE1* (Resnick et al., 2006). The major question that I want to address is how RTE1 regulates ETR1 receptor function. *RTE1* is specific to certain *etr1* dominant ethylene insensitive alleles (Resnick et al., 2008), indicating the possibility that *RTE1* affects the function of ETR1 at the protein level rather than at the level of DNA or transcription. *RTE1* does not affect ETR1 sub-cellular localization (Rivarola and Chang, unpublished). ETR1 is not degraded in the *rte1-2* loss-of-function mutant (Resnick et al., 2008). RTE1 does not have any detectable effect on ethylene binding, nor does RTE1 itself bind ethylene (Michiels and Chang, unpublished). All these results suggest a more subtle regulatory role of RTE1 on ETR1.

A possible mechanism of RTE1 function is that RTE1 may facilitate the correct conformational switch in ETR1 needed for ETR1 signaling. Our lab's previous research results supported this hypothesis. A former graduate student in our lab, Jo Resnick, tested *rte1-2*'s ability to suppress 13 dominant ethylene-insensitive *etr1* mutations that lie within the ETR1 ethylene-binding (EB) domain (defined as residues 1-128) (**Figure 2-1**). These *etr1* mutants confer varying degrees of ethylene insensitivity and ethylene binding ability. Each allele carries a mis-sense mutation located in the ethylene-binding region of ETR1 and is thought to confer a conformational defect that causes the inability to switch the signaling domain off (**Figure 2-1 A**). Among them, some mutations abolish or nearly abolish or reduce ethylene binding, whereas some retain the ability to bind ethylene but confer ethylene insensitivity like the *etr1-2* mutation (Wang et al., 2006). In the model for ETR1 receptor signaling proposed by Wang *et al*.(2006), the ethylene binding (EB) domain's conserved function is to control the signaling domain conformation, and only subtle changes in steric structure are needed for the transition between ETR1 signaling on and off states. Resnick *et al*. revealed that loss of *RTE1* function is able to suppress many but not all *etr1* dominant ethylene-insensitive mutations to varying degrees (**Figure 2-1 B**) (Resnick et al., 2008). In other words, some *etr1* mutations require *RTE1* in order to confer ethylene insensitivity, but some others are *RTE1*-independent. Unfortunately, there is no correlation of RTE1-dependence with ethylene-binding ability, strength of signaling or the location of the mutation (Resnick et al., 2008). Based on these results, it was proposed that *RTE1* promotes the formation of the correct ETR1 EBD conformation required for the ETR1 signaling

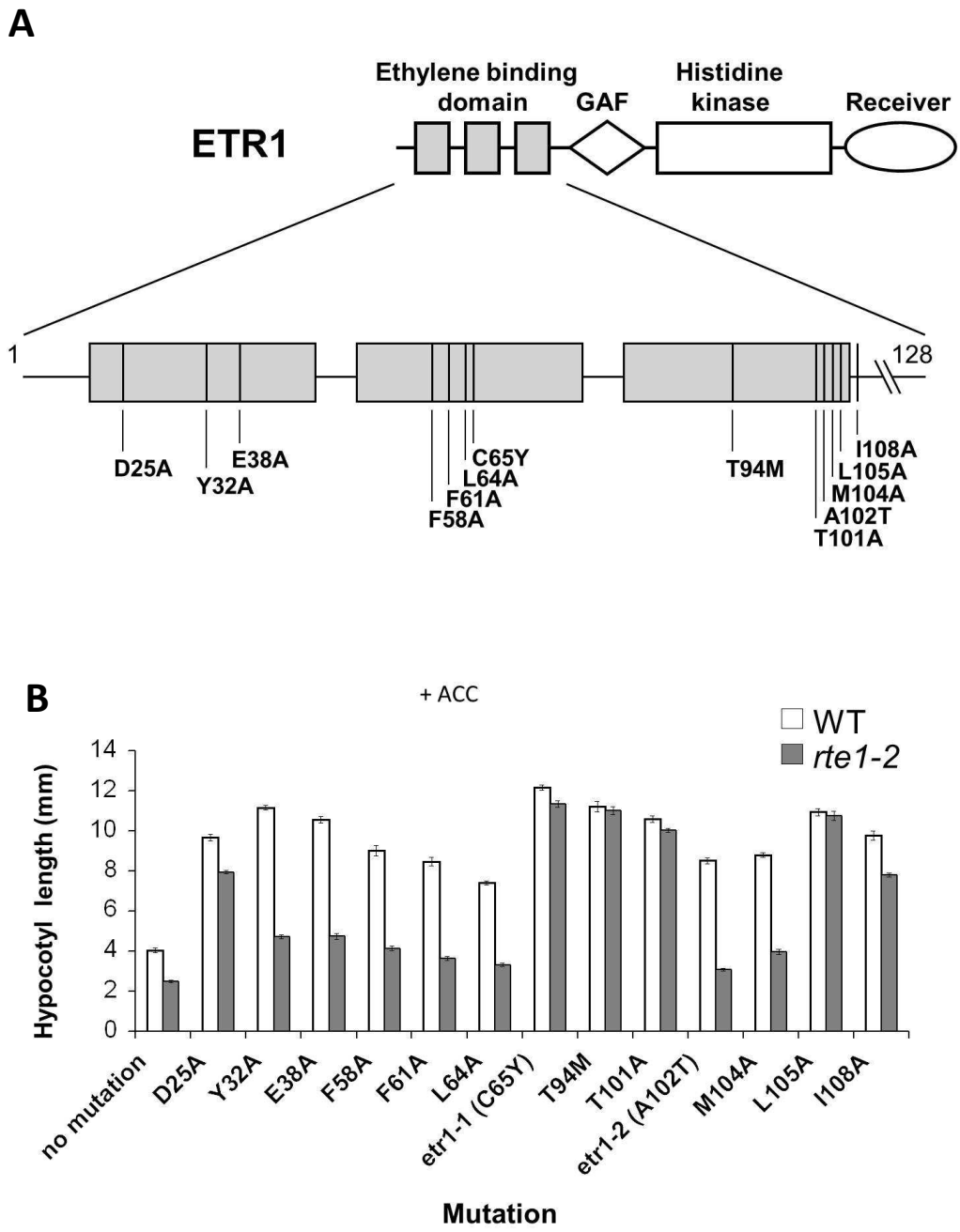


Figure 2-1 Effects of *etr1* dominant mutant transgenes in the wild type and *rte1-2*

The figure is taken from (Resnick et al., 2008). (A) The three transmembrane regions of the ETR1 ethylene-binding domain showing the relative positions of *etr1* mutations tested for suppression in the *rte1-2* background. Vertical lines indicate the

approximate locations of the specified amino acid substitutions carried by *etr1* transgenes transformed into the wild-type and *rte1-2* to test for ethylene insensitivity and suppression of ethylene insensitivity, respectively. In place of transgenes, the *etr1-1* (C65Y) and *etr1-2* (A102T) mutations were tested for suppression using the *etr1-1rte1-2* and *etr1-2 rte1-2* double mutants respectively. (B) Measurements of hypocotyl length conferred by the mutant *etr1* transgenes in wild-type versus *rte1-2* seedlings showing the degree of ethylene insensitivity and suppression, respectively. Hypocotyl lengths (mean ± SE for15–20 seedlings) were measured in representative homozygous lines of 4-day-old dark-grown seedlings on 20 μM ACC. Untransformed seedlings are indicated as 'no transgene'.

"on" state. RTE1 might be required to stabilize certain mutant conformations, while other conformations are stable enough without RTE1. These *etr1* alleles may represent the various states of the ETR1 receptor signaling process. *RTE1* might promote the ETR1 "on" state by altering equilibrium between the various states at points (A), (B) in **Figure 2-2**.

Two other reports support the idea that RTE1 affects N-terminal ETR1. Firstly, both full-length ETR1 and the truncated ETR1 (residues 1-349, including the ethylene binding domain within the membrane and soluble GAF domain) appear to physically associate with RTE1(Dong et al., 2010). Secondly, loss of the *RTE1* overexpression phenotype in the *etr1-7* null mutant is rescued by co-expression of a truncated *ETR1* comprising residues 1-349 (Zhou et al., 2007), which is thought to signal through interaction with the ERS1 ethylene receptor (Xie et al., 2006). These results suggest that ETR1 (1-349) is the target of RTE1 action.

Therefore, for the question of how RTE1 regulates ETR1 signaling, the current model is that RTE1 is involved in regulating ETR1 EBD conformation to promote ETR1 signaling. This chapter provides two additional pieces of evidence to support this hypothesis: cold temperature and silver could rescue the *rte1* mutant phenotype in ethylene response. These rescue experiments may give us some clues about the function of the RTE1 protein.

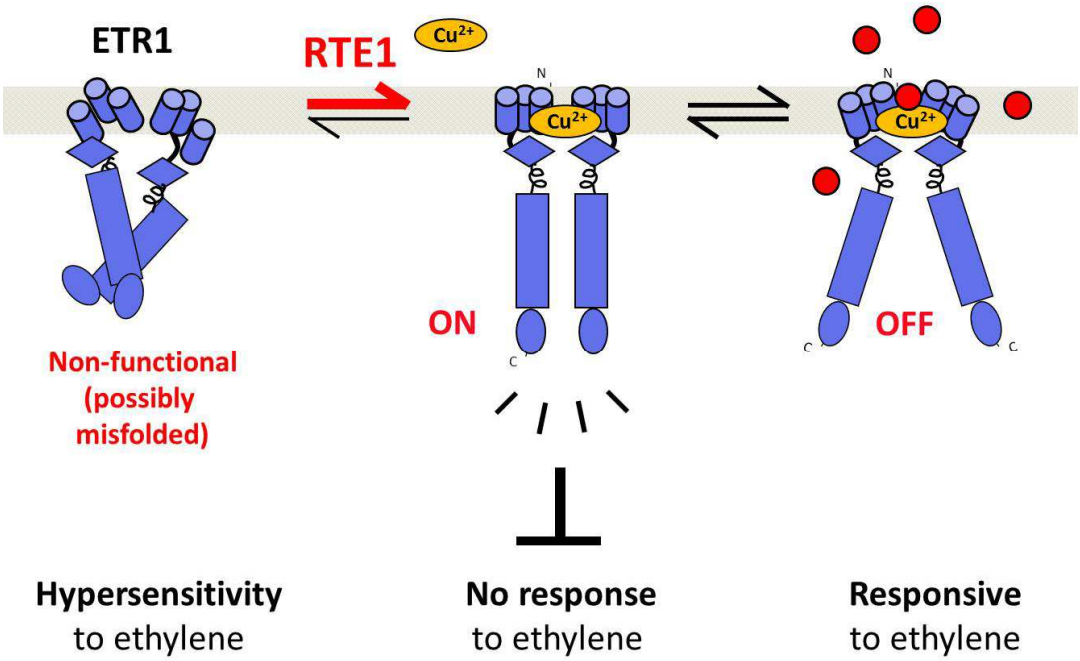


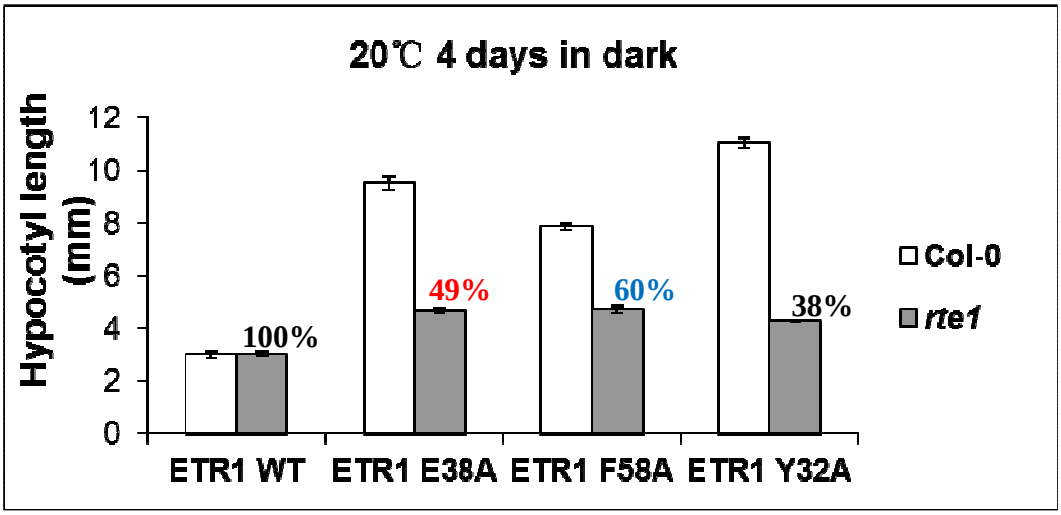
Figure 2-2 Model for the promotion of ETR1 signaling by RTE1

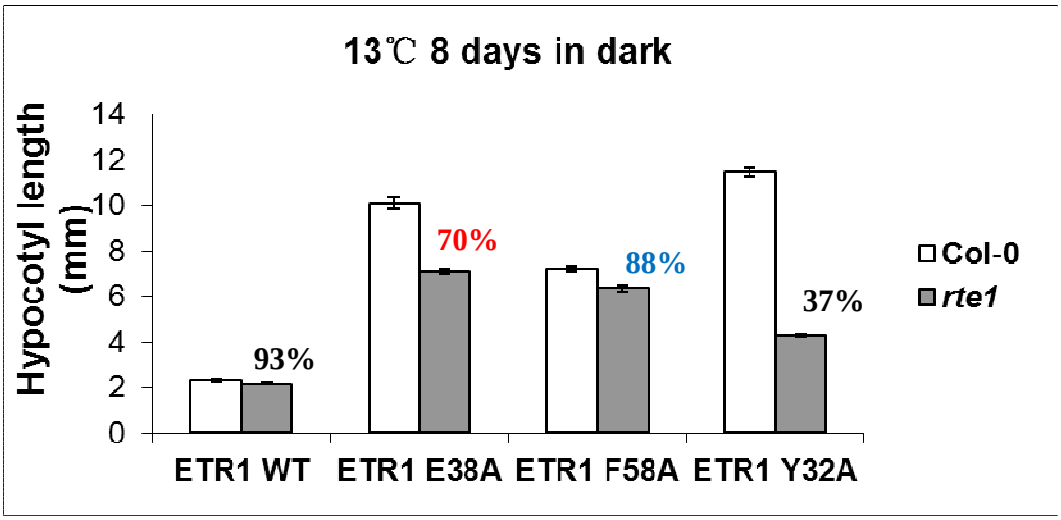
RTE1 acts on the ethylene-binding domain of ETR1 to promote the signaling 'on' state. The nascent non-functional ETR1 protein requires the action of RTE1 to allow transition to the functional 'on' state. The absence of RTE1 causes ethylene hypersensitivity, probably due to the non-functional ETR1 ethylene receptor. The copper cofactor is also required by ethylene receptors to be functional. When ethylene binds, there is presumably a conformational change in the ethylene binding domain, which transmits the signal to the C-terminal signaling output domain, turning signaling off and resulting in the ethylene response.

Results

rte1 can be partially rescued by low temperature

It is well established that temperature alters membrane fluidity, and that the physical state of membrane lipids can directly affect the activity of membrane proteins (Los and Murata, 2004). Temperature also affects protein conformation and thermally induced changes in protein conformation may result from the disruption of the chemical bonds involved in the maintenance of protein structure (Somero, 1978). If *RTE1* plays a role in conformational changes of the ETR1 transmembrane domain, I speculated that ETR1 receptor signaling may be sensitive to membrane fluidity change and/or thermally induced changes of the chemical bonds maintaining the protein structure. Therefore, I tested *rte1*'s ability to suppress the ethylene insensitivity conferred by 11 of the 13 *etr1* dominant alleles in cold condition (**Figure 2-3**). At the optimal growth temperature (20°C), the *rte1-2* mutation suppressed the ethylene insensitivity conferred by 7 of the 11 *etr1* dominant alleles, indicating that the 7 *etr1* dominant mutants require wild-type *RTE1* to confer ethylene insensitivity. 5 of the 7 *RTE1*-dependent *etr1* dominant alleles did not show any changes at 13°C and 9°C. Interestingly, the suppression of 2 of the 7 *RTE1*-dependent *etr1* dominant alleles (E38A and F58A) by *rte1-2* was partially alleviated at 13 °C, and further alleviated at 9 °C, indicating the two *RTE1*-dependent *etr1* dominant alleles (E38A and F58A) are less dependent on *RTE1* to confer ethylene insensitivity when they are grown in cold. In other words, the *rte1* mutation was partially rescued by cold temperature.


20℃ 4 days in dark
Hypocotyl length (mm)
0
2
4
6
8
10
12
100%
49%
60%
38%
ETR1 WT
ETR1 E38A
ETR1 F58A
ETR1 Y32A
Col-0
rte1


13℃ 8 days in dark
Hypocotyl length (mm)
0
2
4
6
8
10
12
14
93%
70%
88%
37%
ETR1 WT
ETR1 E38A
ETR1 F58A
ETR1 Y32A
Col-0
rte1

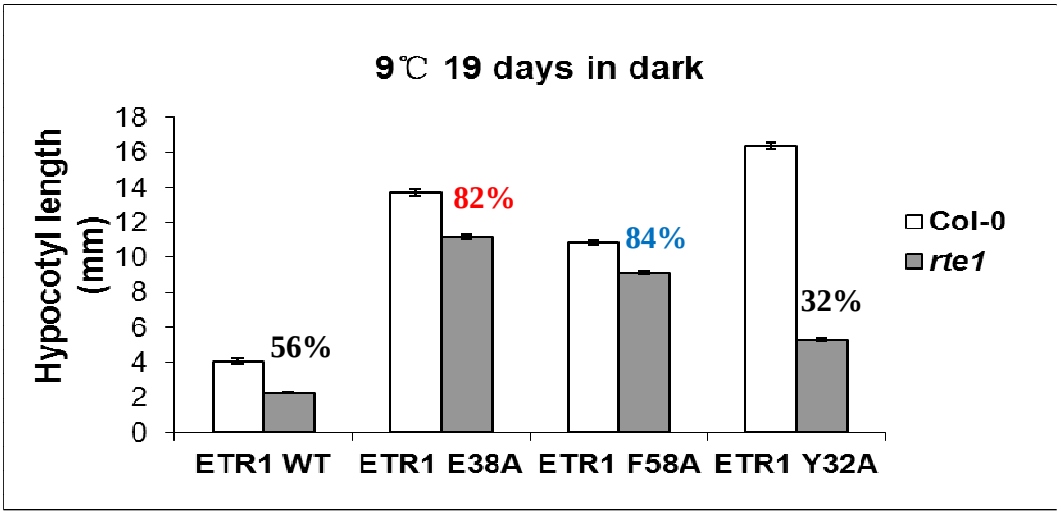

9℃ 19 days in dark
Hypocotyl length (mm)
0
2
4
6
8
10
12
14
16
18
56%
82%
84%
32%
ETR1 WT
ETR1 E38A
ETR1 F58A
ETR1 Y32A
Col-0
rte1

Figure 2-3 Partial alleviation of the *rte1* phenotype by cold temperature

Measurements of hypocotyl length conferred by the mutant *etr1* E38A, F58A and Y32A transgenes in wild-type versus *rte1-2* seedlings showing the degree of ethylene insensitivity and suppression, respectively. The numbers above the columns represent the percentage of the hypocotyl length of the *etr1* transgene in the *rte1-2* mutant background of that in Col-0 background. Hypocotyl lengths (mean±SE for 30-40 seedlings) were measured in representative homozygous lines of 4-day-old dark-grown seedlings in the presence of 20μM ACC in 20°C and 8-day-old dark-grown seedlings in 13°C as well as 19-day-old dark-grown seedlings in 9°C.

Silver can restore signaling of the non-functional ETR1 receptor in an *rte1* null plant

To test whether ETR1 is present but cannot properly function in *rte1-2,* an experiment was carried out to see if silver can rescue the inactive ETR1 protein in *rte1-2*. The rationale for this experiment is that the ETR1 protein is absent in the *etr1-7* null mutant whereas it may be present but inactive in the *rte1-2* null mutant. Silver ions presumably bind to the ethylene receptors in place of copper and lock them in a signaling conformation that cannot be shut off even with ethylene (Binder et al., 2007; Rodriguez et al., 1999). When seedlings were grown in the presence of a high ratio of silver nitrate to ACC, which is readily converted to ethylene in plants by ACC oxidase, both *etr1-7* and *rte1-2* were insensitive to the ethylene provided by ACC and indistinguishable in ethylene response. Presumably, this is because the wild type ethylene receptor family members in both *etr1-7* null and *rte1-2* null mutants were locked ON by Ag(I) ions and the receptor signaling was strong enough to repress all the ethylene response (Resnick et al., 2006). At a lower ratio of Ag (I) to ACC, the plants lacking ETR1 (e.g. *etr1-7*) do not have enough signaling output to repress the ethylene response, and as a result, exhibit triple response, whereas the plants having inactive ETR1 (e.g. *rte1-2*) have enough signaling output to repress the ethylene response since silver could restore the inactive ETR1 to a signaling ON state.

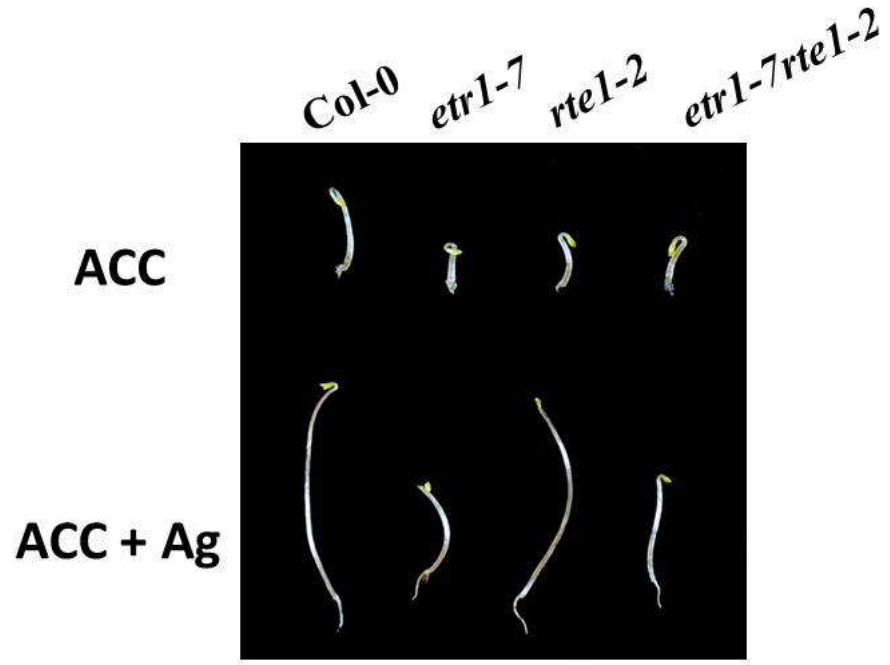
Based on this hypothesis, a former graduate student in our lab, Maximo Rivarola did initial test to look for the optimal ratio of Ag (I) to ACC to rescue the effect of the *rte1-2* mutation on ETR1 (Chang, 2008). We found that a triple response phenotype was elicited in *etr1-7* whereas not in *rte1-2* in the presence of 100 µM ACC and 10

µM silver nitrate (**Figure 2-4**). *rte1-2* behaved just like the wild type, suggesting the ETR1 receptor in *rte1-2* mutant was active just like in the wild type plants when there is silver. In addition, the double *etr1-7 rte1-2* null mutant phenocopied the *etr1-7* null mutant, placing *ETR1* downstream of *RTE1* (**Figure 2-4**). This result suggests that silver can restore ETR1 signaling in an *rte1* null and *RTE1* acts upstream of *ETR1*. The result that silver rescues inactive ETR1 protein in *rte1* mutant supports that RTE1 may play a role in promoting the active conformation of ETR1 for signaling since silver presumably acts on the EBD conformation.

Discussion

The results presented here provide two more pieces of supporting evidence that RTE1 may be involved in regulating the conformational changes to promote ETR1 signaling. Firstly, we showed that cold temperature converted two *RTE1*-dependent ETR1 mutant (E38A and F58A) alleles to being nearly *RTE1*-independent. As described above, the two alleles are presumed to be held in the signaling ON state with the help of RTE1. This conformation cannot be maintained without RTE1 at the normal temperature. But in the cold, the two *etr1* dominant alleles conferred ethylene insensitivity in the absence of RTE1, indicating that the conformation required for signaling ON could be achieved without RTE1. This result suggests that cold temperature could do a similar job or cause the same effect as wild type RTE1 does. It has been well known that protein conformation and membrane fluidity can be affected by temperature. Cold temperature could directly make ETR1 E38A and F58A conformation held in a receptor signaling ‘on’ state by causing the changes of

A


Col-0
etr1-7
rte1-2
etr1-7rte1-2
ACC
ACC + Ag

B

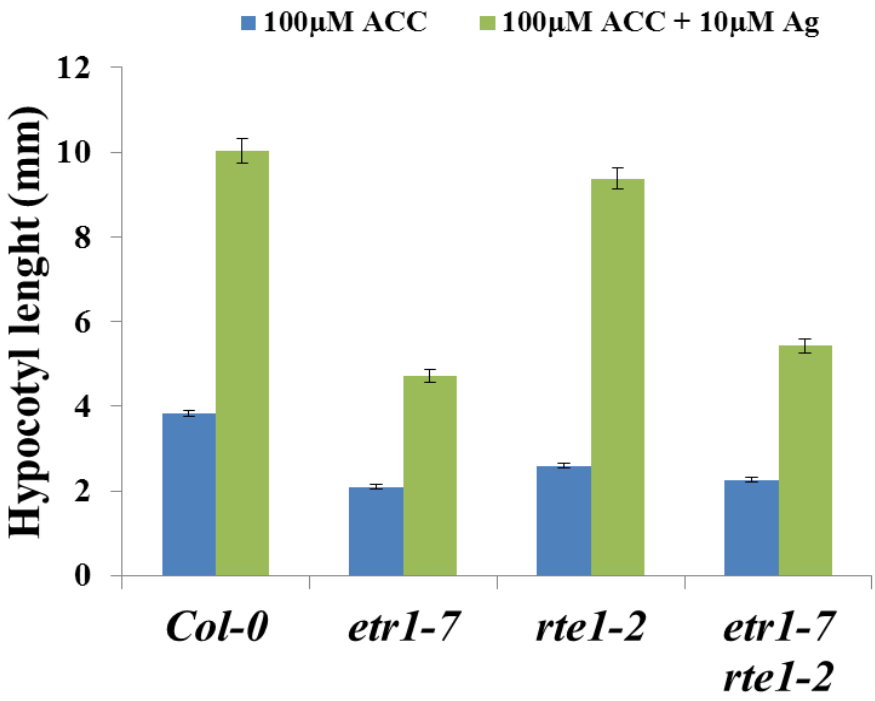

100µM ACC
100µM ACC + 10µM Ag
Hypocotyl lenght (mm)
12
10
8
6
4
2
0
Col-0
etr1-7
rte1-2
etr1-7 rte1-2

Figure 2-4 Silver can restore ETR1 signaling in an *rte1* null

Treatment with the ethylene-response inhibitor silver nitrate ($AgNO_3$) at 10µM dramatically alleviates the ethylene response (100µM ACC) in wild type and the *rte1* loss of function mutant (*rte1-2*), but much less in an *etr1* null mutant (*etr1-7*) and *etr1-7 rte1-2* double mutant. (A) Representative four-day-old dark grown seedlings germinated on MS medium with 10µM silver nitrate plus 100µM ACC. (B) 16 – 26 seedlings were measured per genotype at each condition and mean ± standard error are shown.

chemical bonds in ETR1 transmembrane domains. An alternative explanation is that, cold temperature could cause lower membrane fluidity, so that the more rigid membrane prevents E38A and F58A receptors from shifting toward the OFF signaling state in the absence of RTE1. Thus, I reason that RTE1 may function like a molecular chaperone, facilitating the folding of ETR1, particularly the ethylene binding domain. Alternatively, RTE1 may exert an indirect effect on the ETR1 EBD conformation by affecting the membrane environment where ETR1 EBD resides.

Secondly, silver can rescue the ethylene hypersensitive phenotype of *rte1-2*, but not *etr1-7*, probably because silver restores ETR1 signaling in *rte1-2* whereas there is no ETR1 to act on in the *etr1-7* null mutant. The finding that silver can convert ETR1 to RTE1-independent suggests that the conformation of the ethylene binding domain is likely to be locked into a signaling ON state by silver which overrides the need for RTE1. Therefore, RTE1's function may be related to the ETR1 EBD conformation. However, I have not ruled out the possibility that ETR1 is not totally nonfunctional in the *rte1* mutant. Perhaps a small population of ETR1 proteins can still signal without RTE1. In this case, silver might bind to the functional ETR1 proteins to signal constitutively.

The two results provided in this chapter, together with previously reported evidence, support the hypothesis that RTE1 affects the conformational switch between active and inactive states of the ETR1 receptor. However, the question that remains unanswered is how RTE1 regulates the ETR1 EBD conformation. Does RTE1 play a direct role in the folding of ETR1 EBD, or affect the conformation of the ETR1 EBD indirectly, for example, through regulating the membrane

environment of the ETR1 receptor? The next chapters will continue to address this question.

Materials and Methods

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* Columbia (Col-0) ecotype was used as the wild-type strain in all experiments. All mutant *etr1* transgenes were kindly donated by Dr. Anthony Bleecker's lab, described in Wang et al (2006). The plasmids carrying individual *etr1* transgenes were transformed into Col-0 and *rte1-2* plants by the floral dip method using *Agrobacterium tumefaciens* strain GV3101 as described in (Resnick et al., 2008). The homozygous transgene T3 or T4 seeds were sown on Murashige and Skoog (MS) medium containing 20 μM ACC. Following a 3-day stratification at 4℃, the seeds were placed in light for six hours and then grown in a light tight black plexiglass box at 20℃ for 4 days, or at 13℃ for 8 days, or at 9℃ for 19 days.

For the silver rescue experiment, the wild type, *etr1-7*, *rte1-2* and *etr1-7 rte1-2* double mutants were sown on MS medium with 100μM ACC and 10μM silver nitrate and grown in the dark at 20℃ for 4 days following a 3-day stratification at 4℃ and a six-hour incubation under light.

Seedlings were removed from the MS medium and placed onto a black cloth for digital photography. The hypocotyl length was measured from the digital images using ImageJ software (http://rsbweb.nih.gov/ij/).

Genotyping Markers

Genotyping was carried out using either the Phire Plant Direct PCR kit (Finnzymes) or after isolating total genomic DNA by the CTAB method (Dellaporta et al., 1983).

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

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

Chaper 3 : Cytochrome b5 isoforms interact with and play a similar role as RTE1 in regulating ETR1 receptor signaling

Introduction

In Chapter 2, I discussed the supporting evidence for the model that RTE1 may promote the active conformation of the ethylene binding domain (EBD) of the ETR1 receptor required for ETR1 signaling. Next, I wanted to understand the molecular basis of the effects of RTE1 on ETR1. How does RTE1 affect the conformation of the ETR1 ethylene binding domain (EBD)? It has been speculated that RTE1 may affect ETR1 EBD conformation either in a direct way (e.g. acting as a molecular chaperone involved in protein folding), or in an indirect way (e.g. affecting the membrane environment where ETR1 EBD reside). Since RTE1 is a novel protein, no implications can be obtained from the orthologs in other species. To obtain possible insight into the molecular function of RTE1, I looked for RTE1 interacting proteins. Exploring protein interactions not only extends information about known proteins but also can help to identify functions of unknown proteins. If the RTE1 interacting protein candidate has known functions/functional domains and is shown to be involved in ethylene signaling, it could guide us to dissect the unknown molecular function of RTE1.

There are many methods for detecting protein-protein interaction with different sensitivity and specificity. The yeast two-hybrid assay is one of the easiest approaches, but does not allow for the detection of interactions with membrane spanning proteins. Since RTE1 contains membrane spanning domains, I screened for

protein partners of RTE1 by using the yeast split-ubiquitin assay, based on reconstitution of the ubiquitin (Ub) protein halves (Cub and Nub) in the cytosol (Stagljar et al., 1998). Depending on the different readout of the interaction, there are two split-ubiquitin-based approaches (the Ura3-based system and the transcription-based system) (**Figure 3-1**). I screened existing cDNA libraries using the Ura3-based system and the putative positives were retested using the transcription-based system. Before screening, the Chang lab had demonstrated that the RTE1 bait protein localizes to yeast Golgi and ER membranes and that its C-terminus is cytosolic, as required by the reporter protein (Liesch and Chang, unpublished). The subcellular localization was indicated by the interaction between RTE1 bait and particular yeast membrane marker fused with wild-type Nub (Reichel, 2005; Wittke et al., 1999).

Results

Isolation of putative RTE1 interacting proteins

Using RTE1 as bait, I screened two different *Arabidopsis* cDNA libraries, one made from 6-day old light/dark-grown seedlings (DualSystems Biotech, Switzerland) and the other from inflorescences (kindly provided by Prof. Imre Sommsich, Max-Planck-Institut für Züchtungsforschung, Köln, Germany). The seedling cDNA library yielded 30 putative positives (out of 3.9×10^6 colonies), but unfortunately none were confirmed when the plasmids were isolated and retested. The inflorescence library yielded 16 positives (out of 3.2×10^5 colonies). Three were positive when retested (**Figure 3-2 A**). Two of the clones carried the full length open reading frame of gene

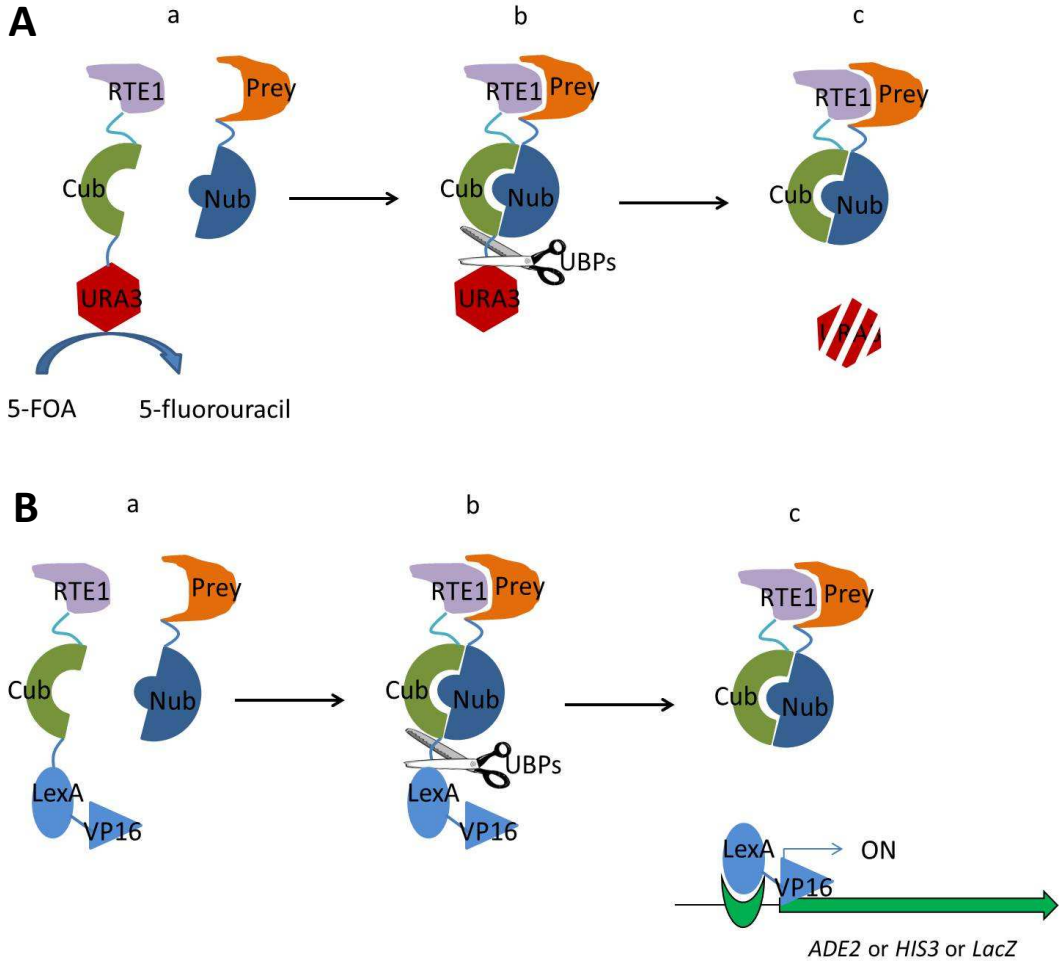


Figure 3-1 The yeast split-ubiquitin system

(**A**). The Ura3-based system. (a) Bait and Prey do not interact. The bait is RTE1 in this Study which is fused to the C-terminal half of ubiquitin (Cub) and URA3 protein which converts the compound 5-fluoroorotic acid (5-FOA) into the toxic product 5-fluorouracil. As a result, yeast cells expressing the RTE1-Cub-URA3 fusion protein will die when plated on media containing 5-FOA. The prey is fused to the N-terminal half of ubiquitin (Nub). (b) The interaction between bait and prey results in the reconstitution of split-ubiquitin. Split-ubiquitin is immediately recognized by UBPs which then cleave the polypeptide chain between Cub and URA3, releasing the

URA3 protein. (c) The released URA3 protein is unstable and rapidly degraded by the 26S proteasome, leading to cells that can grow on medium containing 5-FOA. (**B**). The transcription-based system. (a) Bait and Prey do not interact. The bait is RTE1 in this Study which is fused to the C-terminal half of ubiquitin (Cub) and the artificial transcription factor LexA-VP16. The prey is fused to the N-terminal half of ubiquitin (Nub). (b) The interaction between bait and prey results in the reconstitution of split-ubiquitin. Split-ubiquitin is immediately recognized by UBPs which then cleave the polypeptide chain between Cub and LexA-VP16. (c) The LexA-VP16 transcription factor is released from the membrane and translocates to the nucleus where it binds to the LexA regulated promoters and activate the reporter genes *ADE2*, *HIS3* and *LacZ*. The activation of *ADE2* and *HIS3* enables the yeast to grow on media lacking adenine and histidine. The activation of *lacZ* can be detected via *b*-galactosidase assays.

At1g48750, which is predicted to encode a non-specific lipid transfer protein (ns-LTP). The third clone contained a 3' fragment of the At5g48810 gene encoding an ER-localized cytochrome *b*5 (Cb5) isoform called AtCb5-D. The isolated clone encodes the C-terminal 38 amino acids (of the 140-residue protein) consisting of the predicted transmembrane domain and a luminal polar region (**Figure 3-2 B**). The full length AtCb5-D cDNA was also tested and confirmed for its ability to interact with RTE1. In a test for specificity, I found that both ns-LTP and AtCb5-D were able to interact with RTE1 and the RTE1 homolog (RTH), which shares 51% identity with RTE1, but not with two bait versions of a cation transporter, CHX20 (provided by Dr. Heven Sze, University of Maryland) (**Figure 3-3**).

Analysis of the AtCb5 family: subcellular localization, membrane topology and gene expression

Cytochrome b5 (Cb5) is conserved in plants, animals, fungi and purple phototrophic bacteria (Schenkman and Jansson, 2003). Cb5 is known to be a ubiquitous hemoprotein that functions as an electron transfer protein (Schenkman and Jansson, 2003). Cytochrome b5 is a tail-anchored (TA) membrane protein that is targeted posttranslationally to various organelles. The features of TA membrane proteins include an N-terminal domain exposed to the cytosol, a single hydrophobic segment located near the C-terminus and a short C-terminal tail region that protrudes into the organelle lumen (Kutay et al., 1993).

Arabidopsis has five identified putative cytb5 isoforms (AtCb5-A, At1g26340; AtCb5-B, At2g32720; AtCb5-C, At2g46650; AtCb5-D, At5g48810; AtCb5-E, At5g53560) with 40-70% identity and one Cytochrome b5-like protein (AtCb5LP,

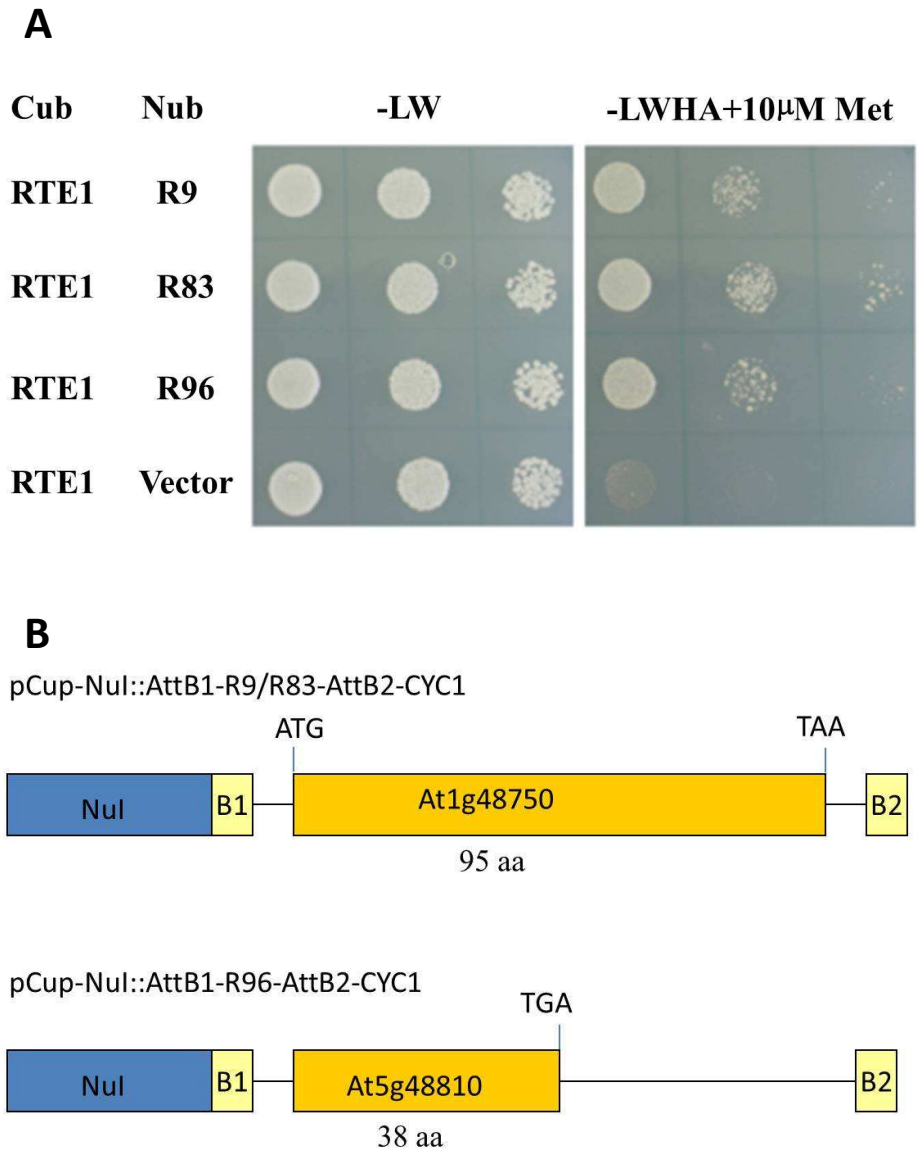
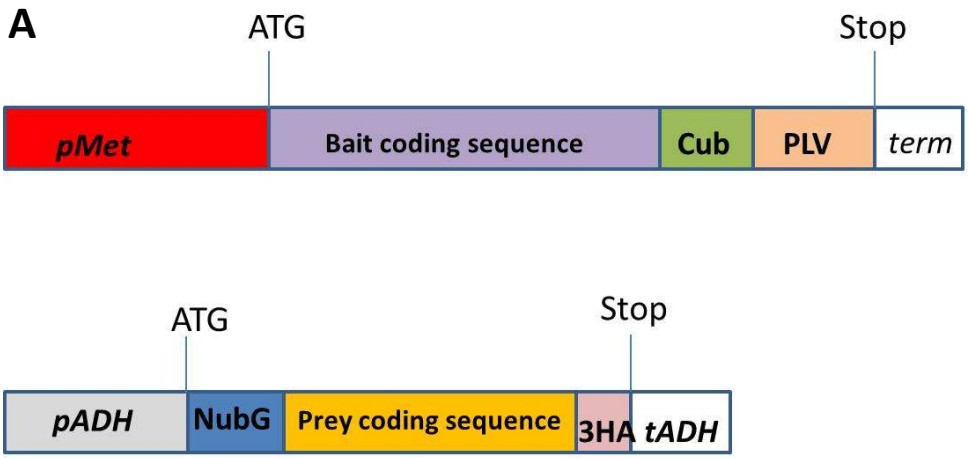


Figure 3-2 Yeast split ubiquitin assay of proteins interacting with RTE1

(**A**). The full length RTE1 bait protein (fused with Cub) interacts with prey clones R9 and R83 (both At1g48750) and R96 (At5g48810) (fused with Nub) isolated from an inflorescence cDNA library using the URA3 reporter system (shown in Figure 3-1 A). Interaction shown here uses the transcriptional reporter system in Obrdlik *et al* (2004). Cells were spotted onto agar medium from 10-fold serial dilutions of liquid overnight cultures, and then grown for 3 days. Medium lacking tryptophan and leucine (-LW)

selects for the Cub and Nub plasmids, respectively. Protein interactions are indicated by growth on medium additionally lacking histidine and adenine (-LWHA). Methionine is used to control the level of the RTE1 fusion, which is expressed under a methionine repressible promoter. (**B**). Diagram of the positive prey clones. Two (R9 and R83) carried the full length open reading frame of gene At1g48750, which is predicted to be a non-specific lipid transfer protein (ns-LTP). One (R96) contained a 3' fragment of the At5g48810 gene encoding an ER-localized cytochrome *b*5 isoform called AtCb5-D. All are in frame with Nub.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PLV
term
ATG
Stop
pADH
NubG
Prey coding sequence
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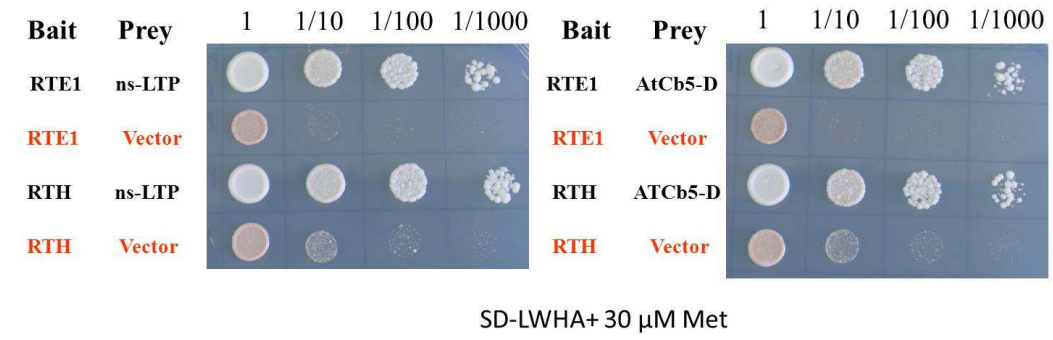
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Bait
Prey
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1/10
1/100
1/1000
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ns-LTP
RTE1
Vector
RTH
ns-LTP
RTH
Vector
Bait
Prey
1
1/10
1/100
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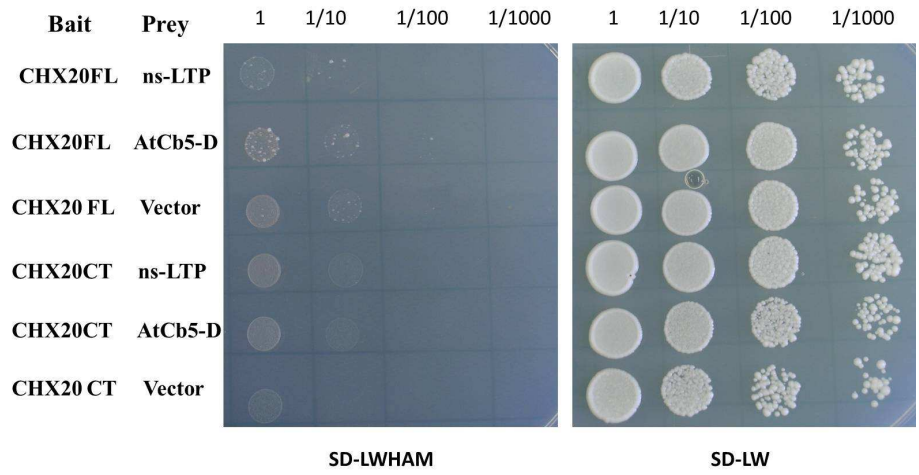
AtCb5-D
RTE1
Vector
RTH
ATCb5-D
RTH
Vector
SD-LWHA+ 30 µM Met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Prey
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1/1000
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CHX20FL
AtCb5-D
CHX20 FL
Vector
CHX20CT
ns-LTP
CHX20CT
AtCb5-D
CHX20 CT
Vector
SD-LWHAM
SD-LW

Figure 3-3 Interaction specificity of ns-LTP and AtCb5-D

(**A**).Bait and prey constructs used for protein interaction assay in the split-ubiquitin yeast two-hybrid system. The expression of the bait protein fused with C-terminal ubiquitin and PLV transcriptional factor is driven by the pMet promoter which is repressed by Methionine. Term marks the terminator. 'ATG 'and 'stop' mark the start and the stop codon in the expression cassette. For the prey construct, pADH is the ADH1 promoter and tADH marks the ADH1 terminator. NubG represents the N-terminal ubiquitin with an isoleucine at position 13 to glycine mutation which abolishes the strong affinity between wildtype Nub and Cub in order to prevent their spontaneous reassembly.3xHA tag is fused with the prey coding sequence at C-terminus. ns-LTP and AtCb5-D interact with RTE1 and RTH (**B**), but not with both full length (FL) and C terminal tail (CT) of CHX20 (**C**). Yeast cells containing indicated bait and prey constructs were tested for their binding on the minimum medium omitting Leucine (L), Trptophan (W), Histidine (H),Adenine (A) and supplemented with indicated concentration of Methionine (M). The results indicate the growth of yeast after incubation at 30℃ for 2 days on -LW plates or for 3 days on -LWHAM plates.

At1g60660) (**Figure 3-4**). Comparison of the amino acids sequences of the five Arabidopsis cytochrome b5 isoforms revealed that they have conserved features of the Cb5 protein family: a predicted N terminal heme-binding domain containing a conserved heme-binding motif (-HPGG-) and a C-terminal transmembrane domain that anchors the protein to the ER or chloroplast membrane (**Figure 3-4**). AtCb5LP has a transmembrane domain at its N-terminus (**Figure 3-4**). AtCb5-D (At5g48810) is localized to the ER membrane, whereas AtCb5-A (At1g26340) is localized to the chloroplast envelope (Maggio et al., 2007). The sequences of AtCb5-B, -C and -E resemble those of Cb5-A, -B and -C in *Aleurites fordii* (tung tree). Hwang et al. have shown the localization of tung Cb5-A,-B and -C on the ER membrane and tung tree Cb5-D is localized to mitochondria both in vitro and in vivo (Hwang et al., 2004). Therefore, AtCb5-B, -C, and –E could be also localized to the ER membrane (**Figure 3-5**).

To analyze the expression patterns of AtCb5 genes based on microarray meta-analysis, we used Genevestigator (http://www.genevestigator.com) (Zimmermann et al., 2005). According to microarray data, *AtCb5-D* and *AtCb5-E* are highly expressed throughout the plant during most stages. *AtCb5-A* and *AtCb5-B* are expressed at a lower level in most organs at most stages of development. *AtCb5-C* is expressed at a lower level than *AtCb5-D* and *AtCb5-E*, but higher level than *AtCb5-A* and *AtCb5-B* in almost all organs throughout development with a peak in young flowers (**Figure 3-6**). Since *AtCb5* genes are expressed in almost all organs throughout all stages of development, they overlap with *RTE1* and *ETR1* expression patterns previously described (Dong et al., 2008; Hua et al., 1998; Raz and Ecker, 1999).

Figure 3-4 Comparison of Arabidopsis cytochrome b5 (Cb5)-A, B, C, D, E and like protein polypeptide sequences

The alignment of Cb5 isoforms in *Arabidopsis thaliana* using the CLUSTALW algorithm (Chenna et al., 2003; Larkin et al., 2007). The following sequences are shown: AtCb5-A (NP_173958.1), AtCb5-B (NP_180831.1), AtCb5-C (NP_182188.1), AtCb5-D (NP_199692.1), AtCb5-E (NP_200168.1) and AtCb5-like protein (NP_176265.1). The box indicates conserved heme-binding motif. The predicted transmembrane domains are underlined.

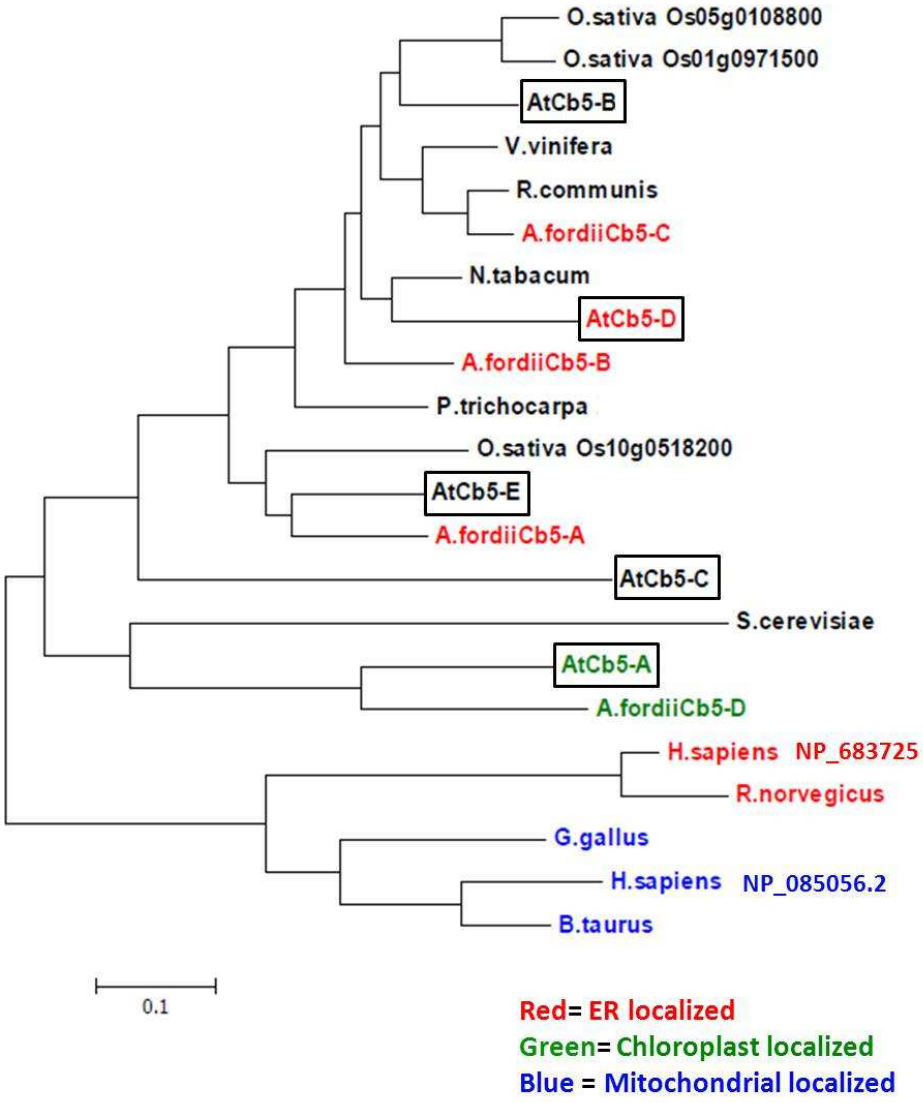
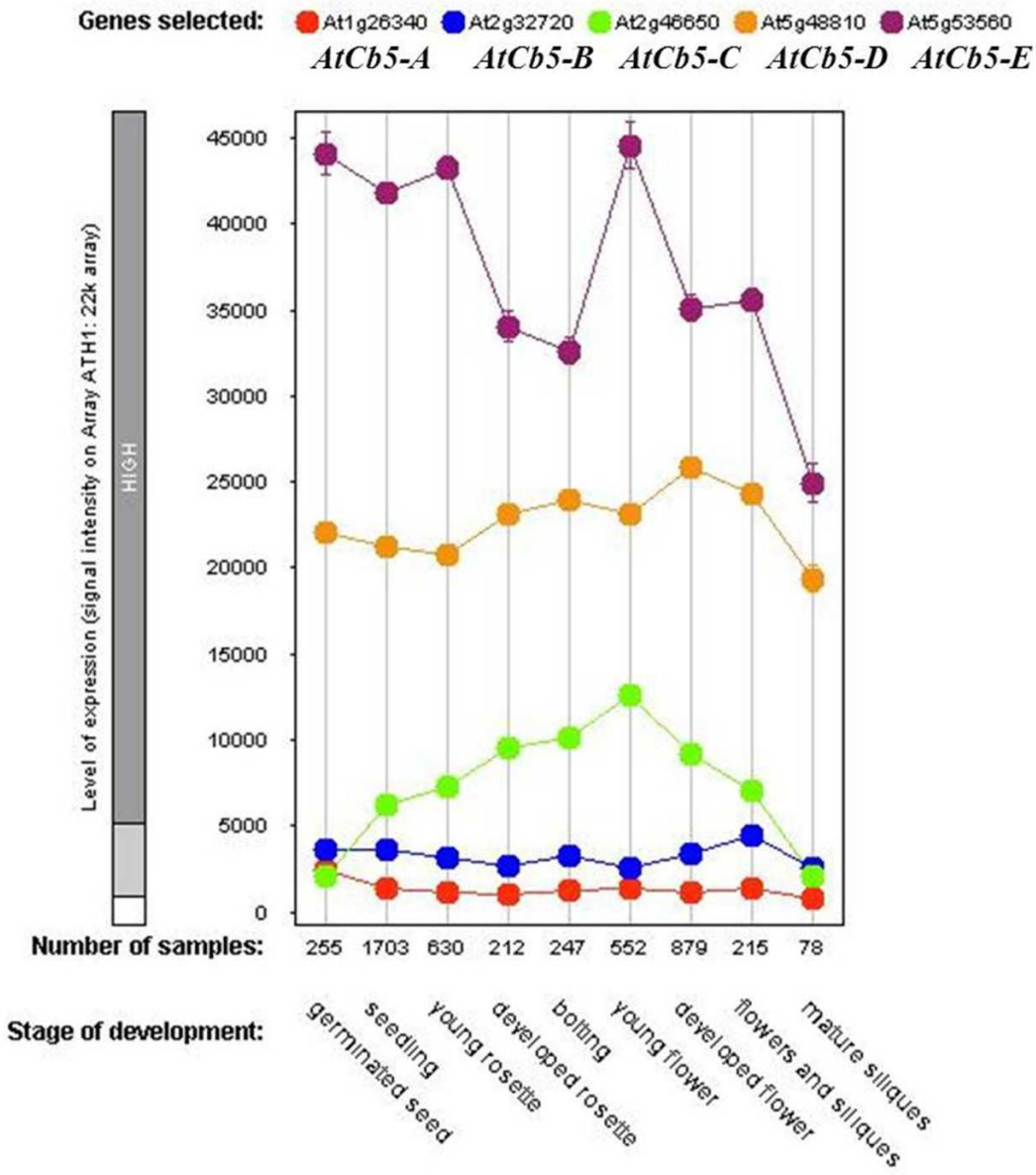


Figure 3-5 A phylogenetic tree of various Cb5 proteins

The following sequences are shown: Arabidopsis thaliana Cb5-A "AtCb5-A" (NP_173958.1), Arabidopsis thaliana Cb5-B "AtCb5-B" (NP_180831.1), Arabidopsis thaliana Cb5-C "AtCb5-C" (NP_182188.1), Arabidopsis thaliana Cb5-D "AtCb5-D" (NP_199692.1), Arabidopsis thaliana Cb5-E "AtCb5-E" (NP_200168.1), Aleurites fordii Cb5-A "A.fordii_Cb5-A" (AAT84458.1), Aleurites fordii Cb5-B

“A.fordii_Cb5-B” (AAT84459.1), Aleurites fordii Cb5-C “A.fordii_Cb5-C” (AAT84460.1), Aleurites fordii Cb5-D “A.fordii_Cb5-D” (AAT84461.1), Bos taurus “B.taurus” (NP_001157254.1), Gallus gallus “G.gallus” (NP_001025752.1), Homo sapiens “H.sapiens” (NP_085056.2), Homo sapiens “H.sapiens” (NP_ 683725.1), Nicotiana tabacum “N.tabacum” (CAA50575.1), Oryza sativa “O.sativa_Os05g0108800” (NP_001054434.1), Oryza sativa “O.sativa_Os01g0971500O” (NP_001045534.1), Oryza sativa “O.sativa_Os10g0518200” (NP_001065073.2), Populus trichocarpa “P.trichocarpa” (XP_002323982.1), Rattus norvegicus “R.norvegicus” (NP_071581.1), Ricinus communis “R.communis” (XP_002521096.1), Saccharomyces cerevisiae “S.cerevisiae” (NP_014288.1), Vitis vinifera “V.vinifera” (XP_002265677.1). A multi-sequence alignment of the above Cb5 protein sequences were generated using CLUSTALW (Larkin et al., 2007). Then the alignment was used to build the phylogenetic tree using *MEGA* version 5 (Tamura et al., 2011). The phylogenetic trees generated using neighbor-joining and UPGMA clustering methods were compared for assessing the reliability of the trees. Shown is the phylogenetic tree constructed using the neighbor-joining method. Some Cb5 proteins have been shown to localize in the endoplasmic reticulum membrane (red), in the chloroplast envelope (green) or in the mitochondrial outer membrane (blue) (Hwang et al., 2004; Maggio et al., 2007; Mitoma and Ito, 1992). The five Arabidopsis Cb5 proteins are boxed.

A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At2g32720
At2g46650
At5g48810
At5g53560
AtCb5-A
AtCb5-B
AtCb5-C
AtCb5-D
AtCb5-E
Level of expression (signal intensity on Array ATH1: 22k array)
HIGH
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40000
35000
30000
25000
20000
15000
10000
5000
0
Number of samples:
255
1703
630
212
247
552
879
215
78
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young rosette
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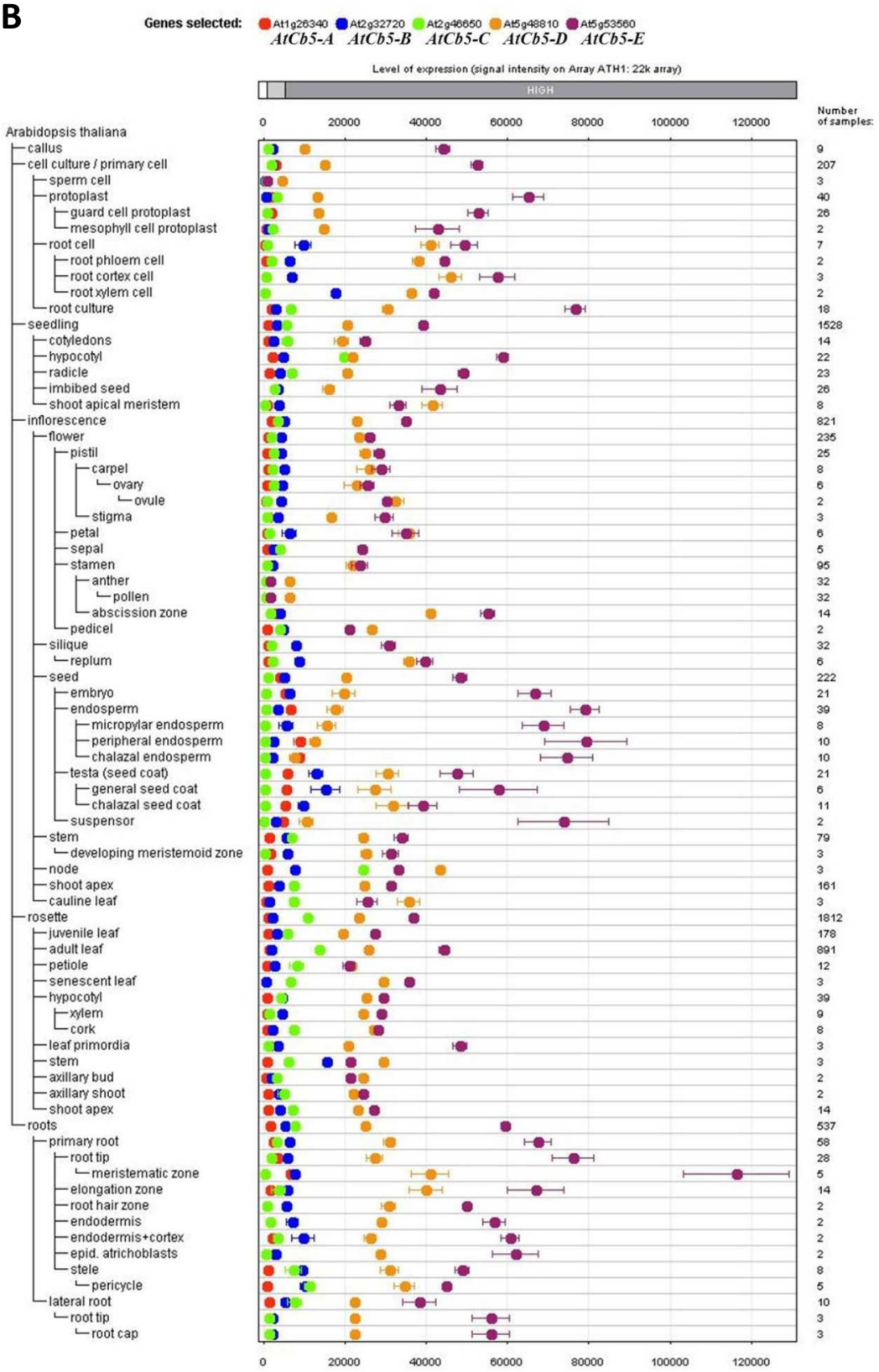
bolting
young flower
developed flower
flowers and siliques
mature siliques

B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AtCb5-A AtCb5-B AtCb5-C AtCb5-D AtCb5-E
Level of expression (signal intensity on Array ATH1: 22k array)
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Number of samples:
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sperm cell 3
protoplast 40
guard cell protoplast 26
mesophyll cell protoplast 2
root cell 7
root phloem cell 2
root cortex cell 3
root xylem cell 2
root culture 18
seedling 1528
cotyledons 14
hypocotyl 22
radicle 23
imbibed seed 26
shoot apical meristem 8
inflorescence 821
flower 235
pistil 25
carpel 8
ovary 6
ovule 2
stigma 3
petal 6
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anther 32
pollen 32
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pedicel 2
silique 32
replum 6
seed 222
embryo 21
endosperm 39
micropylar endosperm 8
peripheral endosperm 10
chalazal endosperm 10
testa (seed coat) 21
general seed coat 6
chalazal seed coat 11
suspensor 2
stem 79
developing meristemoid zone 3
node 3
shoot apex 161
cauline leaf 3
rosette 1812
juvenile leaf 178
adult leaf 891
petiole 12
senescent leaf 3
hypocotyl 39
xylem 9
cork 8
leaf primordia 3
stem 3
axillary bud 2
axillary shoot 2
shoot apex 14
roots 537
primary root 58
root tip 28
meristematic zone 5
elongation zone 14
root hair zone 2
endodermis 2
endodermis+cortex 2
epid. atrichoblasts 2
stele 8
pericycle 5
lateral root 10
root tip 3
root cap 3
0 20000 40000 60000 80000 100000 120000

Figure 3-6 Gene expression map of *AtCb5* family genes obtained using Genevestigator

(A)Scatterplot map showing the levels of gene expression throughout Arabidopsis development. (B) Scatterplot map showing the levels of gene expression in different Arabidopsis tissues. The Meta Analyzer tool of the Genevestigator software was queried with AGI codes of 5 *AtCb5* genes.

Protein-protein interaction between RTE1 and all the AtCb5 isoforms in yeast

Since AtCb5 isoforms share a high degree of sequence similarity and 4 out of 5 isoforms could have the same localization with RTE1 and ETR1 (Dong et al., 2008), it suggests that other AtCb5 isoforms besides AtCb5-D could also interact with RTE1. I examined this possibility using the yeast split-ubiquitin assay. As shown in **Figure 3-7**, all five AtCb5 isoforms interacted with RTE1. AtCb5-B, -C and –E showed the strongest interaction with RTE1, whereas AtCb5-A had the weakest interaction with RTE1. LTP, the other putative RTE1-interacting protein that I isolated from the library screen, did not show as strong an interaction with RTE1 as AtCb5-B, -C and -E.

***etr1-2* ethylene insensitivity is partially dependent on *AtCb5*s**

In order to investigate the biological relevance of the interactions between AtCb5s and RTE1, I examined whether *AtCb5*s play a role in ethylene signaling similar to *RTE1* by analyzing their mutant phenotypes. I obtained T-DNA insertion lines for *AtCb5-D* (from GABI-KAT) and *AtCb5-B* and *-C* (from The Salk Institute). Both *atcb5-b* (Salk_100161) and *atcb5-c* (Salk_027748) carry a T-DNA in the third exon of the *AtCb5-B* and *AtCb5-C* coding sequences, respectively (**Figure 3-8 A**). *atcb5-d* (N376665) carries a T-DNA insertion in the second exon of the *AtCb5-D* coding sequence (**Figure 3-8 A**). An RT-PCR analysis of *atcb5* T-DNA insertion mutants showed that *AtCb5-B* transcript levels are substantially decreased, whereas both full length *AtCb5-C* and *–D* transcripts are eliminated although the transcript fragments upstream of the T-DNA insertion are still present (**Figure 3-8 B**). This

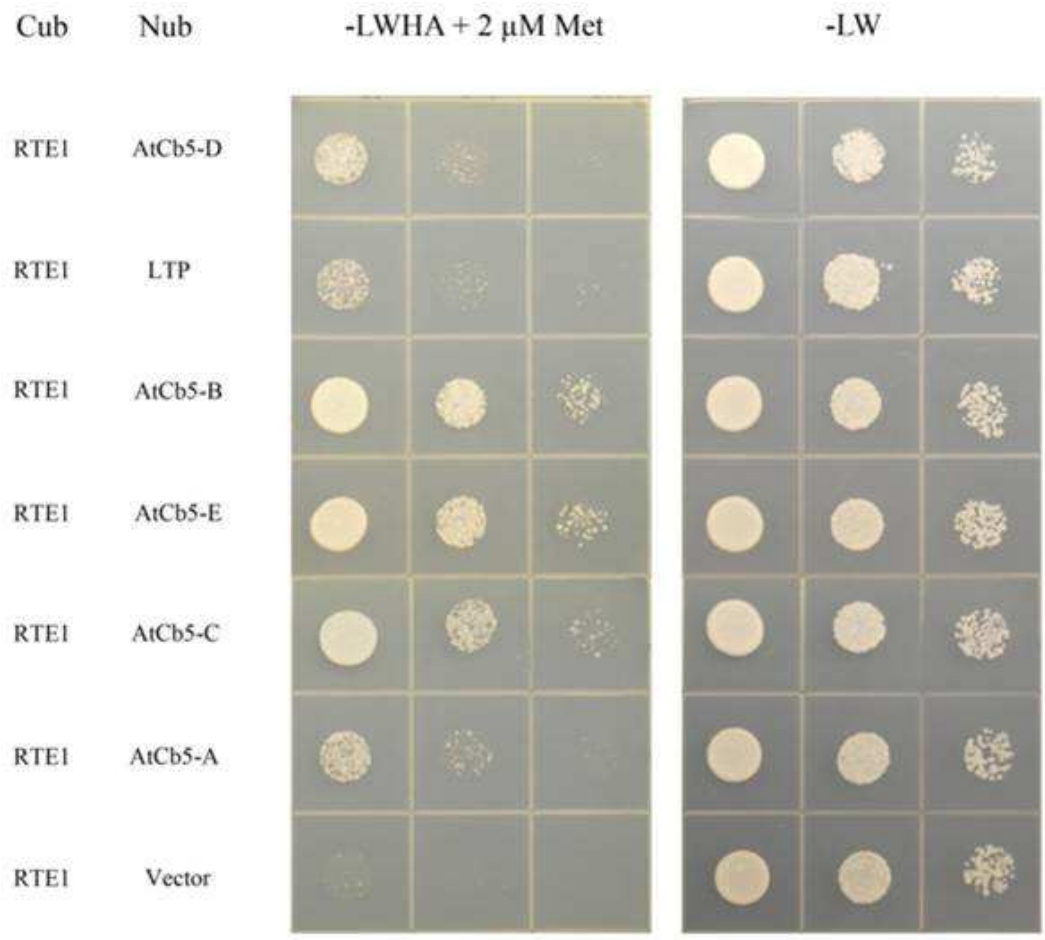


Figure 3-7 Yeast split ubiquitin assay of the interaction between RTE1 and AtCb5 family proteins as well as LTP

The full length RTE1 bait protein (fused with Cub) interacts with prey clones AtCb5-A (At1g26340), AtCb5-B (At2g32720), AtCb5-C (At2g46650), AtCb5-D (At5g48810), AtCb5-E (At5g53560) and Lipid Transfer Protein (LTP, At1g48750) (fused with Nub). Schematic structure of both bait and prey protein are shown in Figure 3-3 (A). Yeast cells were spotted onto agar medium from 10-fold serial dilutions of liquid overnight cultures, and then grown at 30℃ for 3 days. Medium lacking leucine and tryptophan (-LW) selects for the Cub and Nub plasmids,

respectively. Protein interactions are indicated by growth on medium additionally lacking histidine and adenine (-LWHA). Methionine was used to control the level of the RTE1 fusion protein, which was expressed under a methionine repressible promoter.

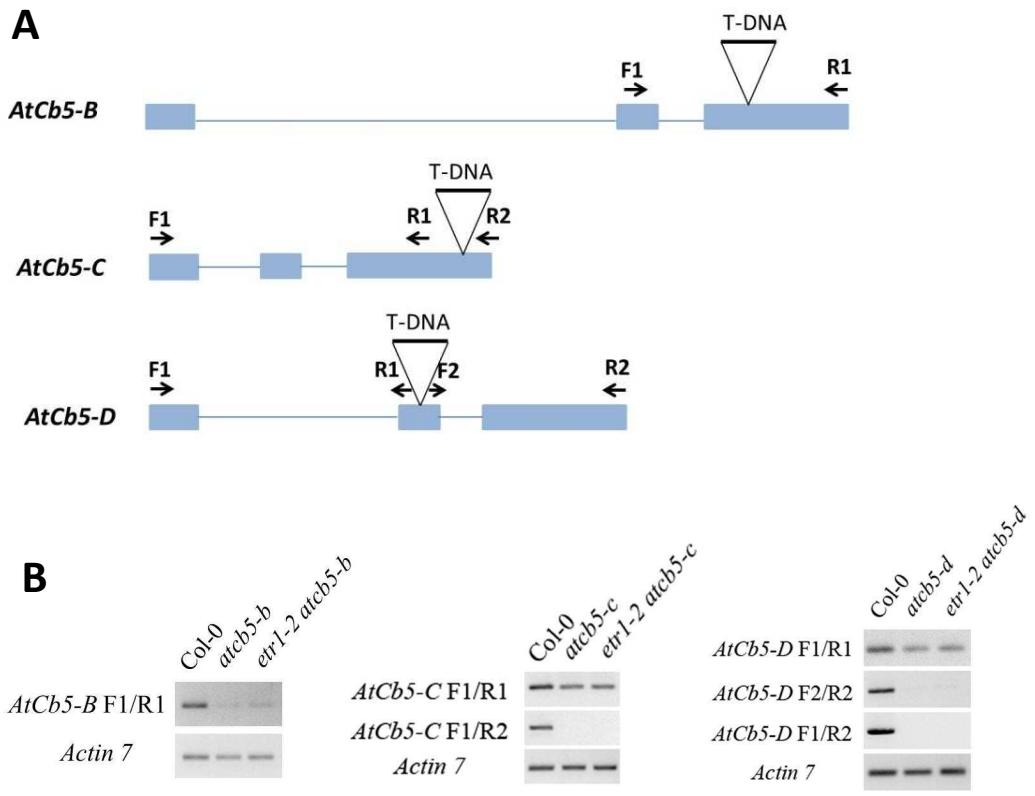


Figure 3-8 *atcb5-b, -c, -d* T-DNA insertion mutants

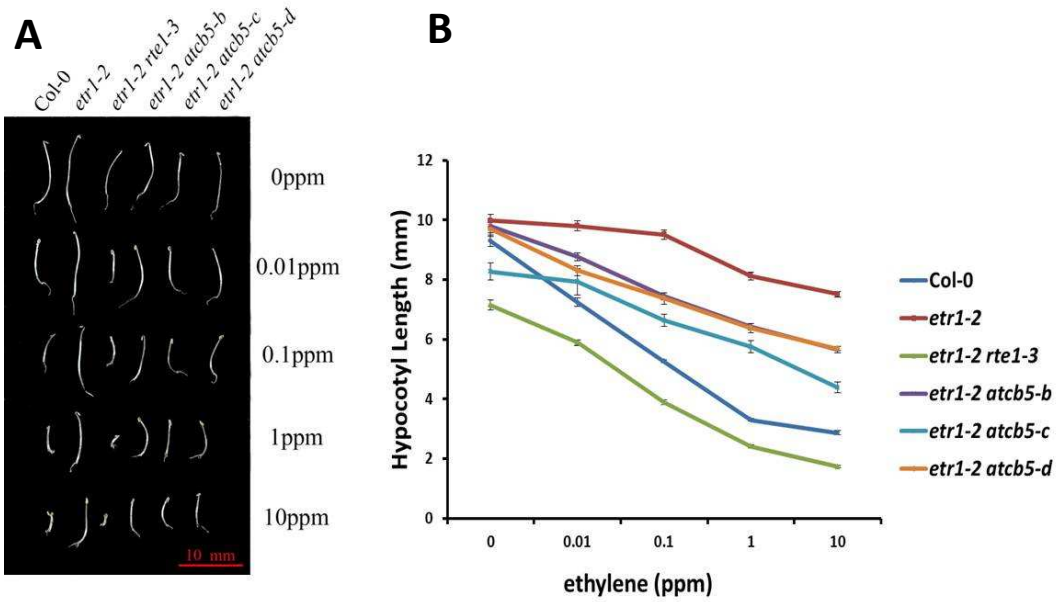
(A). *atcb5-b* and *atcb5-c* mutations are a SALK-derived T-DNA insertion in the 3rd exon of both genes. The *atcb5-d* mutation is a GABI-Kat-derived T-DNA insertion in the 2nd exon of the gene.

(B). RT-PCR showed that transcripts levels are significantly reduced in all the 3 mutants background compared to Col-0 wild-type. RNA levels of *Actin 7* are shown as a loading control. The positions of *AtCb5* isoforms primers used for the RT-PCR are indicated in (A) by black, half arrows. The cDNA was prepared from RNA of 1-2 3-week-old rosette leaves per sample.

suggests that both *atcb5-c* and *atcb5-d* are true loss-of-function alleles and *atcb5-b* is a partial loss-of-function allele.

All mutant *atcb5-b, -c* and-*d* alleles reduced the ethylene insensitivity exhibited by *etr1-2*. This suppression is apparent in the seedling triple response (**Figure 3-9 A**) and in an ethylene dose-response analysis (**Figure 3-9 B**). However, the hypocotyl and root appear to be longer than the wild type and *etr1-2 rte1-3* double mutant under almost all concentrations of ethylene. In adults, *etr1-2* insensitive mutants are resistant to the ethylene-induced senescence that is seen in wild-type plants exposed to ethylene for 3 days. However, all mutant *atcb5-b, -c* and *-d* alleles partially suppressed the *etr1-2* ethylene insensitivity in terms of ethylene-induced leaf senescence, as the mutant lines exhibit signs of leaf senescence after 3-day exposure to ethylene (**Figure 3-9 C**).

In order to confirm that the *atcb5-d* mutation partially suppresses *etr1-2* ethylene insensitivity, I complemented the suppressed phenotype of the *etr1-2 atcb5-d* double mutant with both a 3.27 kb genomic DNA fragment containing the *AtCb5-D* gene (with native promoter) and an *AtCb5-D* cDNA driven by the CaMV 35S promoter. The genomic fragment encompassed a region of 1.9 kb upstream of the 5' UTR plus the entire region of *AtCb5-D* gene from the 5' UTR to the end of the 3'UTR. Transformed *etr1-2 atcb5-d* seedlings expressing either the *AtCb5-D* genomic DNA or the cDNA transgenes exhibited ethylene insensitivity similar to that seen in *etr1-2* seedlings (**Figure 3-10**).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1ppm
10ppm
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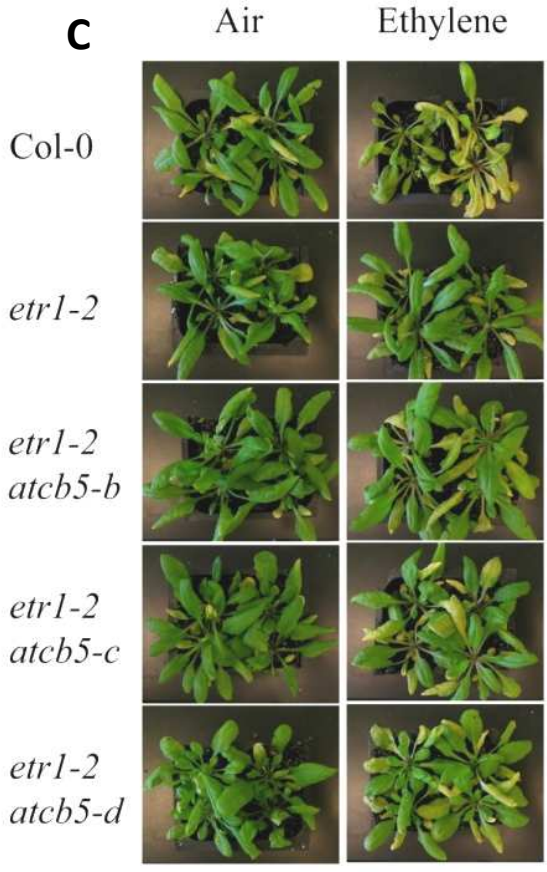

C
Air
Ethylene
Col-0
etr1-2
etr1-2 atcb5-b
etr1-2 atcb5-c
etr1-2 atcb5-d

Figure 3-9 *atcb5* family mutant alleles suppress *etr1-2* ethylene insensitivity to a lesser degree compared with the *rte1-3* null mutant

(A). Analysis of 4-day-old dark-grown etiolated seedlings grown in the presence of different doses of ethylene gas. Representative seedlings of three *atcb5* suppressor lines (*etr1-2 atcb5-b*, *etr1-2 atcb5-c* and *etr1-2 atcb5-d*) are compared with *rte1* suppressor line (*etr1-2 rte1-3*), wild-type Col-0 and the *etr1-2* ethylene insensitive mutant. (B). Ethylene dose-response analysis of hypocotyl length of 4-day-old dark-grown etiolated seedlings. About 20-30 seedlings were measured and mean ± standard error is shown per genotype at each dose. (C) *atcb5* family mutant alleles suppress *etr1-2* ethylene insensitivity to some degree in terms of ethylene-induced leaf senescence (observed as yellowing of the leaves) in ~6-week-old plants treated with or without 100 ppm ethylene for 3 days in a dark airtight chamber.

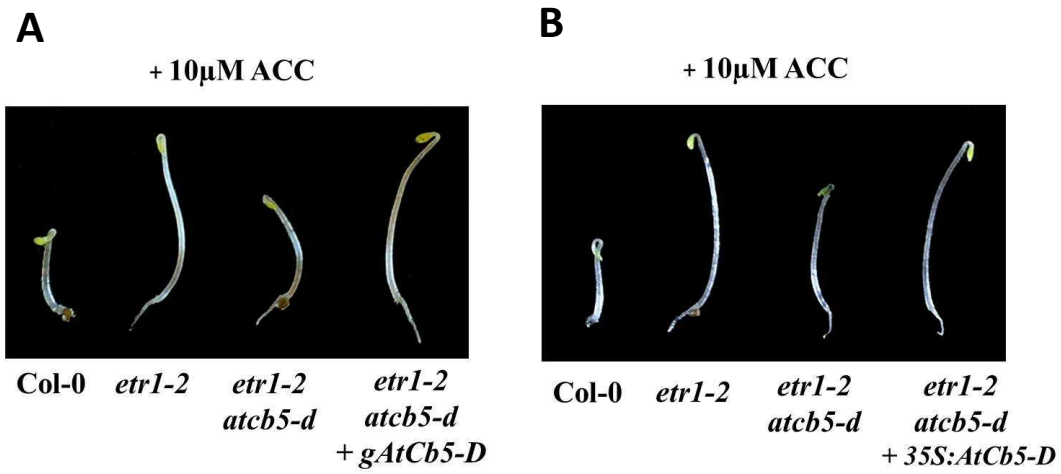


Figure 3-10 Rescue of the *etr1-2 atcb5-d* mutant phenotype, using both an *AtCb5-D* genomic DNA fragment and an *AtCb5-D* cDNA driven by the CaMV 35S promoter

(A) *etr1-2 atcb5-d* mutant phenotype is rescued by a wild-type 3.27 kb genomic DNA fragment 'gAtCb5-D', which incorporates the entire *AtCb5-D* coding region and about 1.9k promoter region. (B) *etr1-2 atcb5-d* mutant phenotype is also rescued by an *AtCb5-D* cDNA driven by the CaMV 35S promoter. Seedlings were grown on MS medium containing 10 μM ACC for 4 days in dark.

Altogether, these results suggest that *AtCb5-B, -C*, and *–D* are partially required by *etr1-2* to confer ethylene insensitivity in both seedling and adult stages. Like *RTE1*, these cytochrome b5 genes could be negative regulators of ethylene response.

AtCb5-D overexpression confers a slight insensitivity to ethylene

The *AtCb5-D* cDNA driven by CaMV 35S promoter was able to complement the suppressed phenotype of the *etr1-2 atcb5-d* double mutant (**Figure 3-10 B**), suggesting this AtCb5-D overexpression construct was functional. When transformed into wild-type plants, this same construct conferred a slight ethylene insensitivity, similar to the phenotype conferred by overexpression of *RTE1* (**Figure 3-11**). RT-PCR analysis of these transgene plants confirmed that *AtCb5-D* is over expressed in these lines (**Figure 3-11 C**). Since the gain-of- *AtCb5-D*-function phenotype is opposite that of the loss-of-function, these results are consistent with *AtCb5-D* being a negative regulator of ethylene response.

atcb5-b/c double and *atcb5-b/d* double mutants are hypersensitive to ethylene

Since the *AtCb5* isoforms share high degree of sequence similarity, there could be functional redundancy among them. In fact I found that all *atcb5-b, -c* and *-d* single mutants display a similar ethylene response phenotype as the wild type. To further examine their effect on ethylene signaling, I made *atcb5-b/c* double and *atcb5-b/d* double mutants. Unlike the single mutants, the hypocotyl of both double mutants was slightly shorter than the wild type in the presence of a low dose of ACC (0.5µM) (**Figure 3-12 A, B**), but neither was as short as the *rte1-3* mutant. In addition, like *rte1-3* and *etr1-7*, both *atcb5-b/c* and *atcb5-b/d* displayed a slightly shorter

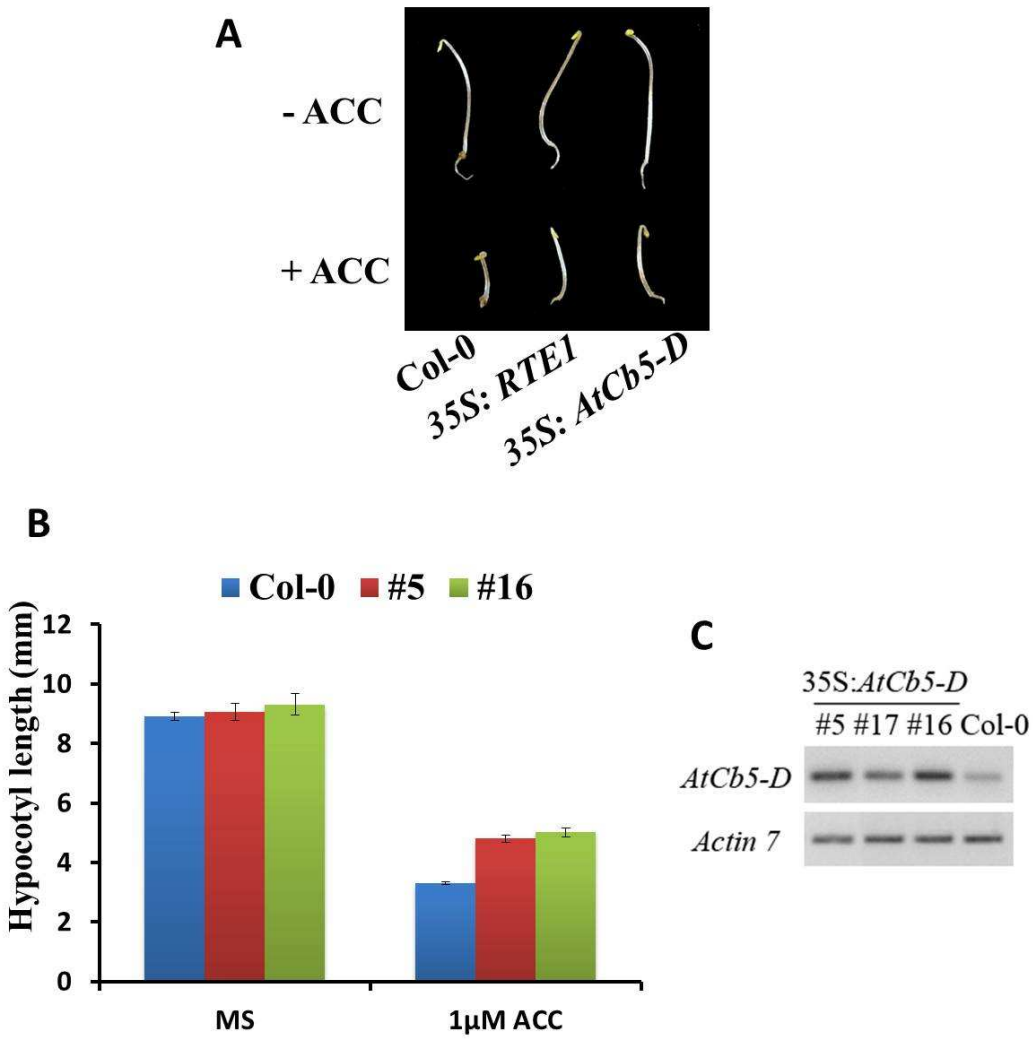
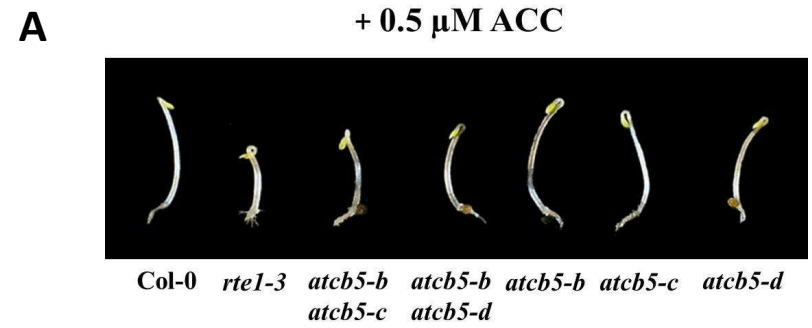
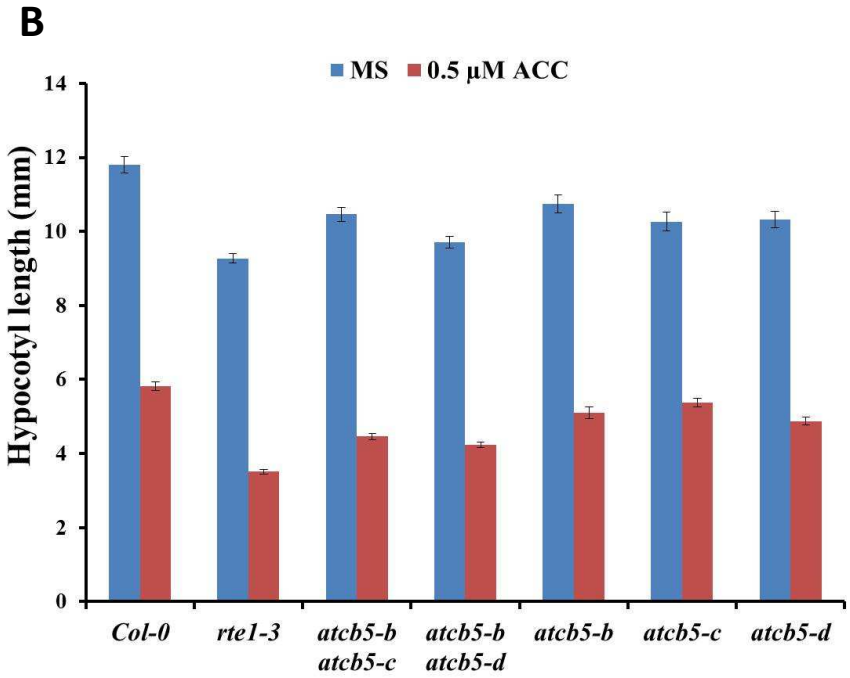


Figure 3-11 Overexpression of *AtCb5-D* confers weak ethylene insensitivity

(A). Representative four-day-old dark grown seedlings of wild type Col-0, an *RTE1* overexpression transgenic line from Resnick et al. (2006), and an *AtCb5-D* overexpression transgenic line (#5), germinated in the presence or absence of 1μM ACC. (B). Measurements of hypocotyl length for wildtype (Col-0) seedlings either untransformed or transformed with *AtCb5-D* over-expression construct in the presence or absence of 1μM ACC. The mean±SE is shown for 15-27 seedlings

measured for wild type (Col-0) and two independent transgenic lines (#5 and #16) at MS and 1µM ACC. (C). RT-PCR showing that *AtCb5-D* is over-expressed in wild-type transgenic lines #5, #17 and #16. Top panel is RT-PCR using *AtCb5-D*-specific primers while the bottom panel is product using *Actin 7*-specific primers showing that all samples had similar levels of RNA. The RNA was prepared from about 1-2 3-week-old rosette leaves per sample.


A
+ 0.5 µM ACC
Col-0
rte1-3
atcb5-b atcb5-c
atcb5-b atcb5-d
atcb5-b
atcb5-c
atcb5-d


B
MS
0.5 µM ACC
Hypocotyl length (mm)
0
2
4
6
8
10
12
14
Col-0
rte1-3
atcb5-b atcb5-c
atcb5-b atcb5-d
atcb5-b
atcb5-c
atcb5-d

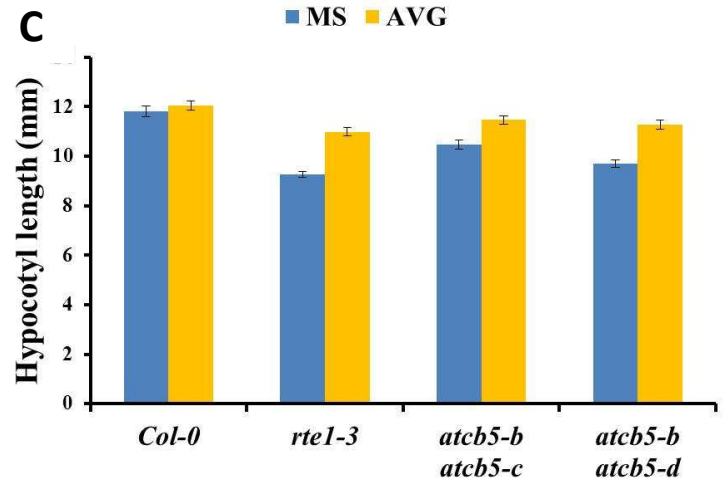

C
MS
AVG
Hypocotyl length (mm)
0
2
4
6
8
10
12
Col-0
rte1-3
atcb5-b atcb5-c
atcb5-b atcb5-d

Figure 3-12 Loss of multiple *AtCb5* isoforms function displays enhanced ethylene sensitivity

(A) Comparison of four-day old dark grown Arabidopsis seedlings grown on 0.5µM ACC. *re1-3, atcb5-b/c* double and *atcb5-c/d* double mutant seedlings exhibit enhanced ethylene sensitivity compared to the wild type and *atcb5-b, c, d* single mutants. (B) Measurement of hypocotyl length for the four-day old dark grown seedlings of indicated genotypes grown on 0.5µM ACC. The mean±SE is shown for 16-33 seedlings measured for each genotype at MS and 0.5µM ACC. (C) The hypocotyl shortening of *rte1-3, atcb5-b/c* double and *atcb5-c/d* double mutant seedlings in the absence of ACC is alleviated by treatment with the ethylene biosynthesis inhibitor 1-aminoethoxyvinylglycine (AVG). The mean±SE is shown for 18-24 seedlings measured for each genotype at MS and 10µM AVG.

hypocotyl in the absence of ethylene than that of the wild type. *rte1-3* and *etr1-7* are known to be shorter than the wild type in the absence of ethylene or ACC (Resnick et al., 2006), due to an enhanced response to endogenous ethylene, since when grown in the presence of the ethylene biosynthesis inhibitor AVG, the hypocotyl shortening is largely alleviated (Cancel and Larsen, 2002). When *atcb5-b/c* and *atcb5-b/d* were grown on 10μM AVG, hypocotyl shortening was alleviated (**Figure 3-12 C)**, suggesting that *atcb5-b/c* and *atcb5-b/d* double mutants are also sensitive to some extent to endogenously produced ethylene similar to *rte1-3* and *etr1-7*.

atcb5-d is unable to suppress other ethylene insensitive receptor mutants

Since the *atcb5-d* loss-of-function mutant can suppress *etr1-2* ethylene insensitivity, I next tested whether the *atcb5-d* loss-of-function mutant could suppress other dominant ethylene insensitive receptor mutants. The *ers1-10* and *etr2-1* mutants were chosen to represent the receptor subfamily I and II dominant ethylene insensitive alleles. *atcb5-d* was unable to suppress *etr2-1* since the *etr2-1 atcb5-d* mutant retained the ethylene insensitivity under all doses of ethylene just like the *etr2-1* single mutant **(Figure 3-13 A).** Interestingly, *atcb5-d* didn't suppress the *ers1-10* mutant either, which is one of the weakest ethylene receptor gain-of-function alleles (Alonso et al., 2003) (**Figure 3-13 B**). Therefore, it appears that the suppression by *atcb5-d* loss-of-function mutation is specific to only *etr1* receptor dominant insensitive alleles, similar to the *rte1* mutant.

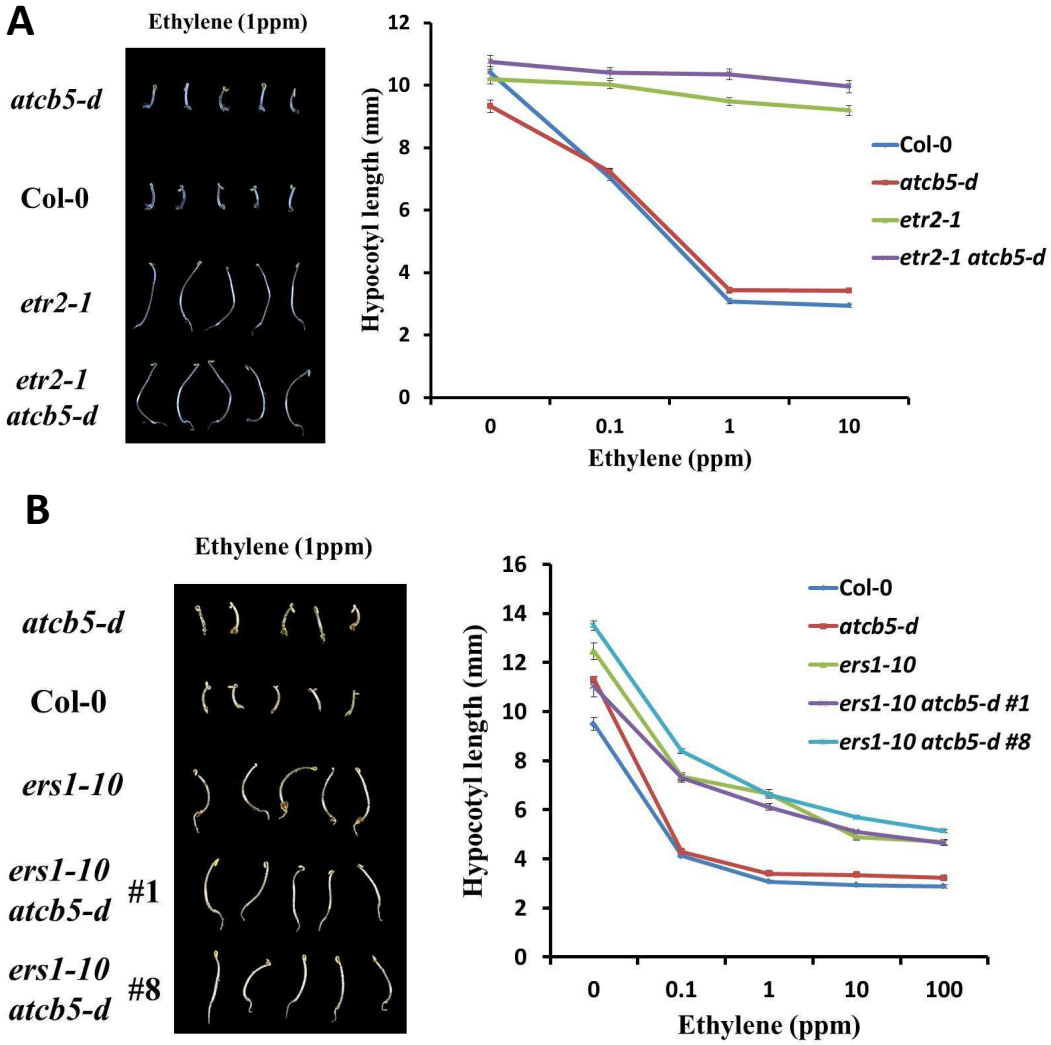


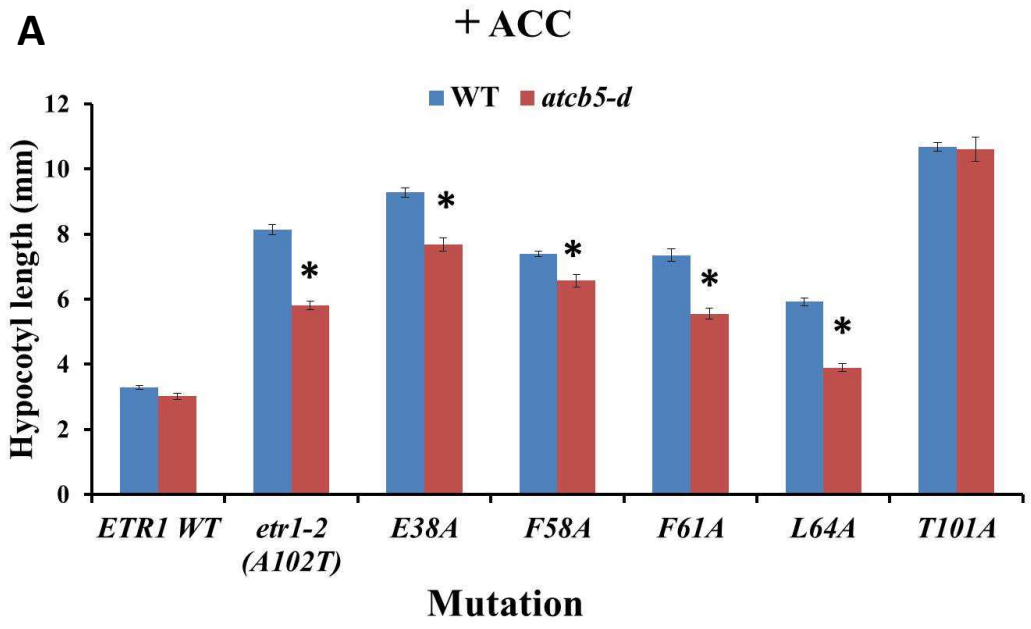
Figure 3-13 *atcb5-d* is unable to suppress other insensitive mutants

(A) *atcb5-d* does not suppress the insensitivity of *etr2-1*, which is a gain-of-function insensitive mutation in the *ETR2* receptor gene. (B) *atcb5-d* does not suppress the insensitivity of *ers1-10*, which is a weak gain-of-function insensitive mutation in the *ERS1* receptor gene. For each graph, ethylene dose-response analysis of hypocotyl length of 4-day-old dark-grown etiolated seedlings was performed. About 15-25 seedlings were measured and the mean ± standard error is shown per genotype at each dose.

atcb5-d can suppress *etr1* dominant ethylene insensitive alleles in a fashion similar to that of *rte1*

Based on above results, we found that *atcb5* loss-of-function mutants exhibited phenotypes in ethylene responses that parallel those of the *rte1* mutant. To further evaluate whether *AtCb5*s behave like *RTE1*, I examined the ability of the *atcb5-d* loss-of-function mutation to suppress additional *etr1* dominant ethylene insensitive alleles. Resnick *et al*. revealed that loss of *RTE1* function is able to suppress many but not all *etr1* dominant ethylene insensitive mutations, though the biochemical basis for the specificity of suppression is unknown (Resnick et al. 2008). If AtCb5s associate with and have a similar role as RTE1, then the *atcb5-d* mutant should suppress the same dominant mutants as *rte1*. I tested this with five existing *etr1* mutant transgenes, each carrying an amino acid substitution that was created through *in vitro* site-directed mutagenesis and is known to confer dominant ethylene insensitivity (Wang et al., 2006); four (E38A, F58A, F61A, L64A) are dependent on *RTE1* for ethylene insensitivity, and one (T101A) is *RTE1*-independent (Resnick et al., 2008; Wang et al., 2006).

I crossed *atcb5-d* with each of the five *etr1* ethylene insensitive transgenic lines. The progeny seedlings homozygous for both *atcb5-d* and the *etr1* mutant transgene were compared with seedlings of the corresponding *etr1* mutant transgenic lines in the presence and absence of 20 µM ACC. *atcb5-d* was able to partially suppress all the four additional *RTE1* dependent insensitive *etr1* mutant transgene ($p < 0.001$) and was unable to suppress the *RTE1*-independent insensitive *etr1* mutant transgene (T101A) (**Figure 3-14**) (**Table II**). Therefore, *atcb5-d* shares the same pattern of


A
+ ACC
WT
atcb5-d
Hypocotyl length (mm)
0
2
4
6
8
10
12
*
*
*
*
ETR1 WT
etr1-2 (A102T)
E38A
F58A
F61A
L64A
T101A
Mutation

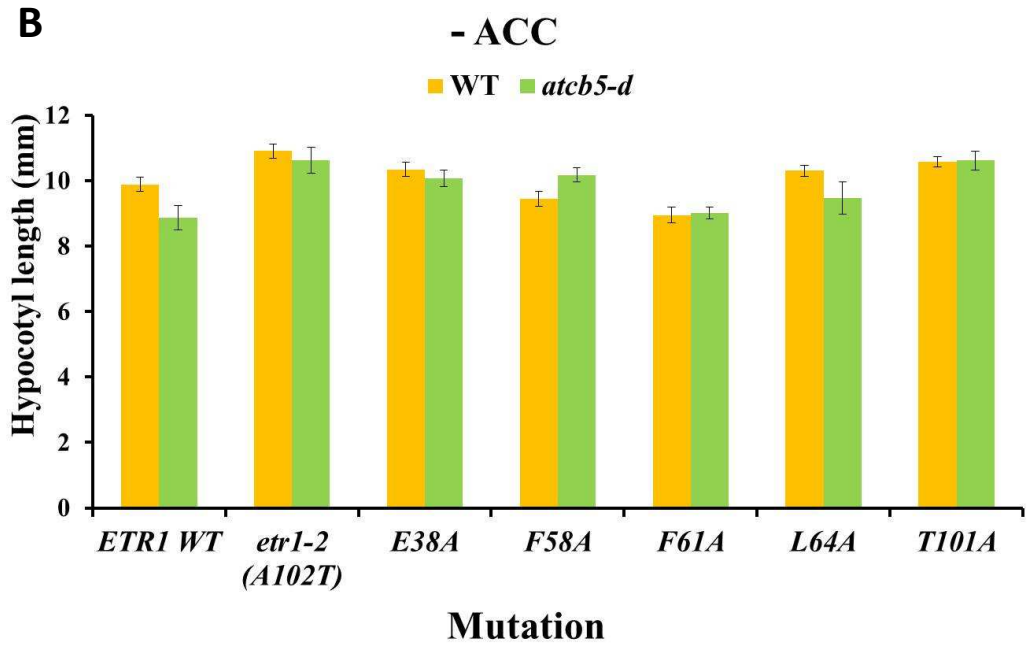

B
- ACC
WT
atcb5-d
Hypocotyl length (mm)
0
2
4
6
8
10
12
ETR1 WT
etr1-2 (A102T)
E38A
F58A
F61A
L64A
T101A
Mutation

Figure 3-14 Effects of *atcb5-d* loss-of-function mutation on *etr1* dominant ethylene insensitive mutant transgenes

Measurements of hypocotyl length of the mutant *etr1* transgenes seedlings with or without *atcb5-d* loss-of-function mutation in the presence (A) and absence (B) of 20 µM ACC. asterisks indicate the significance difference in hypocotyl length between the *etr1* mutant transgene with wild-type *AtCb5-D* and the corresponding *etr1* transgene with *atcb5-d* loss-of-function mutation ($p<0.001$). Hypocotyl length of four-day old dark-grown seedlings homozygous for either *etr1* transgenes only or both *etr1* transgene and *atcb5-d* mutation were measured. The mean ± SE is shown for 15-29 seedlings per genotype under 20µM ACC or MS.

Table II Comparison of the ability of *rte1-2* and *atcb5-d* to suppress a variety of dominant *etr1* mutant alleles

Mutation	Suppressed by *rte1* ? [a]	Suppressed by *atcb5-d* ? (%) [b]
etr1-2 (A102T)	Yes	Yes (71.4%)[c]
E38A	Yes	Yes (82.8%)[c]
F58A	Yes	Yes (88.8%)[c]
F61A	Yes	Yes (75.6%)[c]
L64A	Yes	Yes (65.9%)[c]
T101A	No	No (99.5%)

[a]Results are from Resnick et al. (Resnick et al., 2008).

[b]Values are the percentage hypocotyl length of *etr1* mutant transgene crossed with *atcb5-d* with respect to that of *etr1*transgene only on 20 µM ACC (Figure 3-14 A).

[c]The means of hypocotyl length of *etr1* mutant transgene *atcb5-d* double homozygotes and *etr1* mutant transgene in the wild type background on 20 µM ACC are significantly different ($p < 0.001$) (Figure 3-14 A).

suppression of *etr1* dominant ethylene insensitive alleles as *rte1*, suggesting *AtCb5* may have the same function as *RTE1* in regulating the ETR1 receptor.

AtCb5 and *RTE1* could be in the same pathway

To examine the genetic relationship between *RTE1* and *AtCb5* further, next I wanted to assess whether *rte1* and *atcb5* have synergistic or additive effects on the ethylene response when combined. *rte1* loss-of-function mutations revert the insensitivity exhibited by *etr1-2* to a phenotype comparable to that of the wild type. *atcb5* loss-of-function mutants also make *etr1-2* shorter although not as short as the wild type. I constructed *etr1-2 rte1-3 atcb5-d* triple mutant and compared its phenotype to the *etr1-2 rte1-3* and *etr1-2 atcb5-d* double mutants. The *etr1-2 rte1-3 atcb5-d* triple mutant displayed the same phenotype as the *etr1-2 rte1-3* double mutant (**Figure 3-15 A**), indicating that the *rte1-3* and *atcb5-d* loss-of-function mutations do not have an additive or a synergistic effect. Similarly, the *rte1-3 atcb5-d* double mutant has a phenotype in ethylene response that is the same as the *rte1-3* single mutant (**Figure 3-15 B**). In the ethylene dose response assay, *atcb5-d rte1-3* double mutant is indistinguishable with the *rte1-3* mutant (**Figure 3-15 B**). These results are consistent with the possibility that *RTE1* and *AtCb5-D* act in the same pathway.

As described before, both overexpression of *RTE1* and *AtCb5-D* exhibit a low level of ethylene insensitivity. To test whether RTE1 function is dependent on *AtCb5-D,* the *atcb5-d* loss-of-function mutant was transformed with the *RTE1* over-expression transgene to see if the ethylene insensitivity phenotype conferred by over-

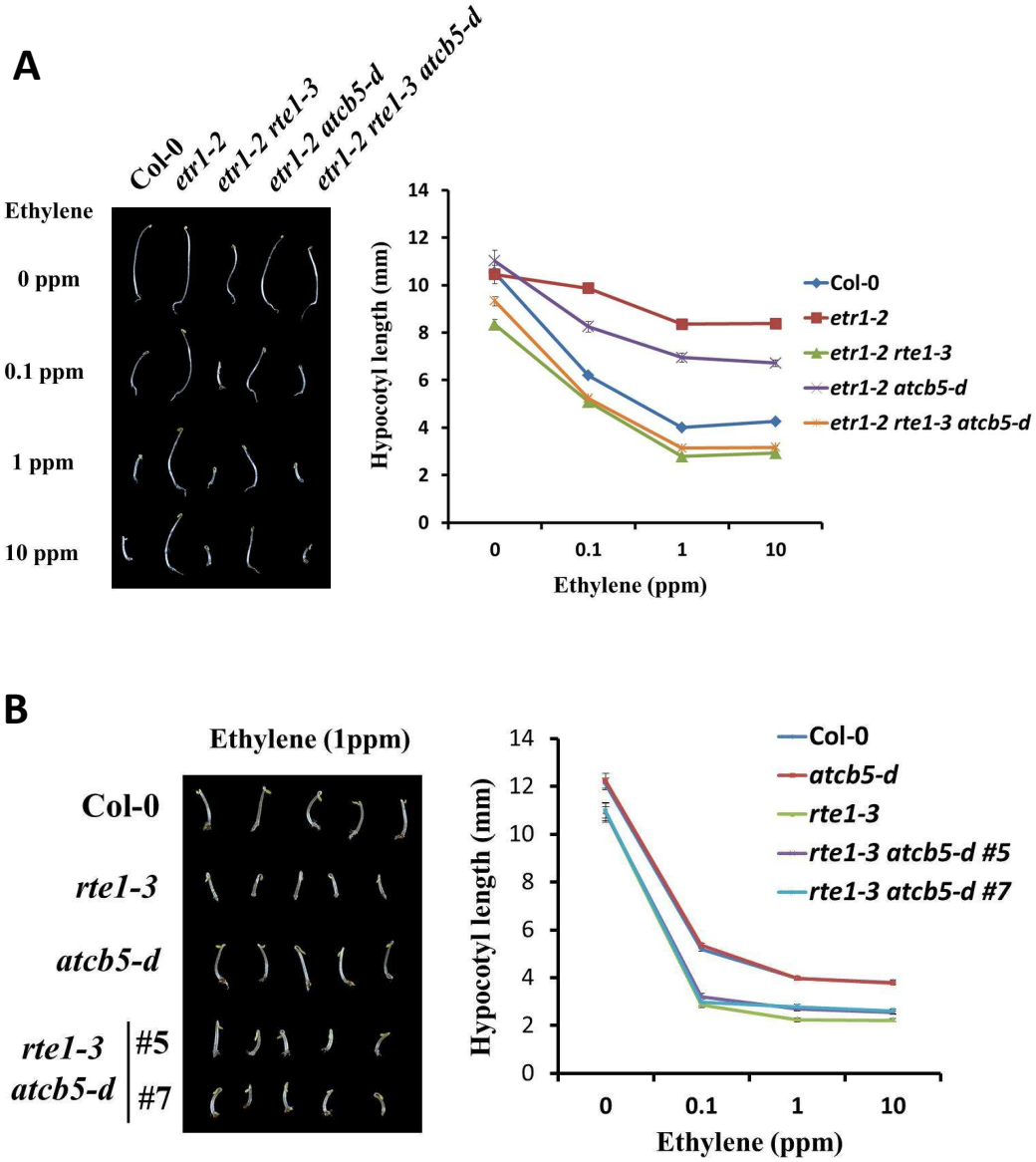


Figure 3-15 *atcb5-d* loss-of-function mutation does not enhance the ethylene sensitivity conferred by *rte1-2* mutation, suggesting *AtCb5-D* may act in the same pathway as *RTE1*

(A) Ethylene dose-response of hypocotyl length in 4-day-old dark grown seedlings indicates that the *etr1-2 rte1-3 atcb5-d* triple mutant does not exhibit an enhanced

phenotype in comparison to *etr1-2 rte1-3* double and *etr1-2 atcb5-d* double mutant. The mean ± SE is shown for 13-30 seedlings per genotype at each dose. (B) Ethylene dose-response of hypocotyl length in 4-day-old dark grown seedlings shows the similarity of ethylene hypersensitivity in *rte1-3 atcb5-d* double mutant and *rte1-3* mutant, in contrast to *atcb5-d* mutant and the wild-type (Col-0). The mean ± SE is shown for 13-26 seedlings per genotype at each dose.

expressing *RTE1* is blocked. 2 out of 2 independent *RTE1* over expression transgenic lines showed no suppression by the *atcb5-d* mutation (**Figure 3-16**). Although the two genes are likely in the same pathway, it is still unclear whether *AtCb5* acts upstream of *RTE1* based on the current data. Since *AtCb5-B* and *AtCb5-C* also affect ethylene signaling, the possibility that *RTE1* is an upstream regulator of several downstream *AtCb5* isoforms cannot be ruled out.

Discussion

In order to gain some possible insights to the molecular function of RTE1, I carried out a screen for RTE1-interacting proteins and isolated an ER-localized cytochrome b5 (AtCb5-D) and an ns-LTP. Because the T-DNA insertion lines for several *AtCb5* family members were available, I performed detailed studies on AtCb5 to investigate the role of AtCb5 in connection to RTE1 function.

Firstly, the interactions of cytochrome b5 family proteins with RTE1 were tested in yeast. The interaction of AtCb5-D was also seen with the RTE1 homolog (RTH), but not detected with two bait versions of a cation transporter CHX20, suggesting AtCb5-D could interact with RTE family proteins specifically. All the five Arabidopsis cytochrome b5 isoforms interact with RTE1 in yeast, but only AtCb5-B, C, D, E interact with RTE1 under high stringency. Since AtCb5-D is and AtCb5-B, C, E are predicted to be localized in the ER membrane where RTE1 and ETR1 reside, the protein interaction between RTE1 and AtCb5-B, C, D, E in yeast could be occurring in planta. The weak interaction between RTE1 and chloroplast localized

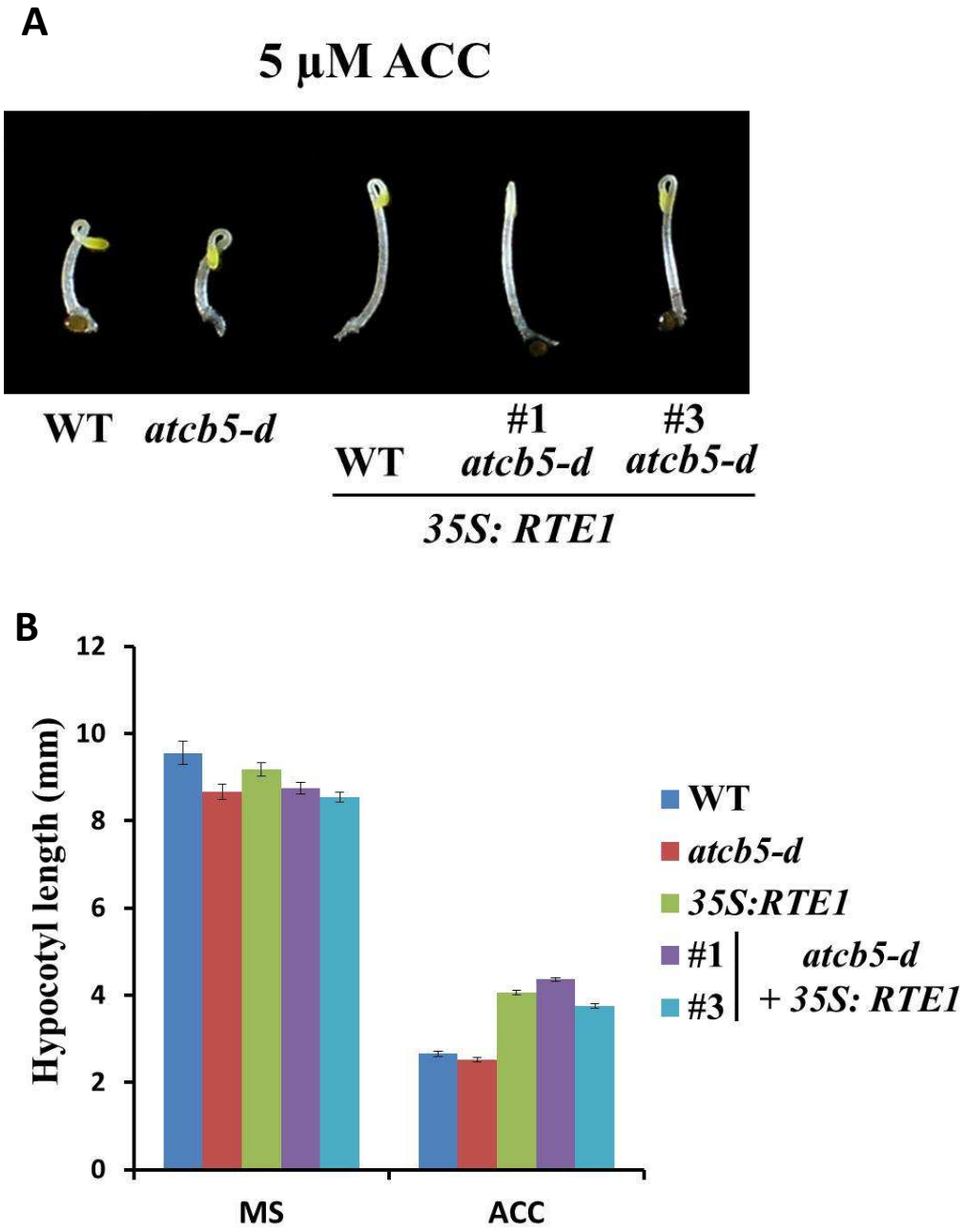


Figure 3-16 The *atcb5-d* loss-of-function mutation does not block the reduced ethylene sensitivity conferred by *RTE1* over-expression

(A) Representative etiolated seedlings of *atcb5-d* and the wild-type (Col-0) either untransformed (-) or transformed (+) with *35S: RTE1*, grown in the dark for 4 days in

the presence or absence of 5µM ACC. (B) Measurements of hypocotyl length for the wild type (Col-0), *atcb5-d*, the *35S: RTE1* transgene line in the wild-type background, and two independent transgene lines (#1 and #3) transformed with *35S: RTE1* construct in the *atcb5-d* background, grown in the presence or absence of 5µM ACC. The mean ± SE is shown for 20-38 seedlings per line for each treatment.

AtCb5-A could be due to the mislocalization of AtCb5-A in ER in yeast. Hwang *et* al. found that the mitochondria localized Tung (*Aleurites fordii*) cytochrome b5 isoform can stimulate the exclusively ER localized fatty acid desaturase 2 (FAD2) activity in yeast, suggesting it could be mislocalized to ER in yeast cells, which is probably due to the different sorting mechanism of cytochrome b5 in plant and yeast (Hwang et al., 2004). The physical interaction in planta needs to be confirmed by using Bimolecular Fluorescence Complementation (BiFC) and co-immuoprecipitation (Co-IP), which are in progress.

To justify the biological relevance of these interactions, using the available T-DNA insertion mutants for *AtCb5-B, C, D,* I performed genetic analyses on the phenotypes of these mutants in ethylene response. Interestingly, the results suggest functional parallels of AtCb5 with RTE1 in ethylene signaling. Firstly, similar to RTE1, both wild type ETR1 and *etr1-2* partially depend on AtCb5 in order to be functional. Due to functional redundancy among AtCb5 isoforms, the single *atcb5-b, c, d* loss-of-function mutant does not revert the ethylene insensitivity exhibited by *etr1-2* to a phenotype comparable to that of the wild-type as *rte1* does, and itself does not exhibit significant ethylene response phenotype. However, losing two *AtCb5* family members show enhanced ethylene sensitivity, although not as hypersensitive as *rte1-3*. Therefore, I could predict a more severe phenotype if losing more *AtCb5* family members. Secondly, overexpression of *AtCb5-D* displays weak ethylene insensitivity, just like over-expressing *RTE1*. The opposite phenotype between overexpressing *AtCb5* and loss-of-*AtCb5* function confirms a negative role of *AtCb5* in ethylene signaling. Thirdly, *AtCb5*s show the same specificity for the ETR1

receptor and certain *etr1* dominant mutant alleles as *RTE1*, suggesting *AtCb5* could play a similar role as *RTE1* in regulating the ETR1 EBD conformation.

Since *atcb5* mutants have phenotypes in ethylene responses that parallel those of the *rte1* mutant, *AtCb5* and *RTE1* could be in the same pathway that regulates the ETR1 receptor. Consistent with this speculation, the simultaneous loss of both *RTE1* and *AtCb5-D* has no obvious additive or synergistic effect on ethylene response compared to single mutants. The order of action for RTE1 and AtCb5 remains unclear. I favor a model in which *AtCb5*s act upstream of *RTE1* based on the finding that the *atcb5-d* loss-of-function mutation cannot block the reduced ethylene sensitivity conferred by overexpressing *RTE1*, although I cannot rule out that *RTE1* is upstream of *AtCb5* and RTE1 can signal through other AtCb5 isoforms when one member is gone, as AtCb5 family members function redundantly. The coming lines of *35S: AtCb5-D* transgene crossed with the *rte1-3* mutant will help to understand the order of action of AtCb5 and RTE1.

The genetic analysis of *AtCb5* places it in the ethylene signaling pathway. It may interact with RTE1 and play a similar role as *RTE1* in positively regulating the ETR1 receptor. The established function of cytochrome b5 may provide insights into the function of RTE1. Cytochrome b5 is known to serve as an electron transfer protein in a number of oxidation/reduction reactions in biological tissues, including fatty acid desaturation (Shimakat.T et al., 1972), fatty acid elongation (Keyes et al., 1979), fatty acid hydroxylation (Kearns et al., 1991; Smith et al., 1992), and cytochrome P450 monooxygenations (Hildebra.A and Estabroo.Rw, 1971). In these reactions, cytochrome b5 can accept an electron either from NAPDH-cytochrome P450

reductase or NADH- cytochrome b5 reductase and transfer them to a variety of electron acceptor proteins such as cytochrome P450, desaturase, hydroxylase, monooxygenase and metmyoglobin and methemoglobin reductase (Jansson and Schenkman, 1977; Schenkman and Jansson, 2003). The cytochrome P450 superfamily, another hemoprotein, is a large group of mixed function oxidases which catalyze the oxidation of numerous molecules.

Since cytochrome b5 can carry out a variety of oxidation/reduction reactions, and redox modifies protein residues such as cysteine (Cys), thus affecting protein structure and precisely regulating protein function, I hypothesize that ETR1 receptor function could be regulated through redox modifications. It has been well known that Cys residues can be modified by a variety of redox-based, post-translational modifications such as S-nitrosylation, sulphenic acid formation, generation of disulphide bridges, S-glutathionylation and sulphinic acid and sulphonic acid formation, which exerts distinct effects on protein functions (Spadaro et al., 2010). There are three conserved cysteines (C4, C6, C65) within the ethylene binding domain of the ethylene receptors which could be the target of redox modification. RTE1 could be also involved in redox modification of its target protein ETR1 based on the genetic suggestion that RTE1 may function similarly as AtCb5. This would be consistent with our hypothesis that RTE1 may play a role in conformational changes of the ETR1 N-terminal transmembrane region that regulates the C-terminal signaling output.

Another possibility is that cytochrome b5 could affect the ER-residing ETR1 EBD conformation through changes in membrane fluidity, which may be a result of

the complex effects of cytochrome P450. It has been well established that cytochrome b5 plays a role in lipid biosynthesis and metabolism by transferring electrons to and thus activating a variety of oxidases such as desaturase and hydroxylase (Schenkman and Jansson, 2003; Vergeres and Waskell, 1995). Notably, the dependence of membrane fluidity on the extent of unsaturation of fatty acids in membrane lipids is a well characterized phenomenon. ETR1, as a membrane protein, may be highly sensitive to subtle membrane environmental changes in composition and fluidity. What is compatible with this possibility is that I also isolated a non-specific lipid transfer protein (ns-LTP) (At1g48750) from cDNA library screening for putative RTE1 interacting proteins using yeast split-ubiquitin assay. ns-LTPs are small, soluble, basic proteins found in animals, plants and microorganisms (Kader, 1996; Wirtz, 1997). They are generally thought to be involved in lipid metabolism, but their cellular roles are unknown (Lai et al., 2008; Wirtz, 1997). ns-LTP bind non-specifically to the acyl chains of fatty acids and can transfer single phospholipids, glycolipids, fatty acids and sterols between membranes in vitro, thus altering membrane lipid composition (Kader, 1996; Wirtz, 1997). The functions of plant ns-LTPs and their regulation are unknown. Arabidopsis has about 23 LTP genes in 3 distinct subfamilies (Arondel et al., 2000). At1g48750 is in a subfamily with two other homologs. At1g48750 is expressed throughout the plant during most stages. The biological relevance of the interaction between RTE1 and ns-LTP remains to be uncovered.

I favor the hypothesis that cytochrome b5 may regulate the ETR1 EBD conformation through redox modification because this would make possible the

specificity of *AtCb5* and *RTE1* for ETR1 receptor and certain *etr1* dominant mutant alleles. Though the basis for the specificity is not yet understood, conceivably there could be a distinct feature in ETR1 EBD which allows it to be the target of redox modification. In contrast, the membrane environmental changes could affect all the membrane residing proteins including other four ethylene receptors which are *RTE1* and *AtCb5* independent, since the five Arabidopsis ethylene receptors form heteromeric protein complexes and should reside in the same subcellular compartment (Gao et al., 2008).

Materials and Methods

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* Columbia ecotype was used as the wild-type strain in all experiments. The AtCb5-D T-DNA line (GABI_328H06) was obtained from the European Arabidopsis Stock Centre (NASC), while the AtCb5-B T-DNA line (Salk_100161) and the AtCb5-C T-DNA line (Salk_027748) were requested from the Arabidopsis Biological Resource Center (ABRC).

Plants were grown in soil under 16-hour light/8-hour dark in a controlled environment chambers under white fluorescent light. For all the seedling analyses, seeds were sown on Murashige and Skoog (MS) medium containing 0.8% agar. Following a 3-day stratification at 4℃, the seeds were placed in light for five to six hours and then grown in the dark at 20℃ for 4 day. For the ethylene triple responses assay, seeds were germinated either in the presence of ethylene gas or on MS medium

containing ACC (Sigma Aldrich) at the indicated concentrations. The ethylene gas (specialty gases of America, Toledo, OH) was injected into the air-tight mason jars.

Seedlings were removed from the MS medium onto a black cloth for photographs. The hypocotyl length was measured from the digital photographs using ImageJ software (http://rsbweb.nih.gov/ij/).

Mutant Genotyping

Genotyping was carried out using either the Phire Plant Direct PCR kit (Finnzymes) or isolating total genomic DNA by the CTAB method (Dellaporta et al., 1983).

The following primers flanking the T-DNA insertion sites were used to detect wild-type *AtCb5*s: AtCB5-D FP: 5'- TAGCTGTGTCAATATCACCCACAT-3', AtCb5-D RP: 5'-GTGCTGCTTAAGATGTCTCTGTGT-3'; AtCb5-B FP: 5'-CACACGACAACGTTTTGAATG-3', AtCb5-B RP: 5'-TCAGAAGTGGATCTTCCCATG-3'; AtCb5-C FP: 5'-AAACATAACGCGTGTGGTCTC-3', AtCb5-C RP: 5'-TTTGTAAGTATGCCCTCACCC-3'. To detect the T-DNA insertion alleles, the reverse primer above and the SALK T-DNA primer LBa: 5'-TGGTTCACGTAGTGGGCCATCG-3' or the GABI-KAT T-DNA primer: 5'-ACGGATCGTAATTTGTCGTTTTAT-3', which anneals to the T-DNA sequence were used.

For genotyping other mutants, cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993) or derived cleaved amplified polymorphic sequence (dCAPS) markers (Neff et al., 1998) were used. The *rte1-3* allele was

detected by CAPS primers 5'-GGAGTTCCTATGATGGACCTGAAA-3' and 5'-GTAAGTAGCAATTATGAACCA-3'. The amplified DNA fragment is cleaved by the restriction enzyme AloI if the fragment is from *rte1-3*, but not from the wild-type RTE1 allele. The *etr1-2* allele was detected by CAPs primers 5'-CCGATTTCTTCATTGCGATT-3' and 5'-ACCGTATACTCCACGGGATG-3' and the amplified DNA fragment is cleaved by the restriction enzyme HpyCH4IV if the fragment is from *etr1-2*, but not from the wild-type ETR1 allele. The *ers1-10* dCAPS primers 5'-GTGGCCACATGTGCCAATTTTGGAAGAATCCATGCGAGCT-3' and 5'-TGATGGCATGCATCGGTGTCCTCATC-3' introduced a SacI site in the wild-type ERS1 fragment but not *ers1-10*. The *etr2-1* dCAPS primers 5'-AACTGCGAAGACGAAGGAAA-3' and 5'-GGAACAACTCACGAAGTAAAGTAACTCACTA-3' introduced a DdeI site in the mutant *etr2-1* fragment but not the wild-type *ETR2* fragment.

RNA Extraction and RT-PCR

RNA was extracted from pooled seedlings or from rosette leaves in all assays using the RNeasy RNA extraction kit (Qiagen). cDNA was synthesized with oligo(dT) primers using the iScript cDNA synthesis kit (BioRad). For analysis of transcript levels in *atcb5*s T-DNA mutants, the following primers were used.

AtCb5-D F1: 5'-ATGGGCGGAGACGGAAAAGTTTTCAC-3'

AtCb5-D R1: 5'-GAACTTTGTCACATCATAAAC-3'

AtCb5-D F2: 5'-GAGGTTATCTTGACTTCTACAG-3'

AtCb5-D R2: 5'-TCAAGAAGAAGGAGCCTTGGTCTTAGTG-3'

AtCb5-B F1: 5'- TTTCTTGAAGACCATCCAGGTG-3'

AtCb5-B R1: 5'-TTGGTGTAGATACGGATTCCG-3'

AtCb5-C F1: 5'-GGCGAATCTAATTTCGTTTCACG-3'

AtCb5-C R1: 5'-TGGTCAACGTCACCGATACAG-3'

AtCb5-C R2: 5'-CTACTTGTTGTTGTAGAATCTG-3'

Control primers used for RT-PCR were primers annealing to *ACTIN7*. ACT7 F: 5'-GGAACTGGAATGGTGAAGGCTG-3' and ACT7 R: 5'-CGATTGGATACTTCAGAGTGAGGA-3'.

Transgene Constructs and Plant Transformation

AtCb5s coding sequences were amplified (plus or minus the stop codon) from the cDNA clones obtained from ABRC with primers carrying attB sites for cloning via homologous recombination into the Gateway pDONR221 entry vector (Invitrogen) (the $attB_1$ in the sense primer and $attB_2$ site in the antisense primer are underlined):

B1AtCb5-D For: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGCGGAGACGGAAAAGTTTTCACCTT-3'

B2AtCb5-D Rev (+SC):

5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAAGAAGAAGGAGCCTTGGTCTTAGTG-3'

B2AtCb5-D Rev(-SC):

5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCAGAAGAAGGAGCCTTGGTCTTAGTGTAG-3'

B1AtCb5-B For:

5'-

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGAGACGAAGCAAAG

ATCTTCACTCT-3'

B2AtCb5-B Rev(-SC):

5'-

GGGGACCACTTTGTACAAGAAAGCTGGGTCCCCTGATTTGGTGTAGATAC

GGATTCCG-3'

B2AtCb5-B Rev(+SC):

5'-

GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACCCTGATTTGGTGTAGA

TACGGATTC-3'

B1AtCb5-C For:

5'-

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGAATCTAATTTCGT

TTCACGATGT-3'

B2AtCb5-C Rev(-SC):

5'-

GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTGTTGTTGTAGAATCTGA

GAGCGAAAG-3'

B2AtCb5-C Rev(+SC):

5'-

GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACTTGTTGTTGTAGAATC

TGAGAGCG-3'

The *AtCb5*s cDNA clones are:

GC00075: At1g26340: AtCb5-A

U17257: At2g32720: AtCb5-B

G83412: At2g46650: AtCb5-C

U09651: At5g48810: AtCb5-D

G10548: At5g53560: AtCb5-E

The *AtCb5-D* genomic sequence was amplified from Arabidopsis genomic DNA with primers B1gCytB5-D For: 5'-

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGAAAGAGACCCAATT

GAGAGGTTCAC-3' and B2gCytB5-D Rev: 5'-

GGGGACCACTTTGTACAAGAAAGCTGGGTCCTCAGTGTTTATCATTTTCTC

TTACCTG-3' and cloned into the Gateway pDONR221 entry vector (Invitrogen).

AtCb5s coding sequences and *AtCb5-D* genomic sequence were transferred from the pDONR221 (entry) vector (Invitrogen) into various destination vectors such as pN3F6H (the over-expression vector) (Made by Dr. Ruiqiang Chen) and pBGW7 (binary vector allowing gene expression under the control of the native promoter).

The DH5α E.coli strain was used for subcloning. The agrobacterium strain GV3101 was used for plant transformations by the floral dip method(Clough and Bent, 1998). Transformants were selected with either the herbicide Finale (active ingredient glufosinate) (Bayer) or gentamycin (Sigma).

All mutant *etr1* transgenes (in plasmid pPZP211) were kindly provided by the laboratory of Anthony Bleecker (University of Wisconsin, Madison, WI, USA). The *etr1* mutant transgenes were transformed into the wild type by Dr. Jo Resnick (Resnick et al., 2008). *etr1* mutant transgenes were crossed with *atcb5-d* mutant and F2 progeny was genotyped for homozygous *atcb5-d*. F3 seeds were tested on MS medium containing 50mg/l kanamycin for the homozygous *etr1* transgene.

Yeast Split-ubiquitin System

In this study, two yeast split ubiquitin systems using different reporter systems were used. Plasmids, vectors, and yeast cells (JD53) in the first system were kindly provided by Prof. Nils Johnsson, Univ. of Muenster, ZMBE, Germany. The Arabidopsis inflorescence cDNA library was kindly provided by Prof. Imre Sommsich, Max-Planck-Institut für Züchtungsforschung, Köln, Germany. In this system, the bait plasmid pMet-KZ-*RTE1*-Cub-URA3-CYC1 and the prey plasmid pCup-NuI-cDNA-CYC1 contain a His+ or Trp+ marker, respectively. Ura3 in bait plasmid is used as a reporter to demonstrate an interaction (Wittke et al., 1999). We used this system to screen the Arabidopsis inflorescences cDNA library for the RTE1 interacting proteins. In the other system, the artificial transcription factor A-LexA-VP16 (PLV) fused with Cub is released to activate lexA-driven reporter genes *HIS3* and *lacZ* in the nucleus upon bait and prey interaction (Ludewig et al., 2003; Obrdlik et al., 2004). The bait plasmid pMet-RTE1-Cub-PLV-terminator and the prey plasmid pADH-NubG-cDNA-3HA-terminator contain a Leu+ or Trp+ marker, respectively. The bait and prey plasmids as well as yeast strains THY.AP4 and THY.AP5 were kindly provided by Prof. Wolf B. Frommer, Carnegie Institution of Washington,

Stanford, CA,USA. The cDNA library from 6-day old light/dark-grown seedlings (DualSystems Biotech, Switzerland) was screened and the protein interaction was analyzed using the Frommer system.

The RTE1 coding sequence was amplified from a cDNA clone described in (Resnick et al., 2006) with primers atRTE1 SplUbi For: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCACGTGGAAGAGGAGTTCC-3' and atRTE1 SplUbi Rev: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCAGTAATTATGTTCTTAAAACAG-3' (the attB1 in the sense primer and attB2 site in the antisense primer are underlined). The PCR product carrying attB sites was cloned via homologous recombination into the Gateway pDONR221 entry vector (Invitrogen). The RTE1 coding sequence then was transferred from the pDONR221 entry vector into the yeast split ubiquitin assay bait vectors. The AtCb5s coding regions without the stop codon were transferred from the entry clones described in 'Plant Transformation Constructs' into the prey vector pADH-NubG-cDNA-3HA-terminator. The CHX20 C-Tail and CHX20 Full Length bait plasmids were kindly provided by Dr. Heven Sze lab.

Yeasts were maintained on enriched yeast extract-peptone-dextrose (YPD) plates or YPD liquid medium at 30°C. Solid and liquid synthetic complete (SC) media comprised 0.17% yeast nitrogen base (YNB, USBiological), 2% dextrose, 0.5% (NH4)2SO4, and amino acids omitting the indicated ones. For the activation of URA3 activity based system, the SC media was supplemented with 5-FOA (Fluoro-orotic acid, USBiological).

General protocols for yeast transformation and screening were performed as described in "Methods in Yeast Genetics" (Burke et al., 2000) and in "Current Protocols in Molecular Biology" (Ausubel et al., 1989).

Chaper 4 : RTE1 homologs are able to bind heme in vitro

Introduction

In the last chapter, I presented evidence that cytochrome b5 associates with and acts in the same pathway as the novel regulator RTE1 to positively regulate the ETR1 receptor. I speculated that cytochrome b5 could regulate ETR1 function through redox modification since cytochrome b5 is known to be involved in diverse oxidation/reduction reactions. Cytochrome b5 can carry out electron transfer reactions, because the iron atom in the heme prosthetic group alternates between a reduced ferrous (Fe^{2+}) state and an oxidized ferric (Fe^{3+}) state. The genetic analyses in Chapter 3 indicate that cytochrome b5 could play a similar role as RTE1 in ethylene signaling, raising the possibility that RTE1 may be also involved in oxidation/reduction reactions. RTE1 could conceivably regulate the ETR1 EBD conformation via oxidative folding since redox modifications in general are known to affect protein structure and function (Spadaro et al., 2010). Interestingly, the result that human RTE1 binds heme in vitro was firstly uncovered by Dr. Iqbal Hamza's lab when they used human RTE1 as a presumed negative control in testing CeHRG proteins for the ability to bind heme (Rao and Hamza, unpublished). This is consistent with our hypothesis that RTE1 might have the ability to carry out redox reactions, since many proteins involved in redox reactions are heme-binding proteins.

Analysis of the RTE1 amino acid sequence did not reveal any known heme binding motifs. Investigation of other hemoproteins, however, has shown that heme-

binding motifs are variable. Basically, there are two most common ligation motifs: the 5-coordinate mono-histidine, as observed in myoglobin and hemoglobin, and the 6-coordinate bis-histidine, as found in cytochrome *c* (Reedy and Gibney, 2004; Tsiftsoglou et al., 2006). Overall, histidine, cysteine, tyrosine, proline are known to be critical for heme binding in different hemoproteins. For example, CXXCH is the unique heme-binding motif for cytochrome *c* (Stevens et al., 2004; Thony-Meyer, 2000). His-Pro-Gly-Gly (HPGG) forms the core of the heme-binding domain of Cytb5 (Lederer, 1994). A dipeptide motif of cysteine and proline (CP motif) is the heme binding sequence within the heme responsive motifs (HRM) of a variety of heme-regulated proteins (Zhang and Guarente, 1995). The d_1 heme of cyt cd_1 is bound by His-Tyr in the ferric state (Gordon et al., 2003). RTE1 has conserved amino acids such as histidine, cysteine, tyrosine, proline, which could potentially be involved in heme binding. The Arabidopsis RTE1 sequence has 40.5% identity with human RTE1 (hRTE1) over 156 amino acids, and 51% identity to Arabidopsis RTH (AtRTH) over 209 amino acids, suggesting that human RTE1 and Arabidopsis RTH may have a similar molecular function as Arabidopsis RTE1. To gain some insights into the question whether RTE1 can carry out redox reactions, we investigated whether RTE1 binds heme.

In addition, it is worth noting that GAF domains bind small molecules such as cyclic GMP and chromophores (which are synthesized from heme) (Aravind and Ponting, 1997). The GAF domain is present in phytochromes and ethylene receptors, which are both derived from cyanobacterial two-component receptors and related in structure (Mount and Chang, 2002). GAF in phytochromes binds a tetrapyrrole

chromophore, suggesting that ethylene receptor GAF domain could bind heme. The GAF domains of the ethylene receptors have two conserved histidine residues (His^{160} and His^{309}), which might be involved in heme binding.

Results

Human RTE1, RTE1-1 and Arabidopsis RTH bind heme in vitro

To test the binding of hRTE1, AtRTE1 and AtRTH to heme, expression constructs fused with a Hemagglutinin (HA) epitope were transiently expressed in HeLa cells and then hemin agarose affinity chromatography was performed on cell lysates. The affinity chromatography showed binding of heme to a positive control, CeHRG4, which is a newly identified heme transporter (Rajagopal et al., 2008), as well as to hRTE1 and AtRTH (**Figure 4-1**). In contrast, essentially no binding was observed for human ZIP4, an eight-transmembrane-domain zinc transporter, suggesting that the binding was not due to nonspecific hydrophobic interactions (**Figure 4-1**). Unfortunately, I was unable to test the ability of AtRTE1 to bind heme, because the expression of AtRTE1 in HeLa cells was too low to carry on the hemin agarose affinity chromatography.

I next tested a mutated version of hRTE1 to see whether the mutation would disrupt the heme binding ability of hRTE1 (**Figure 4-1**). *rte1-1* is a loss of function mutation in Arabidopsis, which is a G-to-A missense mutation that results in a Cys-Tyr substitution at the conserved Cys^{161} residue. *rte1-1* was isolated from the genetic screening for suppressors of the weak ethylene-insensitive mutant *etr1-2*. The corresponding mutation encoding the same Cys to Tyr substitution was introduced

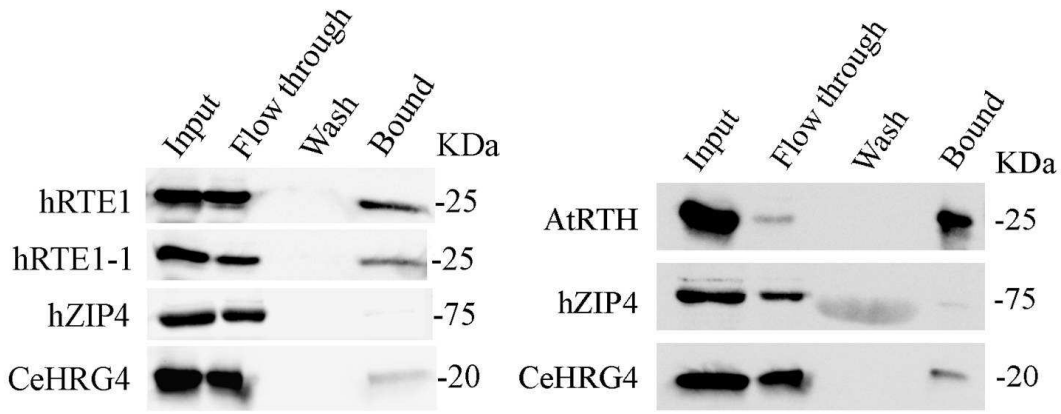


Figure 4-1 hRTE1, hRTE1-1 and AtRTH proteins interact with heme in the hemin-agarose pull down assay

Cell lysates from HeLa cells expressing the indicated HA-tagged proteins were incubated with hemin-agarose. Equivalent proportions of input lysates (Input), the supernatant after binding and before washing (Flow-through), the final wash before elution (Wash) and the eluates (Bound) were subjected to SDS-PAGE followed by immunoblotting with anti-HA antisera . *C.elegans* heme transporter CeHRG4 and Human zinc transporter hZIP4 were used as positive and negative controls respectively.

into hRTE1 by in vitro site-directed mutagenesis and tested for heme binding ability. hRTE1 carrying the *rte1-1* mutation did not detectably affect the heme binding ability of hRTE1 (**Figure 4-1**). This result indicates that the conserved Cys161 may not be required for heme binding in hRTE1 and suggests that the *rte1-1* loss-of-function phenotype caused by the C161Y substitution may not be due to heme binding disruption.

Since the GAF domain of ETR1 could conceivably bind heme, I tried to test whether Arabidopsis ETR1 protein can bind heme. Unfortunately, the hemin-agarose affinity chromatography was unable to be performed for the AtETR1 protein due to its poor expression in the transfected HeLa cells.

Human RTE1, Arabidopsis RTE1 and Arabidopsis RTH fail to rescue the growth of a heme-deficient yeast strain

Since hRTE1 and AtRTH bind heme in the above hemin agarose pull-down assay, we next tested whether they are able to transport heme using a heme-deficient yeast rescue assay. The *hem1Δ* yeast strain lacks the gene encoding δ-aminolevulinic acid (ALA) synthase, which is the rate-limiting enzyme in the heme biosynthesis pathway (Crisp et al., 2003), and therefore requires an external heme source for growth. CeHRG-1 is a heme importer (Rajagopal et al., 2008) and was used as a positive control. In comparison to the empty vector control, the positive control CeHRG-1 significantly increased the growth of *hem1Δ* yeast at 1μM heme, whereas the expression of AtRTE1, AtRTH and hRTE1 showed no difference from the negative control (**Figure 4-2**).

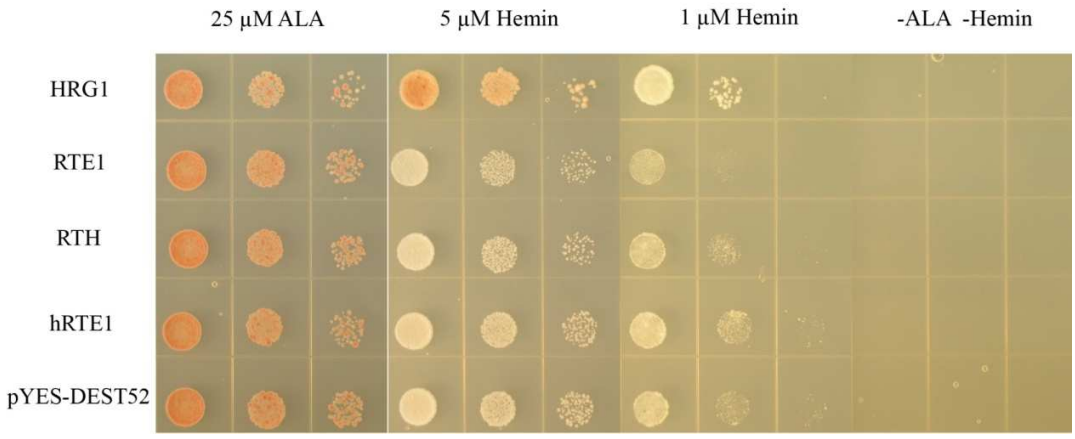


Figure 4-2 RTE1, RTH and hRTE1 cannot rescue the growth of hem1Δ yeast at low heme concentrations

The DY1457 *hem1Δ (6D)* yeast strain transformed with indicated constructs was spotted in 10-fold serial dilutions on synthetic complete medium plates lacking uracil. The heme transporter HRG-1 and the empty vector pYES-DEST52 were used as controls. The plate containing glucose and 25µM δ-aminolevulinic acid (ALA) which rescues the *hem1Δ* defect, shows the amounts of cells plated. Plates with different concentrations of heme were supplemented with 0.4% galactose to induce expression of the transformed genes. Yeast grown on the ALA positive control plate and a plate with 5 µM heme displayed red pigment accumulation due to a mutation in the ADE2 locus.

Therefore, unlike CeHRG-1, AtRTE1 and AtRTH cannot import heme into yeast cells though they have in vitro heme binding ability.

Discussion

The above affinity chromatography results suggest that RTE family proteins could be hemoproteins. However, it still remains unknown whether RTE1 can bind heme in vivo. It is possible that RTE1 binds some other porphyrins in plants like chlorophylls, bilins, and corrins, since they are structurally similar. Therefore, a heme specificity test and an in vivo heme binding test are needed. The C161Y substitution caused by the *rte1-1* mutation didn't disrupt the in vitro heme-binding ability of hRTE1. There are two kinds of explanation for this result. One is that C161 is not the residue responsible for heme binding. The other one is that C161 can bind heme because it has been known that tyrosine could also bind heme. So it is necessary to convert C161 to other amino acids that are known not to bind heme and then test the heme-binding ability of the mutant.

Although AtRTE1 and AtRTH failed to rescue the heme-deficient yeast strain, this result cannot rule out the possibility that RTE proteins could mediate intracellular heme transport. Since RTE1 is localized to the ER membrane, RTE1 may not be able to mediate the heme uptake through plasma membrane from outside of the yeast cell. In addition, a western blot is needed to show that the inability of AtRTE1, AtRTH and hRTE1 to rescue the heme-deficient yeast strain is not due to poor expression in the *hem1Δ* yeast strain.

If RTE1 is truly a hemoprotein, what is the possible molecular function of RTE1 as a hemoprotein? Is heme involved in regulating the ETR1 receptor function? Investigation of these questions will help to understand the molecular mechanisms by which RTE1 regulates ETR1.

What is heme and what kinds of biological roles does heme play? Heme is a prosthetic group that consists of an iron atom contained in the center of a large heterocyclic organic ring called a porphyrin. Hemoproteins have been shown to be involved in diverse crucial biological functions such as oxygen transport (hemoglobin, myoglobin), oxygen metabolism (oxidases, peroxidases, catalases), electron transfer (cytochromes), the circadian clock control (Rev-erb α), micro RNA processing (DGCR8), transcription factors (HAPs) and biosynthesis of some signal molecules like NO (nitric oxide synthase), steroid hormones (hydroxylases), cyclic GMP (guanylate cyclase) (Faller et al., 2007; Guarente and Mason, 1983; Ponka, 1999; Tsiftsoglou et al., 2006; Yin et al., 2007). In addition, since heme is hydrophobic and cytotoxic, heme transporters (HRGs) are required to bind heme and deliver heme to subcellular destinations (Chen et al., 2011a; Rajagopal et al., 2008).

It is particularly interesting that RTE1 could physically associate with another hemoprotein AtCb5, which has a similar function in ethylene signaling. Since cytochrome b5 is known to carry out electron transfer, we propose that RTE1 may also carry out redox reactions and there might be redox communication between RTE1 and AtCb5. It is unclear which protein is upstream in the pathway that regulates ETR1 receptor function; for example, RTE1 could accept electrons from AtCb5 or provide electrons to AtCb5. I favor the possibility that RTE1 may act

downstream of AtCb5 for several reasons. Firstly, as described in Chapter 3, the weak ethylene insensitivity conferred by overexpressing *RTE1* cannot be blocked by loss of *AtCb5-D* function. However, it is possible that loss of one AtCb5 member function is not sufficient to block RTE1 signaling since other functional redundant AtCb5 members are present. Therefore it is the key to test whether the weak ethylene insensitivity conferred by overexpressing *AtCb5-D* is blocked by the loss of *RTE1* function. Secondly, it has been demonstrated that RTE1 physically associates with ETR1 (Dong et al., 2010). Thus I speculate that ETR1 could be the direct substrate of RTE1. I don't know whether AtCb5 interacts with ETR1 in yeast and planta. I was unable to test the interaction of AtCb5 and ETR1 in the yeast split-ub assay due to non-functional ETR1 in yeast when fused with Cub or Nub. This could be tested in planta using BiFC. Thirdly, it is easier to explain the specificity of RTE1 and AtCb5 for ETR1. I know AtCb5 transfers electrons to numerous electron acceptor proteins such as cytochrome P450 enzymes; therefore AtCb5 can regulate various oxidation/reduction reactions. If RTE1 were downstream of AtCb5, RTE1 could be a specific adaptor protein for redox of ETR1 by AtCb5. In other words, RTE1 accepts electrons from AtCb5 and specifically modifies ETR1 directly or through regulating certain oxidase/reductase which modifies ETR1.

It is also possible that AtCb5 and/or RTE1 are involved in electron transfer affecting the redox state of the copper cofactor required for ethylene binding by the receptors and that the copper redox state affects receptor conformation. However, the evidence showed that RTE1 function seems to be unrelated to copper (Resnick et al., 2008). Alternatively, RTE1 could deliver heme to cytochrome b5, which exerts a

redox effect on ETR1. Another speculation is that RTE1 may function to deliver heme to the ETR1 receptor, which conceivably could bind heme.

Materials and Methods

Human Cell Culture and Transfection

HeLa cells were maintained in RPMI medium (GIBCO/BRL) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin/glutamine and cultured at 37℃ in a humidified incubator with 5% CO2. DNA constructs were transiently transfected into Hela cells using FuGENE 6 (Roche) for western blotting and hemin-agarose affinity chromatography.

Construct Cloning

The AtRTH coding sequence was PCR-amplified from an existing RTH cDNA clone using primers carrying flanking *Eco*RI and *Xba*I restriction sites. The sequence for the Hemagglutinin (HA) epitope tag was included in the reverse primers to generate HA-tagged proteins. The PCR product was first ligated into the pGEM-T vector (Promega). Following restriction digestion and DNA purification, the digested DNA fragment was ligated into the pCDNA3.1 (+) Zeo vector (Invitrogen).

For the hRTE1-1 clone (in pcDNA3.1(+) zeo), a G to A mutation was introduced into pcDNA3.1(+)-hRTE1 clone(from caren) by in vitro site-directed mutagenesis using the OuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sequences of the mutagenic primers are hRTE1-1 MG F:

ctctgctgtgacaactaccactcgcacgtggc and hRTE1-1 MG R:

gccacgtgcgagtggtagttgtcacagcagag

The CeHRG4 and hZIP4 constructs (in vector pcDNA3.1(+) zeo) were kindly provided by the laboratory of Dr. Iqbal Hamza (University of Maryland, College Park).

Hemin-agarose Pull-down Assays

Hemin-agarose pull-down assays were performed according to the procedure as described in Rajagopal et al. (2008). In brief, transfected HeLa cells were lysed with rocking for 30 min in MS buffer (210 mM mannitol, 70 mM sucrose and 10 mM HEPES, pH 7.4) in the presence of 2.5% protease inhibitor cocktail (Sigma) in cold room. The lysates were centrifuged at 100 X *g* for 5 min, and the post-nuclear supernatants were quantified by Bradford assay (Bio-rad). The same proportion of proteins from each sample was subjected to SDS-PAGE and immunoblotting with a rabbit anti-HA antibody (Sigma). Chemiluminescence images were captured with a Fujifilm luminescent image analyzer LAS-4000. The expression of individual proteins was quantified by measuring band intensities with Multi Gauge software (Version 3.1; Fujifilm).

Next, the equivalent amount of target protein was mixed with untransfected Hela cell lysates to obtain 500μg of total protein for the binding reaction. The samples were incubated with gentle rocking at room temperature for 30 min in the presence of 300 nmol hemin-agarose (Sigma, Cat No.: H6390). The binding reaction mixture was centrifuged at 800 X *g* for 3 min, and the resulting agarose pellets were washed three times with 1 ml of wash buffer (150 mM NaCl, 1 % NP-40, and 50 mM Tris-HCl, pH

8.0). The final pellets were incubated with Laemmli sample-loading buffer containing 100 mM DTT for 10 min at room temperature and then boiling for 5 min to elute the bound proteins.

Equivalent amounts of input protein, flow-through after binding reaction, flow-through after the final time wash and the eluted protein were loaded on a 12 % SDS/PAGE and immunoblotting with HA antibodies. The hemin agarose assay on hRTE1 and was done in three biological replicates and on hRTE1-1 and AtRTH was done in two biological replicates.

Yeast Strains, Growth and Transformation

The heme-deficient *S. cerevisiae* strain, DY1457 *hem1Δ (6D),* was provided by the laboratory of Dr. Iqbal Hamza (University of Maryland, College Park). This *hem1Δ* strain lacks the gene encoding δ-aminolevulinic acid (ALA) synthase, which is the rate-limiting enzyme in the heme biosynthesis pathway (Crisp et al., 2003).Yeast were maintained on enriched yeast extract-peptone-dextrose (YPD) plates or YPD liquid medium at 30°C. Solid and liquid synthetic complete (SC) media comprised 0.17% yeast nitrogen base (YNB, USBiological), 2% dextrose, 0.5% $(NH_4)_2SO_4$, and amino acids, as described in Sherman, 2002 (Sherman, 2002). The regular growth medium was supplemented with 250μM δ-aminolevulinic acid (ALA).

RTE1, RTH, hRTE1 ORFs were cloned into the vector pYES-DEST52 (Invitrogen) by Gateway cloning. *CeHRG1* in pYES-DEST52 was provided by the laboratory of Dr. Iqbal Hamza's lab (University of Maryland, College Park). All of these constructs were transformed into *hem1Δ* yeast using polyethylene glycol and

lithium acetate at 42°C for 20 min. Positive clones were isolated by plating the transformants onto the SC medium minus uracil. 250μM ALA was supplemented in the medium.

Yeast Heme Rescue Assay

Individual transformed yeast colonies were transferred onto SC-Ura agar plates containing 2% raffinose instead of glucose. Residual ALA was removed by growing the transformants in SC-Ura liquid medium without ALA for 16 h. Equal amount of transformed yeast were spotted in 10-fold serial dilutions onto growth assay plates containing 4% galactose for gene induction and different concentrations of hemin chloride or ALA. Yeast growth was analyzed after incubation at 30°C for 3-5 days.

Chaper 5 : A unique proline (P9) conserved only in ETR1 orthologs is involved in the specificity of RTE1 for ETR1

Introduction

Previous genetic studies suggest that ETR1 is the only ethylene receptor dependent on RTE1 in Arabidopsis and RTE1 is believed to act on the ethylene binding domain (EBD) of ETR1 (Resnick et al., 2008; Resnick et al., 2006; Rivarola et al., 2009; Zhou et al., 2007). However, the EBD is the most conserved region of Arabidopsis ethylene receptors (Rodriguez et al., 1999). It remained unknown how the EBD of ETR1 is distinct from other ethylene receptors to specifically require RTE1.

The current model of ethylene receptor signaling is that ethylene binding at the transmembrane EBD causes EBD conformational changes, which are presumed to be propagated to the cytoplasmic transmitter domain to affect the receptor signaling status. The dominant missense mutations at many conserved amino acids within the EBD of the ethylene receptors, which confer ethylene insensitivity, have been proposed to result in the various structural defects within EBD that inhibit the conformational transition to shut off receptor signaling (Wang et al., 2006). Previously, it has been observed that such a dominant mutation identified in one receptor could generally cause the same ethylene insensitivity when it is carried by another receptor at the corresponding position. For example, when a missense

mutation encoded by *etr1-4*, which causes an Ile to Phe substitution at residue 62, is introduced into the *ERS1* and *ERS2 g*ene, it confers dominant ethylene insensitivity (Hua et al., 1995; Hua et al., 1998). This is even true for transferring a dominant mutation to the ethylene receptors of different species. For example, the tobacco *ERS* transgene carrying the *Never-ripe* (*Nr*) mutation identified in tomato results in ethylene insensitivity in tobacco (Terajima et al., 2001). These facts suggest a conserved mechanism of ethylene receptor signaling among different species.

Interestingly, a former graduate student in our lab, Maximo Rivarola, revealed a correlation of dominant missense mutation transferability with *RTE1*-dependence (Rivarola et al., 2009). As described by Resnick et al.(2008), the dominant missense mutations within ETR1 EBD which confer ethylene insensitivity can be classified into two categories: *RTE1*-dependent and *RTE1*-independent. Maximo found that only those *RTE1*-independent dominant mutations in the ETR1 receptor can be transferred into the identical conserved positions in other Arabidopsis ethylene receptors, whereas the *RTE1*-dependent ones fail to cause ethylene insensitivity when carried by another ethylene receptor, even *ERS1*, which is closest to *ETR1* (they share 75% amino acid identity in the EBD). Consistent with this finding, several existing ethylene insensitive mutations in the four other ethylene receptor genes were tested and shown to be independent of *RTE1* (Resnick et al., 2006; Rivarola et al., 2009). Therefore, the ETR1 receptor is distinct from other ethylene receptors in terms of *RTE1*-dependence. As suggested in Chapter 3, AtCb5, a putative RTE1 interacting protein in the same pathway as RTE1, also appears to be specific to the ETR1 receptor, not other receptors.

Why does ETR1 uniquely require *RTE1* to properly function? Does ETR1 have a distinct signaling regulation mechanism? Since *RTE1*-dependent *etr1* mutations are presumed to cause altered EBD conformations which have defects in switching receptor signaling off, *RTE1* is thought to maintain such an altered conformation (Resnick et al., 2008). The specificity of RTE1 for ETR1 is probably due to the unique steric structure of the ETR1 EBD. In addition, the specificity of RTE1 is not likely based on differences in expression patterns of ethylene receptors (Rivarola et al., 2009). To investigate the basis for this specificity and reveal the structural and functional differences between ETR1 and other receptors, I sought to identify the ETR1 residue(s) required for *RTE1* dependence. Because such residue(s) could be potential target sites for RTE1 action and this would help to not only understand the molecular function of RTE1 and the structure-function relationship of the ETR1 receptor, but also predict other protein targets of RTE1 homologs in other species.

Based on the high degree of conservation (75% identity and 83% similarity) over the N terminal 128 amino acids (EBD) between Arabidopsis ETR1 and ERS1, it is surprising that the two ethylene receptors exhibit opposite dependence on RTE1: ETR1 is RTE1 dependent, whereas ERS1 is RTE1-independent. As shown in **Figure 5-1**, in this region of the polypeptide, a few residues are not conserved between ETR1 EBD and ERS1 EBD. A strategy of screening for the residue(s) essential for *RTE1* dependence is to interchange each non-conserved amino acid between ETR1 and ERS1 by means of in vitro site-directed mutagenesis and analyze which amino acid substitution can convert ETR1 to a *RTE1*-independent receptor and/or ERS1 to a *RTE1*-dependent receptor.

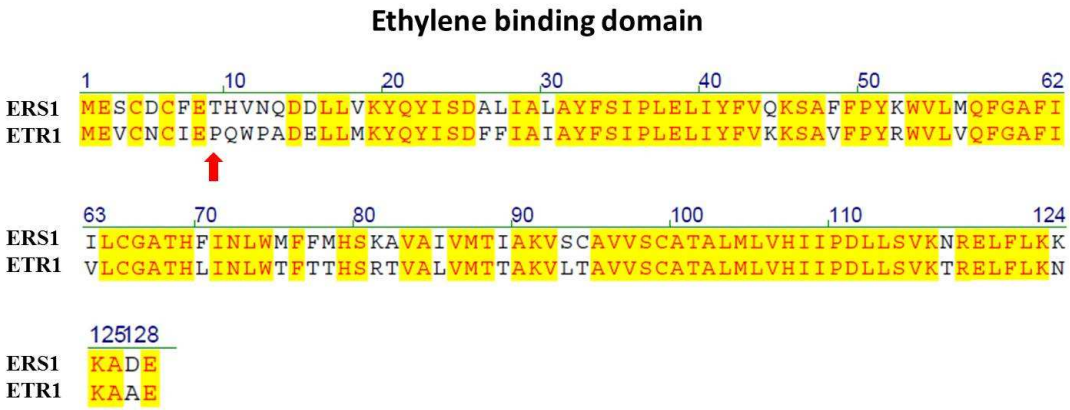


Figure 5-1 Comparison of amino acid sequences of the Arabidopsis ETR1 EBD and ERS1 EBD

The N-terminal 128 amino acid residues of ETR1 and ERS1 are aligned with the Vector NTI software (Invitrogen). The identical residues between ETR1 and ERS1 are highlighted in yellow. The amino acid at the position 9 that is a threonine in ERS1 and a proline in ETR1 is pointed out by a red arrow.

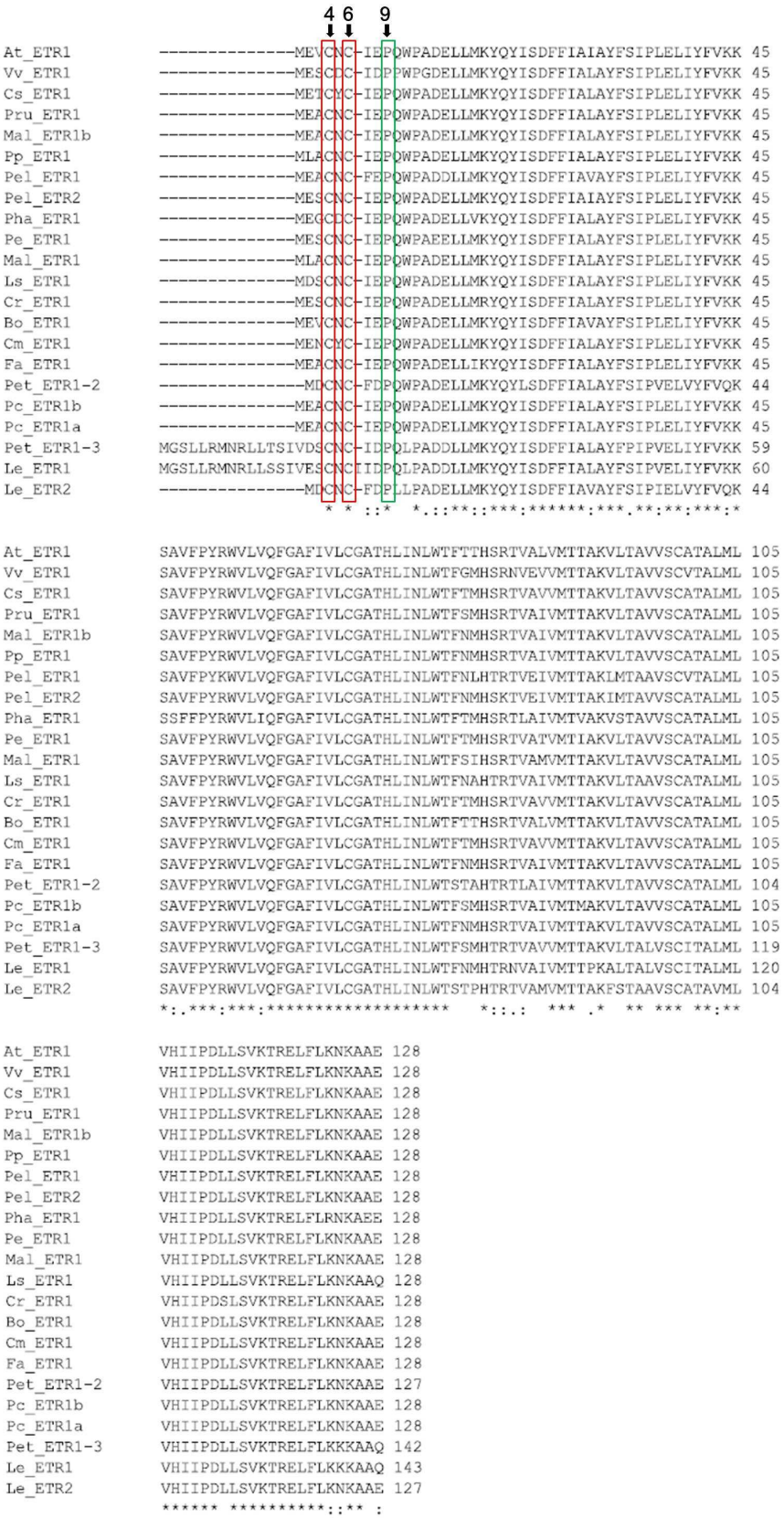
In this chapter, I report that proline at position 9, which is conserved only in ETR1 orthologs, is largely responsible for the specificity of RTE1 for ETR1.

Results

A unique proline (P9) is conserved only in ETR1 orthologs

It has been known that only ETR1 depends on RTE1 and the other Arabidopsis ethylene receptors are not. Therefore, to uncover the basis of RTE1 specificity, I sought to look for the residue(s) in Arabidopsis ETR1 that are absent in ERS1 and the three other Arabidopsis ethylene receptors. Proline 9 in ETR1 is one of such non-conserved residues. Wang et al**.** (2006) searched available sequence data for the presence of genes containing the Ethylene Binding Domain (EBD)-like sequences in a variety of organisms . Of 113 candidate EBD-containing genes uncovered by Wang et al. (2006), we chose the 61 that are annotated subfamily I or subfamily II ethylene receptor homologs from various plants (**Table III**) to perform EBD sequences alignment. Proline 9 (P9) in Arabidopsis ETR1 is the only one residue that is almost exclusively conserved in subfamily I ETR orthologs, not found in subfamily I ERS and subfamily II ethylene receptors (**Figure 5-2**), though there are three ethylene receptors (OsERS, PaERS1 and PhaERS), which are classified into subfamily I ERS based on the gene structure, also contain the P9 (**Figure 5-2 B**). The phylogenetic tree of the 61 candidate ethylene receptors indicates that the three subfamily I ERS-like ERS ethylene receptors are closer to subfamily I ETR ethylene receptors (**Figure 5-3**), suggesting that they may behave more like ETR1 than ERS1. P9 sits close to two cysteines (C4 and C6 in Arabidopsis ETR1), which are conserved among all 61 annotated ethylene receptors.

A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Vv_ETR1 ----------MESCIC-IDPPWPGDELLMKYQYISDFFIALAYFSIPLELIYFVKK 45
Cs_ETR1 ----------METCYC-IEPQWPADELLMKYQYISDFFIALAYFSIPLELIYFVKK 45
Pru_ETR1 ----------MEACNC-IEPQWPADELLMKYQYISDFFIALAYFSIPLELIYFVKK 45
Mal_ETR1b ----------MEACNC-IEPQWPADELLMKYQYISDFFIALAYFSIPLELIYFVKK 45
Pp_ETR1 ----------MLACNC-IEPQWPADELLMKYQYISDFFIALAYFSIPLELIYFVKK 45
Pel_ETR1 ----------MEACNC-FEPQWPADDLLMKYQYISDFFIAVAYFSIPLELIYFVKK 45
Pel_ETR2 ----------MESCNC-IEPQWPADELLMKYQYISDFFIAIAYFSIPLELIYFVKK 45
Pha_ETR1 ----------MEGCIC-IEPQWPADELLVKYQYISDFFIALAYFSIPLELIYFVKK 45
Pe_ETR1 ----------MESCNC-IEPQWPAEELLMKYQYISDFFIALAYFSIPLELIYFVKK 45
Mal_ETR1 ----------MLACNC-IEPQWPADELLMKYQYISDFFIALAYFSIPLELIYFVKK 45
Ls_ETR1 ----------MDSCNC-IEPQWPADELLMKYQYISDFFIALAYFSIPLELIYFVKK 45
Cr_ETR1 ----------MESCNC-IEPQWPADELLMRYQYISDFFIALAYFSIPLELIYFVKK 45
Bo_ETR1 ----------MEVCNC-IEPQWPADELLMKYQYISDFFIAVAYFSIPLELIYFVKK 45
Cm_ETR1 ----------MENCYC-IEPQWPADELLMKYQYISDFFIALAYFSIPLELIYFVKK 45
Fa_ETR1 ----------MEACNC-IEPQWPADELLIKYQYISDFFIALAYFSIPLELIYFVKK 45
Pet_ETR1-2 ----------MDCNC-FDPQWPADELLMKYQYLSDFFIALAYFSIPVELVYFVQK 44
Pc_ETR1b ----------MEACNC-IEPQWPADELLMKYQYISDFFIALAYFSIPLELIYFVKK 45
Pc_ETR1a ----------MEACNC-IEPQWPADELLMKYQYISDFFIALAYFSIPLELIYFVKK 45
Pet_ETR1-3 MGSLLRMNRLLTSIVDSCNC-IDPQLPADDLLMKYQYISDFFIALAYFPIPVELIYFVKK 59
Le_ETR1 MGSLLRMNRLLSSIVESCNCIIDPQLPADDLLMKYQYISDFFIALAYFSIPVELIYFVKK 60
Le_ETR2 ----------MDCNC-FDPLLPADELLMKYQYISDFFIAVAYFSIPIELVYFVQK 44
* * ::* *.::**::***:******:***.**:**:***:*
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Vv_ETR1 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFGMHSRNVEVVMTTAKVLTAVVSCVTALML 105
Cs_ETR1 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFTMHSRTVAVVMTTAKVLTAVVSCATALML 105
Pru_ETR1 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFSMHSRTVAIVMTTAKVLTAVVSCATALML 105
Mal_ETR1b SAVFPYRWVLVQFGAFIVLCGATHLINLWTFNMHSRTVAIVMTTAKVLTAVVSCATALML 105
Pp_ETR1 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFNMHSRTVAIVMTTAKVLTAVVSCATALML 105
Pel_ETR1 SAVFPYKWVLVQFGAFIVLCGATHLINLWTFNLHTRTVEIVMTTAKLMTAAVSCVTALML 105
Pel_ETR2 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFNMHSKTVEIVMTTAKIMTAVVSCATALML 105
Pha_ETR1 SSFFPYRWVLIQFGAFIVLCGATHLINLWTFTMHSRTLAIVMTVAKVSTAVVSCATALML 105
Pe_ETR1 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFTMHSRTVATVMTIAKVLTAVVSCATALML 105
Mal_ETR1 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFSIHSRTVAMVMTTAKVLTAVVSCATALML 105
Ls_ETR1 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFNAHTRTVAIVMTTAKVLTAAVSCATALML 105
Cr_ETR1 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFTMHSRTVAVVMTTAKVLTAVVSCATALML 105
Bo_ETR1 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFTTHSRTVALVMTTAKVLTAVVSCATALML 105
Cm_ETR1 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFTMHSRTVAVVMTTAKVLTAVVSCATALML 105
Fa_ETR1 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFNMHSRTVAIVMTTAKVLTAVVSCATALML 105
Pet_ETR1-2 SAVFPYRWVLVQFGAFIVLCGATHLINLWTSTAHTRTLAIVMTTAKVLTAVVSCATALML 104
Pc_ETR1b SAVFPYRWVLVQFGAFIVLCGATHLINLWTFSMHSRTVAIVMTMAKVLTAVVSCATALML 105
Pc_ETR1a SAVFPYRWVLVQFGAFIVLCGATHLINLWTFNMHSRTVAIVMTTAKVLTAVVSCATALML 105
Pet_ETR1-3 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFSMHTRTVAVVMTTAKVLTALVSCITALML 119
Le_ETR1 SAVFPYRWVLVQFGAFIVLCGATHLINLWTFNMHTRNVAIVMTTPKALTALVSCITALML 120
Le_ETR2 SAVFPYRWVLVQFGAFIVLCGATHLINLWTSTPHTRTVAMVMTTAKFSTAAVSCATAVML 104
*:.***:***:******************** *::.: *** .* ** *** **:**
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Vv_ETR1 VHIIPDLLSVKTRELFLKNKAAE 128
Cs_ETR1 VHIIPDLLSVKTRELFLKNKAAE 128
Pru_ETR1 VHIIPDLLSVKTRELFLKNKAAE 128
Mal_ETR1b VHIIPDLLSVKTRELFLKNKAAE 128
Pp_ETR1 VHIIPDLLSVKTRELFLKNKAAE 128
Pel_ETR1 VHIIPDLLSVKTRELFLKNKAAE 128
Pel_ETR2 VHIIPDLLSVKTRELFLKNKAAE 128
Pha_ETR1 VHIIPDLLSVKTRELFLRNKAEE 128
Pe_ETR1 VHIIPDLLSVKTRELFLKNKAAE 128
Mal_ETR1 VHIIPDLLSVKTRELFLKNKAAE 128
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Fa_ETR1 VHIIPDLLSVKTRELFLKNKAAE 128
Pet_ETR1-2 VHIIPDLLSVKTRELFLKNKAAE 127
Pc_ETR1b VHIIPDLLSVKTRELFLKNKAAE 128
Pc_ETR1a VHIIPDLLSVKTRELFLKNKAAE 128
Pet_ETR1-3 VHIIPDLLSVKTRELFLKKKAAQ 142
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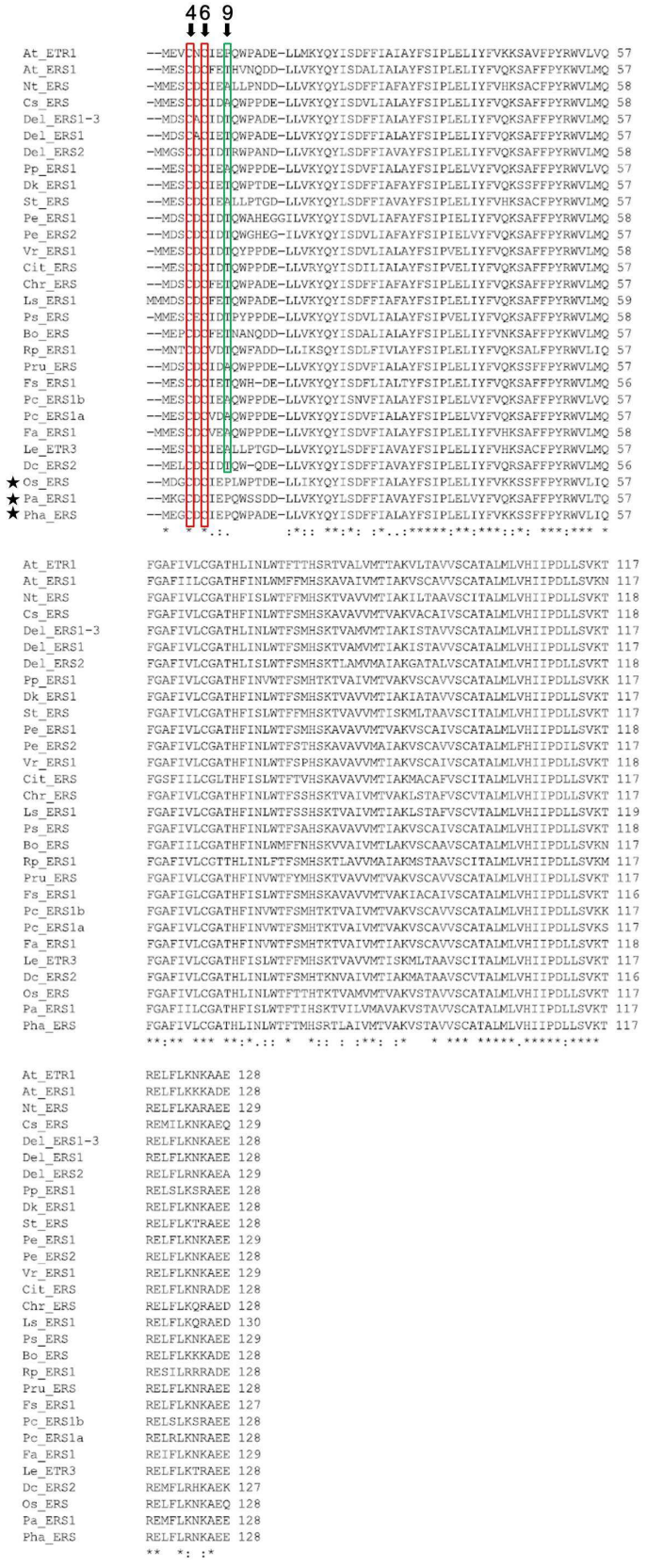
B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Nt_ERS -MMESCDCIEALLPNDD-LLVKYQYLSDFFIAFAYFSIPLELIYFVHKSACFPYRWVLMQ 58
Cs_ERS -MMESCDCIDAQWPPDE-LLVKYQYISDVLIALAYFSIPLELIYFVQKSAFFPYRWVLMQ 58
Del_ERS1-3 --MDSCACIDTQWPADE-LLVKYQYISDFFIALAYFSIPLELIYFVQKSAFFPYRWVLMQ 57
Del_ERS1 --MDSCACIEITQWPADE-LLVKYQYISDFFIALAYFSIPLELIYFVQKSAFFPYRWVLMQ 57
Del_ERS2 -MMGSCDCIDIRWPAND-LLVKYQYLSDFFIAVAYFSIPLELIYFVQKSAFFPYRWVLMQ 58
Pp_ERS1 --MESCDCIEAQWPPDE-LLVKYQYISDVFIALAYFSIPLELVYFVQKSAFFPYRWVLVQ 57
Dk_ERS1 --MESCDCIEDQWPTDE-LLVKYQYISDFFIAFAYFSIPLELIYFVQKSSFFPYRWVLMQ 57
St_ERS --MESCDCIEALLPTGD-LLVKYQYLSDFFIAVAYFSIPLELIYFVHKSACFPYRWVLMQ 57
Pe_ERS1 --MDSCDCIDIQWAHEGGILVKYQYISDVLIAFAYFSIPIELIYFVQKSAFFPYRWVLMQ 58
Pe_ERS2 --MDSCDCIDIQWGHEG-ILVKYQYISDVLIAFAYFSIPIELVYFVQKSAFFPYRWVLMQ 57
Vr_ERS1 -MMESCDCIDTQYPPDE-LLVKYQYISDVLIALAYFSIPVELIYFVQKSAFFPYRWVLMQ 58
Cit_ERS --MESCDCIDTQWPPDE-LLVRYQYISDILIALAYFSIPVELIYFVQKSAFFPYRWVLMQ 57
Chr_ERS --MDSCDCFEIQWPADE-LLVKYQYISDFFIAFAYFSIPLELIYFVQKSAFFPYRWVLMQ 57
Ls_ERS1 MMMDSCDCFEIQWPADE-LLVKYQYISDFFIAFAYFSIPLELIYFVQKSAFFPYRWVLMQ 59
Ps_ERS -MMESCDCIDTPYPPDE-LLVKYQYISDVLIALAYFSIPVELIYFVQKSAFFPYRWVLMQ 58
Bo_ERS --MEPCDCFEINANQDD-LLVKYQYISDALIALAYFSIPLELIYFVNKSAFFPYKWVLMQ 57
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Fa_ERS1 -MMESCDCVEAQWPPDE-LLVKYQYISDVFIALAYFSIPLELIYFVHKSAFFPYRWVLMQ 58
Le_ETR3 --MESCDCIEALLPTGD-LLVKYQYLSDFFIAVAYFSIPLELIYFVHKSACFPYRWVLMQ 57
Dc_ERS2 --MELCDCIDIQW-QDE-LLVKYQYISDFFIAVAYFSIPLELIYFVQRSAFFPYRWVLMQ 56
★Os_ERS --MDGCDCIEPLWPTDE-LLIKYQYISDFFIALAYFSIPLELIYFVKKSSFFPYRWVLIQ 57
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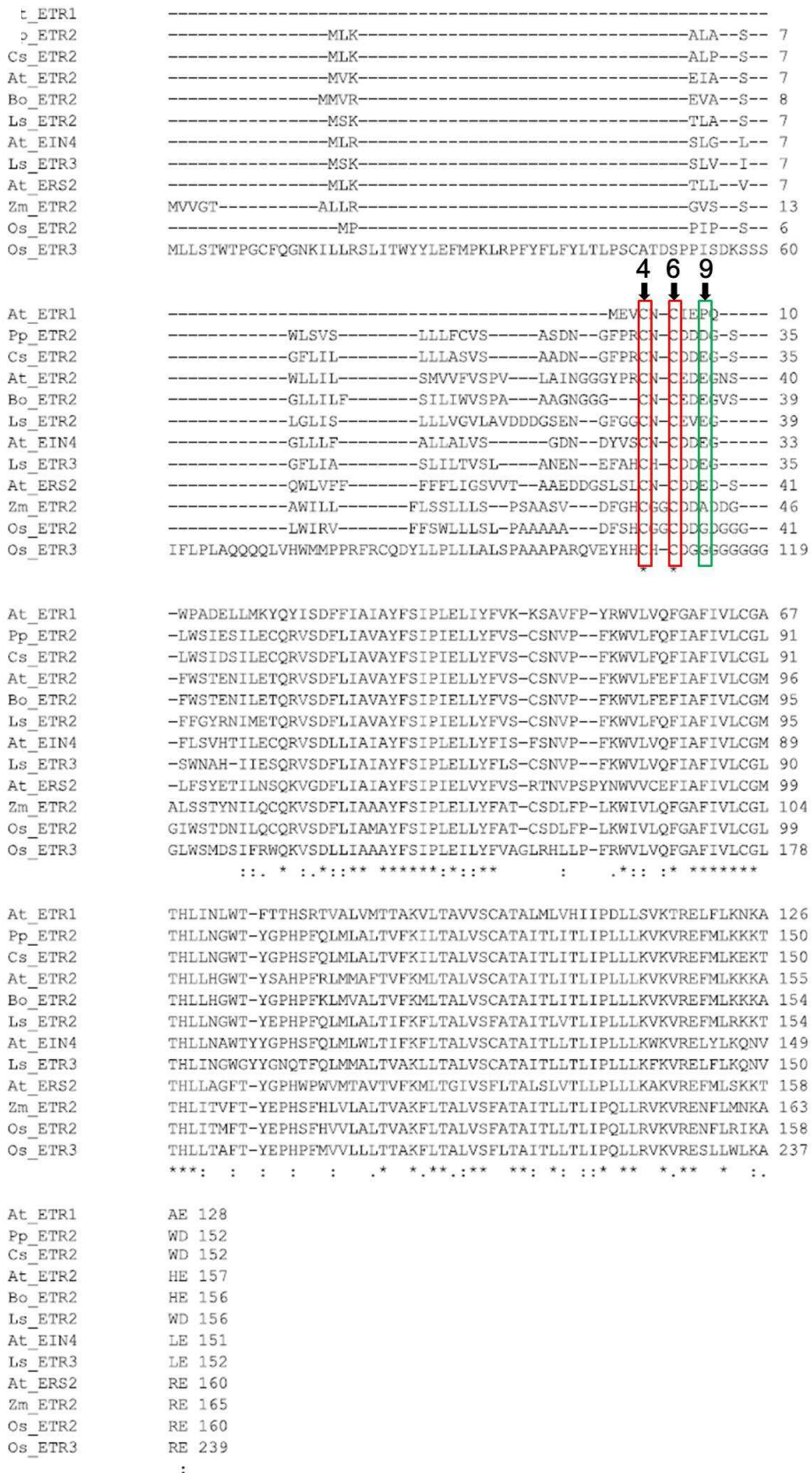
C

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Cs_ETR2         ----------------MLK---------------------------------ALP--S-- 7
At_ETR2         ----------------MVK---------------------------------EIA--S-- 7
Bo_ETR2         ---------------MMVR---------------------------------EVA--S-- 8
Ls_ETR2         ----------------MSK---------------------------------TLA--S-- 7
At_EIN4         ----------------MLR---------------------------------SLG--L-- 7
Ls_ETR3         ----------------MSK---------------------------------SLV--I-- 7
At_ERS2         ----------------MLK---------------------------------TLL--V-- 7
Zm_ETR2         MVVGT----------ALLR---------------------------------GVS--S-- 13
Os_ETR2         -----------------MP---------------------------------PIP--S-- 6
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Cs_ETR2         ------------GFLIL--------LLLASVS-----AADN--GFPRCN-CDDEG-S--- 35
At_ETR2         ------------WLLIL--------SMVVFVSPV---LAINGGGYPRCN-CEDEGNS--- 40
Bo_ETR2         ------------GLLILF-------SILIWVSPA---AAGNGGG---CN-CEDEGVS--- 39
Ls_ETR2         ------------LGLIS--------LLLVGVLAVDDDGSEN--GFGGCN-CEVEG----- 39
At_EIN4         ------------GLLLF--------ALLALVS------GDN--DYVSCN-CDDEG----- 33
Ls_ETR3         ------------GFLIA--------SLILTVSL----ANEN--EFAHCH-CDDEG----- 35
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Zm_ETR2         ------------AWILL-------FLSSLLLS--PSAASV---DFGHCGGCDDADDG--- 46
Os_ETR2         ------------LWIRV-------FFSWLLLSL-PAAAAA---DFSHCGGCDDGDGGG-- 41
Os_ETR3         IFLPLAQQQQLVHWMMPPRFRCQDYLLPLLLALSPAAAPARQVEYHHCH-CDGGGGGGGG 119
                                                            *   *

At_ETR1         -WPADELLMKYQYISDFFIAIAYFSIPLELIYFVK-KSAVFP-YRWVLVQFGAFIVLCGA 67
Pp_ETR2         -LWSIESILECQRVSDFLIAVAYFSIPIELLYFVS-CSNVP--FKWVLFQFIAFIVLCGL 91
Cs_ETR2         -LWSIDSILECQRVSDFLIAVAYFSIPIELLYFVS-CSNVP--FKWVLFQFIAFIVLCGL 91
At_ETR2         -FWSTENILETQRVSDFLIAVAYFSIPIELLYFVS-CSNVP--FKWVLFEFIAFIVLCGM 96
Bo_ETR2         -FWSTENILETQRVSDFLIAVAYFSIPIELLYFVS-CSNVP--FKWVLFEFIAFIVLCGM 95
Ls_ETR2         -FFGYRNIMETQRVSDFLIAVAYFSIPIELLYFVS-CSNVP--FKWVLFQFIAFIVLCGM 95
At_EIN4         -FLSVHTILECQRVSDLLIAIAYFSIPLELLYFIS-FSNVP--FKWVLVQFIAFIVLCGM 89
Ls_ETR3         -SWNAH-IIESQRVSDFLIAIAYFSIPLELLYFLS-CSNVP--FKWVLVQFIAFIVLCGL 90
At_ERS2         -LFSYETILNSQKVGDFLIAIAYFSIPIELVYFVS-RTNVPSPYNWVVCEFIAFIVLCGM 99
Zm_ETR2         ALSSTYNILQCQKVSDFLIAAAYFSIPLELLYFAT-CSDLFP-LKWIVLQFGAFIVLCGL 104
Os_ETR2         GIWSTDNILQCQRVSDFLIAMAYFSIPLELLYFAT-CSDLFP-LKWIVLQFGAFIVLCGL 99
Os_ETR3         GLWSMDSIFRWQKVSDLLIAAAYFSIPLEILYFVAGLRHLLP-FRWVLVQFGAFIVLCGL 178
                       ::. * :.*::** ******:*::**      :    .*:: :* *******

At_ETR1         THLINLWT-FTTHSRTVALVMTTAKVLTAVVSCATALMLVHIIPDLLSVKTRELFLKNKA 126
Pp_ETR2         THLLNGWT-YGPHPFQLMLALTVFKILTALVSCATAITLITLIPLLLKVKVREFMLKKKT 150
Cs_ETR2         THLLNGWT-YGPHSFQLMLALTVFKILTALVSCATAITLITLIPLLLKVKVREFMLKEKT 150
At_ETR2         THLLHGWT-YSAHPFRLMMAFTVFKMLTALVSCATAITLITLIPLLLKVKVREFMLKKKA 155
Bo_ETR2         THLLHGWT-YGPHPFKLMVALTVFKMLTALVSCATAITLITLIPLLLKVKVREFMLKKKA 154
Ls_ETR2         THLLNGWT-YEPHPFQLMLALTIFKFLTALVSFATAITLVTLIPLLLKVKVREFMLRKKT 154
At_EIN4         THLLNAWTYYGPHSFQLMLWLTIFKFLTALVSCATAITLLTLIPLLLKWKVRELYLKQNV 149
Ls_ETR3         THLINGWGYYGNQTFQLMMALTVAKLLTALVSCATAITLLTLIPLLLKFKVRELFLKQNV 150
At_ERS2         THLLAGFT-YGPHWPWVMTAVTVFKMLTGIVSFLTALSLVTLLPLLLKAKVREFMLSKKT 158
Zm_ETR2         THLITVFT-YEPHSFHLVLALTVAKFLTALVSFATAITLLTLIPQLLRVKVRENFLMNKA 163
Os_ETR2         THLITMFT-YEPHSFHVVLALTVAKFLTALVSFATAITLLTLIPQLLRVKVRENFLRIKA 158
Os_ETR3         THLLTAFT-YEPHPFMVVLLLTTAKFLTALVSFLTAITLLTLIPQLLRVKVRESLLWLKA 237
                ***:  :  :  :   :  .*  *.**.:**  **: *: ::* **  *.**  *  :.

At_ETR1         AE 128
Pp_ETR2         WD 152
Cs_ETR2         WD 152
At_ETR2         HE 157
Bo_ETR2         HE 156
Ls_ETR2         WD 156
At_EIN4         LE 151
Ls_ETR3         LE 152
At_ERS2         RE 160
Zm_ETR2         RE 165
Os_ETR2         RE 160
Os_ETR3         RE 239
                 :
```

Figure 5-2 Sequence alignments of EBDs from various plants

(A)Amino acid sequences of the transmembrane region of subfamily I ETR are aligned. (B) Amino acid sequences of the transmembrane region of subfamily I ERS and AtETR1 are aligned. (C) Amino acid sequences of the transmembrane region of subfamily II ethylene receptors and AtETR1 are aligned. All the alignments are performed using the ClustalW tool (Larkin et al., 2007). The accession numbers of all the protein sequences used for alignment are listed in Table III. The amino acid residues of each protein are numbered at the right. Red boxes indicate conserved disulfide cysteines at the N-terminal end of ethylene receptors. Green boxes indicate amino acids residues at positions corresponding to the proline residue at the ninth residue of AtETR1. Stars mark three subfamily I ERS receptors (Os_ERS, Pa_ERS1 and Pha_ERS) which possess the proline residue conserved in subfamily I ETR1 receptors.

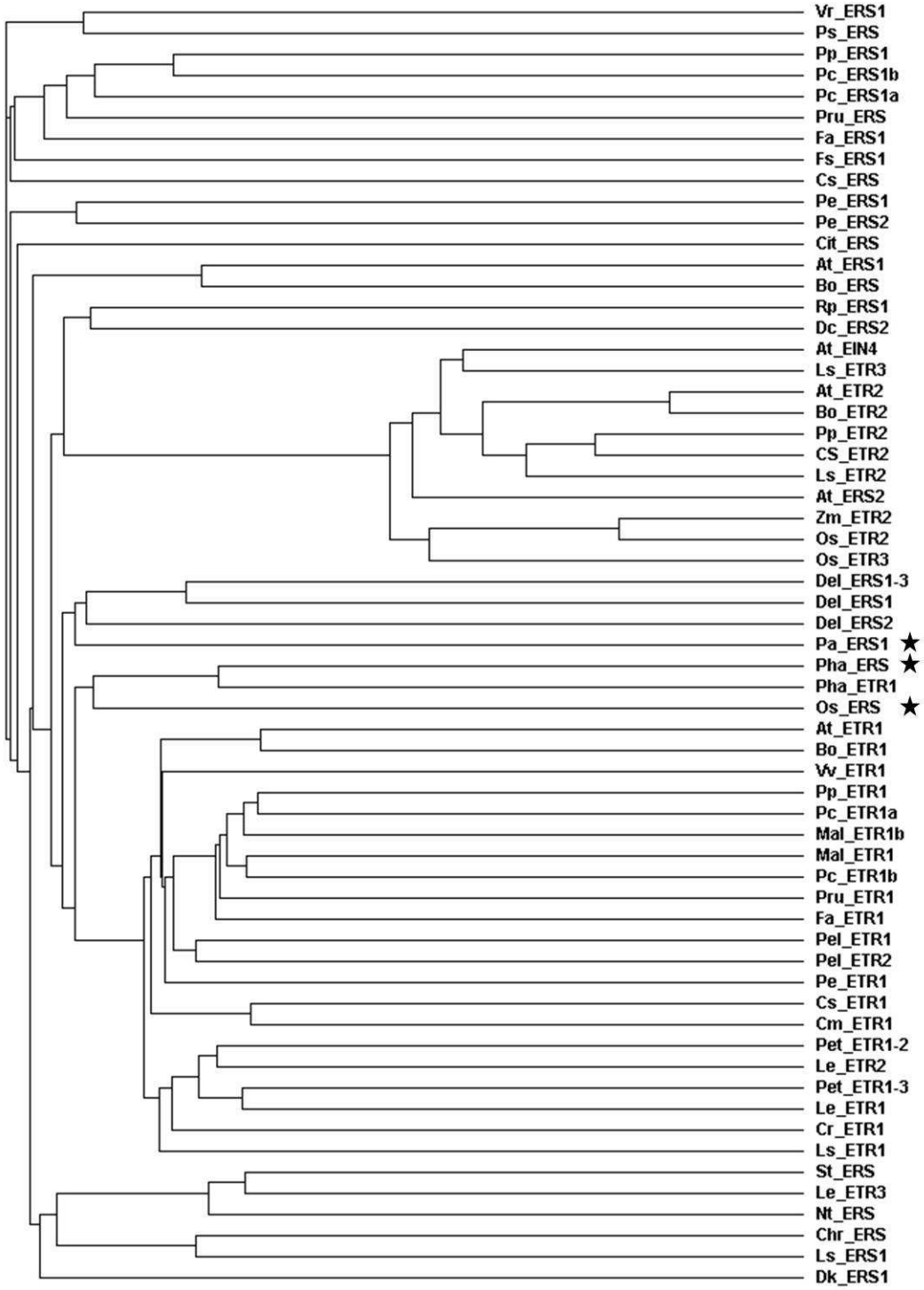


Figure 5-3 Phylogenic relationships of ethylene receptors from various plants

The full length ethylene receptors sequences listed in Table III are aligned and used to generate a phylogenic tree using the neighbor-joining algorithms of the ClustalW web tool (Larkin et al., 2007). Stars indicate the three subfamily I ERS receptors (Os_ERS, Pa_ERS1 and Pha_ERS) which possess the proline residue conserved in subfamily I ETR1 receptors.

Table III Sequences used for alignments in Figure 5-2 and the phylogenic tree in Figure 5-3

Species	Abbrevations	Subfamily	Acronym	GeneBank Description	GeneBank GI	Accession
Nicotiana tabacum	Nt	I-ERS	ERS	ethylene receptor ERS homolog	7652766	AAB96765
Arabidopsis thaliana	At	I-ETR	ETR1		902360	AAA70047
Vitis vinifera	Vv	I-ETR	ETR1	putative ethylene receptor	7547007	AAF63755
Prunus persica	Pru	I-ETR	ETR1	ethylene receptor	6841075	AAF28893
Phalaenopsis equestris	Pha	I-ERS	ERS	ethylene response sensor	64967421	CAD91247
Cucumis sativus	Cs	I-ERS	ERS	ethylene receptor CS-ERS	6136816	BAA85818
Cucumis sativus	Cs	I-ETR	ETR1	ethylene receptor CS-ETR1	6136814	BAA85817
Delphinium x belladonna	Del	I-ERS	ERS2	ethylene receptor	58530712	BAD89292
Delphinium x belladonna	Del	I-ERS	ERS1-3	ethylene receptor	58530710	BAD89291
Malus x domestica	Mal	I-ETR	ETR1b	ethylene receptor	58293963	AAW69924
Pyrus pyrifolia	Pp	I-ERS	ERS1	ethylene receptor	54260392	BAD61002
Pyrus pyrifolia	Pp	I-ETR	ETR1	ethylene receptor	54260390	BAD61001
Lycopersicon esculentum	Le	I-ERS	ETR3	ethylene receptor never ripe	52222392	AAU34075
Pelargonium x hortorum	Pel	I-ETR	ETR2	ethylene receptor homolog	5006606	AAD37577
Pelargonium x hortorum	Pel	I-ETR	ETR1	ethylene receptor homolog	5006604	AAD37576
Phalaenopsis cv. 'True Lady'	Pha	I-ETR	ETR1	ethylene receptor homolog	4650821	AAD26899
Diospyros kaki	Dk	I-ERS	ERS1	ethylene receptor	44885996	BAD11810
Solanum tuberosum	St	I-ERS	ERS	ethylene receptor homolog	4210924	AAD12777
Passiflora edulis	Pe	I-ERS	ERS1	ethylene response sensor	4164161	BAA37137
Passiflora edulis	Pe	I-ETR	ETR1	ethylene receptor	4164159	BAA37136
Vigna radiata	Vr	I-ERS	ERS1	ethylene response sensor	4138853	AAD03598

Species	Abbrevations	Subfamily	Acronym	GeneBank Description	GeneBank GI	Accession
Citrus sinensis	Cit	I-ERS	ERS	putative ethylene receptor	4092526	AAC99435
Chrysanthemum x morifolium	Chr	I-ERS	ERS	ethylene responsive sensor	39725602	BAD04924
Malus x domestica	Mal	I-ETR	ETR1	ethylene receptor	3411051	AAC31123
Lactuca sativa	Ls	I-ETR	ETR1	putative ethylene receptor ETR1	33341112	AAQ15122
Lactuca sativa	Ls	I-ERS	ERS1	putative ethylene receptor ERS1	33341110	AAQ15121
Catharanthus roseus	Cr	I-ETR	ETR1	putative ethylene receptor	33330872	AAQ10679
Pisum sativum	Ps	I-ERS	ERS	ethylene receptor	3123666	CAA06723
Delphinium 'MagicFountains dark blue'	Del	I-ERS	ERS1	ethylene response sensor 1	29365505	BAC66450
Brassica oleracea	Bo	I-ERS	ERS	ethylene response sensor	2896031	AAC31157
Brassica oleracea	Bo	I-ETR	ETR1	ethylene receptor	2896029	AAC39497
Dianthus caryophyllus	Dc	I-ERS	ERS2	putative ethylene receptor	2662475	AAC03716
Arabidopsis thaliana	At	I-ERS	ERS1	ethylene response sensor (ERS)	23197902	AAN15478
Oryza sativa	Os	I-ERS	ERS	ethylene responsive factor	2281705	AAB72193
Rumex palustris	Rp	I-ERS	ERS1	ethylene response sensor protein	2243158	CAA69646
Prunus persica	Pru	I-ERS	ERS	ethylene-responsive sensor	22036194	AAM89517
Persea americana	Pa	I-ERS	ERS1	ethylene response sensor 1	20386499	AAM21684
Passiflora edulis	Pe	I-ERS	ERS2	ethylene receptor	19032291	BAB85633
Fagus sylvatica	Fs	I-ERS	ERS1	ethylene receptor 1	18496061	CAD21848
Pyrus communis	Pc	I-ERS	ERS1b	putative ethylene receptor	18252345	AAL66204
Pyrus communis	Pc	I-ERS	ERS1a	putative ethylene receptor	18252335	AAL66199
Petunia x hybrida	Pet	I-ETR	ETR1-3	ethylene receptor	17646117	AAL40903
Fragaria x ananassa	Fa	I-ERS	ERS1	ethylene receptor	15131531	CAC48385
Lycopersicon esculentum	Le	I-ETR	ETR2	ethylene receptor	52222390	AAU34074

Species	Abbrevations	Subfamily	Acronym	GeneBank Description	GeneBank GI	Accession
Lycopersicon esculentum	Le	I-ETR	ETR1		1163081	AAA85479
Cucumis melo var.reticulatus	Cm	I-ETR	ETR1	ethylene receptor	11611626	BAB18937
Fragaria x ananassa	Fa	I-ETR	ETR1	ethylene receptor	15131529	CAC48384
Petunia x hybrida	Pet	I-ETR	ETR1-2	ethylene receptor	17646115	AAL40902
Pyrus communis	Pc	I-ETR	ETR1b	putative ethylene receptor	18252341	AAL66202
Pyrus communis	Pc	I-ETR	ETR1a	putative ethylene receptor	18252351	AAL66207
Arabidopsis thaliana	At	II	ERS2	putative ethylene receptor	3687656	AAC62209
Zea mays	Zm	II	ETR2	ethylene receptor homologue	10241927	BAB13718
Oryza sativa (indica cultivar-group)	Os	II	ETR3	putative ethylene receptor	38045981	AAR08915
Oryza sativa (indica cultivar-group)	Os	II	ETR2	putative ethylene receptor	32699977	AAN15203
Lactuca sativa	Ls	II	ETR2	putative ethylene receptor ETR2	33341114	AAQ15123
Lactuca sativa	Ls	II	ETR3	putative ethylene receptor ETR3	33341116	AAQ15124
Arabidopsis thaliana	At	II	ETR2	putative ethylene receptor	3687654	AAC62208
Arabidopsis thaliana	At	II	EIN4	putative ethylene receptor	4105633	AAD02485
Pyrus pyrifolia	Pp	II	ETR2	ethylene receptor	54260394	BAD61003
Cucumis sativus	Cs	II	ETR2	ethylene receptor CS-ETR2	6136818	BAA85819
Brassica oleracea	Bo	II	ETR2	ethylene receptor	29824097	BAB84007

Proline 9 converts *ERS1* (*A102T*) into an *RTE1*-dependent ethylene-insensitive allele

The A102T (*etr1-2*) substitution, which lies within the third predicted transmembrane domain of the ethylene binding domain, is known to confer dominant ethylene insensitivity when present in the ETR1 receptor, and is dependent on RTE1 for ethylene insensitivity (Resnick et al., 2006). In contrast, the A102T substitution, when was introduced to the corresponding conserved position in the *ERS1* coding sequence using in vitro site-directed mutagenesis, fails to cause ethylene insensitivity (Rivarola et al., 2009). We proposed that the different effect of A102T substitution on ETR1 and ERS1 is due to the unique intrinsic steric structure of ETR1 EBD, which may be maintained by RTE1. As described above, P9 is a unique residue that can be found only in ETR1 orthologs, not in other ethylene receptors. ERS1 possesses a threonine at the corresponding position of ETR1 proline 9 (**Figure 5-1**). To investigate whether this unique P9 is responsible for the distinction of ETR1 from other ethylene receptors, by means of site-directed mutagenesis, I introduced P9 to *ERS1* carrying the A102T substitution and driven by the native *ETR1* promoter, to see whether it can render *ERS1* (*A10T*) transgenic plants ethylene insensitive (**Figure 5-4**). The construct was stably transformed into wild-type (Col-0) plants. 13 out of 16 independent transgenic lines showed ethylene insensitivity to varying degrees. Four representative lines are shown in **Figure 5-5**. In contrast, all 17 independent *ERS1* (*A102T*) transgene lines showed the same phenotype as the wild type (Col-0). Since the A102T substitution in ETR1 causes an *RTE1*-dependent ethylene insensitivity, I predicted that the ethylene insensitivity conferred by the *ERS1* (*A102T*, *T9P*)

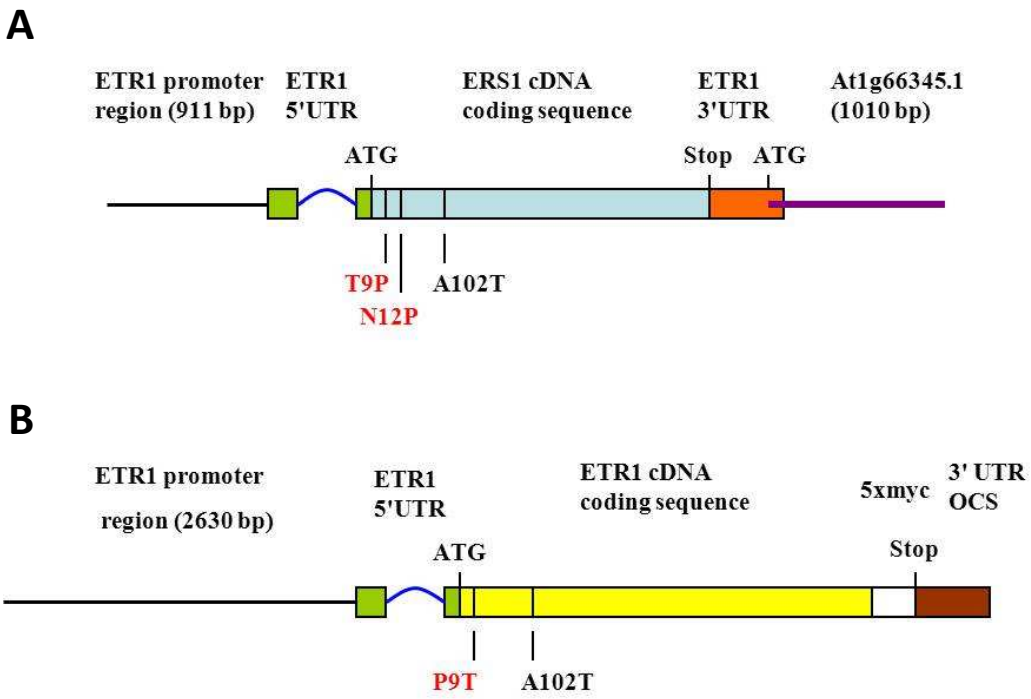


Figure 5-4 Diagrams of *ERS1* and *ETR1* transgene constructs

(A) Diagram of *ERS1* transgenes carrying A102T mutation only or both T9P and A102T mutations or all T9P, T12P and A102T mutations. *ERS1* cDNA coding sequence (light blue) is driven by the *ETR1* native promoter region (black line) and carries the *ETR1* 5' UTR (green) which has an intron (blue line) and 3' UTR (orange) in the binary vector pPZP221. A small 5' portion of the gene that is situated downstream of *ETR1* in the chromosome (purple line) is also present in each construct. (B) Diagram of *ETR1* transgenes (Dong et al., 2008) carrying only the A102T mutation or both P9T and A102T mutations. The *ETR1* cDNA coding sequence (yellow) with a C-terminal 5 x myc tag (white) is driven by the *ETR1* native promoter region (black line) and carries the *ETR1* 5'UTR (green) which has an intron (blue line) and 3' OCS terminator sequence (dark red) in the binary vector pMLBart.

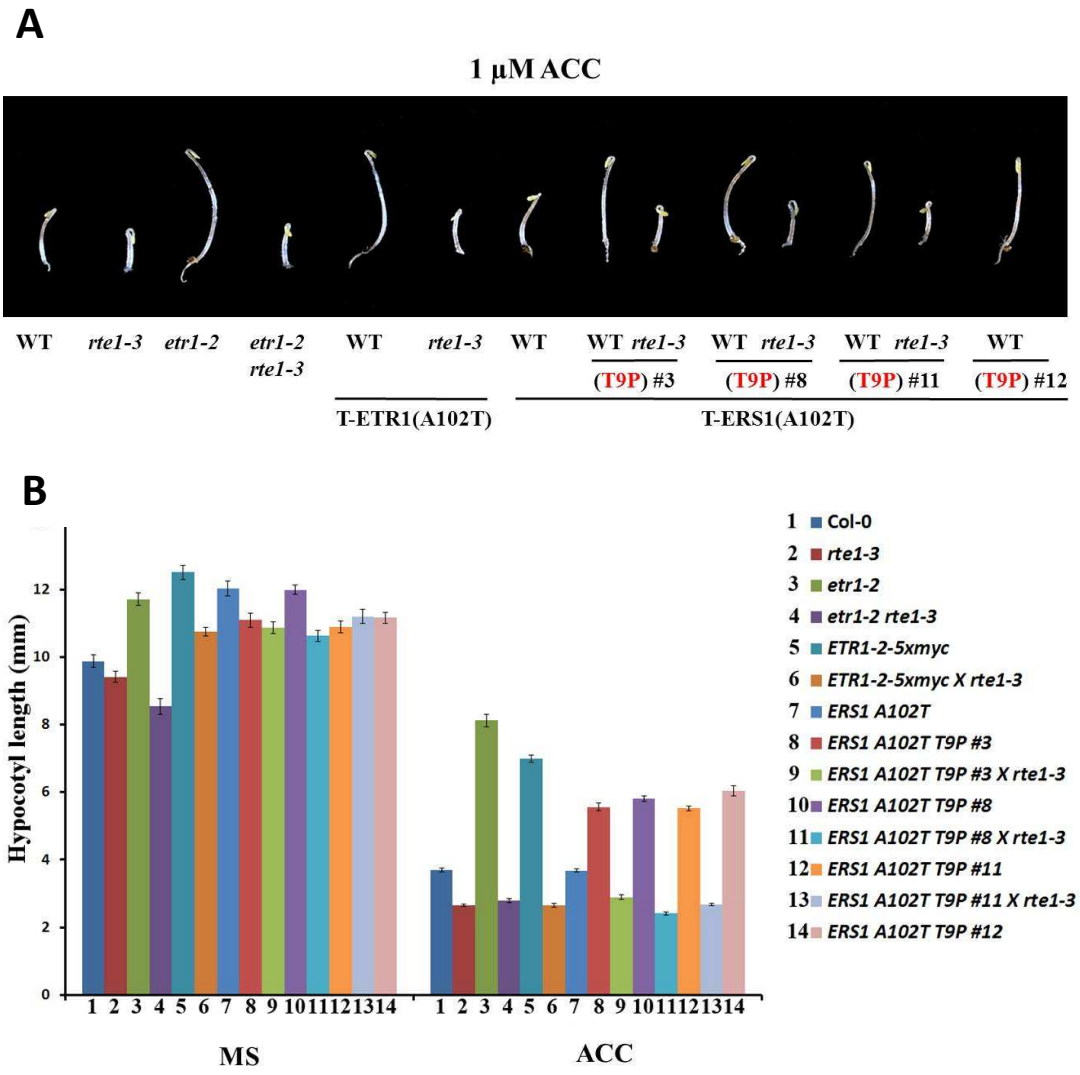


Figure 5-5 Proline 9 converts *ERS1* (*A102T*) into an *RTE1*-dependent ethylene-insensitive allele

The seedling triple-response assay shows the presence or absence of ethylene insensitivity conferred by ethylene receptor *ETR1* and *ERS1* transgenes carrying the indicated amino acid substitutions in WT vs. *rte1-3* background. The wild type Arabidopsis transformed with the *ETR1* and *ERS1* transgene driven by the native *ETR1* promoter (shown in Fig 5-4) carrying A102T substitution display ethylene

insensitivity and triple response respectively. The ethylene insensitivity conferred by the *ETR1* transgene carrying A102T substitution is suppressed when crossed with *rte1-3*. The wild type Arabidopsis transformed with the *ERS1* transgene driven by the native *ETR1* promoter (shown in Fig 5-4) carrying both A102T and T9P substitutions displays ethylene insensitivity, but triple response when crossed with *rte1-3*. Four independent *ERS1* (*A102T, T9P*) transgenic lines in the wild type background (#3, #8, #11 and #12) were shown, three of which (#3, #8, #11) were crossed with *rte1-3* respectively. (A) Shown are representative four-day-old dark grown seedlings homozygous in either the indicated transgene or both the indicated transgene and *rte1-3* loci in the presence of 1 µM ACC. (B) Measurements of hypocotyl length for the homozygous seedlings shown in (A) in the presence or absence of 1µM ACC. The mean±SE is shown for 18-32 seedlings measured for each genotype at MS and 1µM ACC.

transgene is also dependent on *RTE1*. I crossed three representative *ERS1* (*A102T, T9P*) transgenic lines with the *rte1-3* null mutant to see whether the ethylene insensitivity can be suppressed by *rte1-3*. As I predicted, *rte1-3* reverts the insensitivity exhibited by all the three *ERS1* (*A102T, T9P*) transgenic lines to a phenotype comparable to that of the wild type (**Figure 5-5**). When I also transformed the *ERS1* (*A102T, T9P*) construct directly into the *rte1-3* mutant, I found that all 10 independent lines exhibited the ethylene triple response just like the wild type. These results suggest that ERS1 (A102T) is converted into an *RTE1*-dependent ethylene receptor by introducing P9.

In addition to P9, there is another proline (P12) that is present in ETR1 but absent in ERS1 throughout the EBD. To determine whether P12 plays a similar role as P9, I introduced both P9 and P12 into the *ERS1* (*A102T*) transgene. My results showed that introducing P12 does not enhance the *RTE1*-dependent ethylene insensitive phenotype conferred by the *ERS1* (*A102T, T9P*) transgene line, indicating that P12 does not play a role in RTE1 dependence (data not shown).

Loss of proline 9 is not sufficient to convert *ETR1* (*A102T*) into an ethylene sensitive allele

Next, I wanted to test whether ETR1 (A102T) can be converted into an *RTE1*-independent ethylene receptor by replacing P9. I created the reciprocal substitution (P9T) in the *ETR1* coding sequence transgene carrying the A102T substitution and driven by the *ETR1* promoter (**Figure 5-4**). The construct was stably transformed into both the wild type (Col-0) and *etr1-7* null mutant. 9 out of 15 independent transgenic lines in the wild type background and 14 out of 27 independent transgenic lines in the

etr1-7 null mutant background still exhibited ethylene insensitivity when grown on MS media containing ACC (**Figure 5-6**). This result suggests that losing P9 is not sufficient to convert the ETR1 (A102T) receptor to an ethylene receptor like ERS1.

RTE1 could be involved in regulating ETR1 dimerization through affecting disulfide bond formation cooperating with P9

The above results indicate that P9 is important for *RTE1* dependence, but the basis for this dependence remained unknown. We proposed that P9 might be involved in the steric conformation of the ETR1 EBD, which requires RTE1. Since P9 presumably lies close to the known inter-molecular disulfide bond-forming cysteines (C4, C6) in the ETR1 receptor homodimer (Schaller et al., 1995), and we know that disulfide bond formation can affect protein folding, I next wanted to investigate whether ETR1 dimerization is affected in the absence of *RTE1*. I isolated the microsomal protein fraction from existing ETR1-5xmyc transgenic lines in the *etr1-7* null mutant background and the *etr1-7 rte1-2* double null mutant background, which were described in (Resnick et al., 2008). N-ethylmaleimide (NEM) was used to prevent free sulfhydryls from forming disulfide bridges in the process of protein extraction. ETR1-5xmyc proteins were visualized on a Western blot using an anti-Myc antibody. In the presence of reducing reagent (DTT), the band of predicted ETR1-5xmyc monomer size was detected in both the *etr1-7* mutant and the *etr1-7 rte1-2* double mutant (**Figure 5-7**). In the absence of reducing reagent (DTT), ETR1-5xmyc was found to form homo-dimers in both mutants (**Figure 5-7**). However, a small portion of monomer was observed in the *etr1-7 rte1-2* double mutant, than in the *etr1-7* mutant, in the absence of reducing reagent (DTT) (**Figure 5-7**). The above

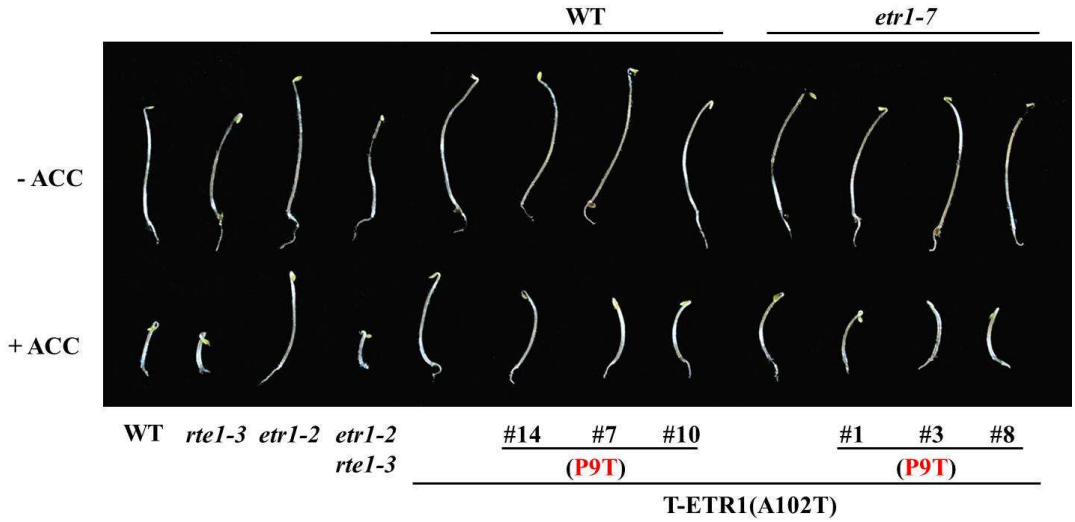


Figure 5-6 Loss of proline 9 is not sufficient to convert *ETR1* (*A102T*) into an *RTE1*-independent ethylene-sensitive allele

Representative four-day-old dark grown seedlings transformed with the indicated transgene in the absence or presence of 1 µM ACC are shown. *ETR1* transgenes under the control of the native *ETR1* promoter (shown in Fig 5-4) carrying either A102T only or both A102T and P9T substitutions were transformed into the wild type Arabidopsis and the *etr1-7* null mutant. Three independent *ETR1* (*A102T P9T*) transgenic lines in the wild type (#14, #7, #10) or *etr1-7* (#1, #3, #8) background are shown.

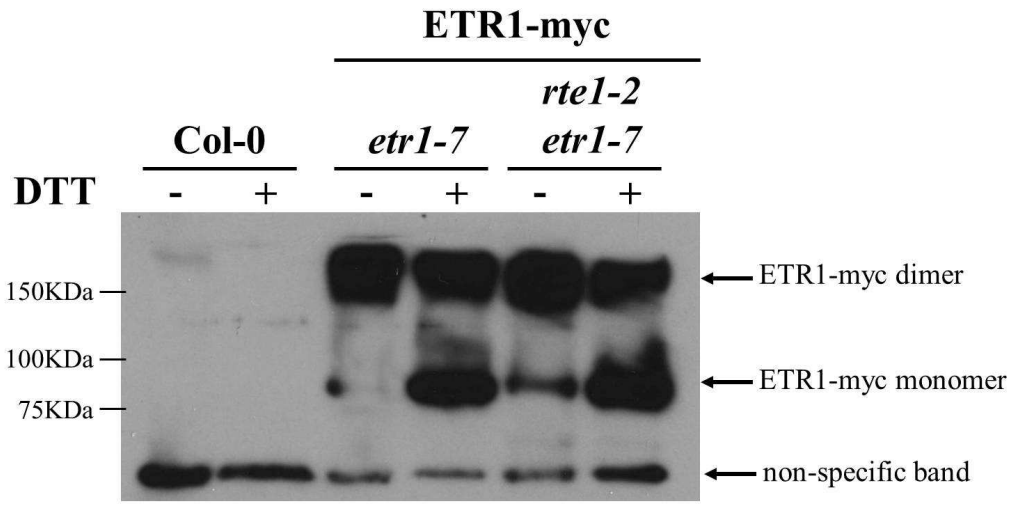


Figure 5-7 Disulfide-linked dimerization of ETR1 may be affected in *rte1-2*

Membrane fractions from 9 day-old Arabidopsis seedlings were incubated in the absence (-) or presence (+) of 150 mM DTT for 1 hour at 37℃. Protein was subjected to 10% SDS-PAGE. ETR1-5x myc was detected by the anti-*c*-myc antibody. In the absence of DTT, a small portion of ETR1-5x myc monomer is seen in the *etr1-7 rte1-2* mutant, but almost not seen in the *etr1-7* mutant. The ETR1-5x myc monomer is visualized at approximately 80 kDa and the ETR1-5x myc homo-dimer is detected at about 150 KDa. Neither the ETR1-5x myc monomer nor homo-dimer band can be detected in the wild type lacking the ETR1-5x myc transgene. A non-specific band of lower molecular weight is detected in all samples and used for the loading control.

findings suggest that ETR1disulfide bonds at C4 and C6 could be partially disrupted in the absence of *RTE1*.

The yeast split-ubiquitin assay of the interaction of ERS1 (A102T, T9P) and RTE1

Dong *et al*. (2010) found that RTE1 and ETR1 associate when expressed transiently in tobacco cells using BiFC, whereas a very weak signal was detected for ERS1 and RTE1, suggesting that RTE1 may specifically interact with ETR1. Therefore, the specificity of RTE1 for ETR1 might be due to the specific interaction of RTE1 with ETR1. Moreover, the above tests indicate that the introduction of P9 converts ERS1 into an *RTE1*-dependent ethylene receptor. To test the possibility that the specific interaction of RTE1 with the ethylene receptors depends on P9, I tried to test the interaction of RTE1 with ERS1 (A102T) with/without P9 as well as ETR1 (A102T) with/without P9 using the yeast split ubiquitin assay. However, unfortunately, the Nub/Cub fused ERS1 and ETR1 proteins appear to be non-functional when expressed in yeast, since ERS1 and ETR1 didn't interact with themselves in this assay, which are supposed to be homo-dimers.

Discussion

Our results show that the introduction of a proline at position 9 of ERS1 A102T confers *RTE1*-dependent ethylene insensitivity to ERS1, suggesting an important role of P9 in the specificity of RTE1 for ETR1. It is likely that the molecular state of ERS1 (A102T T9P) mimics that of ETR1 (A102T), which is *RTE1* dependent.

Proline's side chain has a distinctive cyclic structure, which gives proline an exceptional conformational rigidity compared to other amino acids. Therefore P9 could be critical to form a distinct EBD conformation that requires RTE1 to properly function. However, P9 seems to be essential, but not sufficient, to render RTE1 dependence, since I found that the absence of P9 does not result in the complete loss of RTE1-dependent ethylene insensitivity conferred by the *ETR1* (*A102T*) allele. Another non-conserved proline (P12) does not appear to be involved in *RTE1* dependence. Thus, there might be residue(s) other than P12 cooperating with P9 to build the unique EBD structure.

It is noteworthy that P9 sits close to two conserved cysteines (C4 and C6), which form two intermolecular disulfide bonds in the ethylene receptor dimers. Interestingly, we observed a small portion of ETR1 monomer in the *rte1* mutant in the absence of the reducing reagent, suggesting the disulfide links of a small portion of ETR1 is disrupted in the absence of RTE1. The large proportion of the ETR1 receptor is still present in the covalently linked dimer, but it is possible that the ETR1 dimer on the western blot might not be the correctly formed dimer because ETR1 is largely non-functional, based on prior genetic analyses of *rte1*. This result suggests that the *rte1* mutation does not result in the loss of all ETR1 dimerization. However, it is still likely that RTE1 has effects on the disulfide bonds since we indeed observed a small portion of monomerized ETR1 in *rte1-2*.

How can a proline residue affect a protein's structure and function? I propose several hypotheses here. There is a possible parallel in the role of some specific proline in the folding of Superoxide Dismutase 1 (SOD1) with that in the ETR1

receptor function. In the process of activation of SOD1, a copper cofactor and oxidation are required for proper folding. Compared with mammalian SOD1, which doesn't need a chaperone protein for maturation, yeast SOD1 depends on Copper Chaperone for SOD1 (CCS) to obtain copper and fold correctly. The specificity of CCS on the yeast SOD1 homolog is largely due to the presence of a proline (P144) at the C-terminus of yeast SOD1 inhibiting the oxidation of a critical intramolecular disulfide bond required for the active SOD1 conformation (Carroll et al., 2004). The conformation block by P144 is overcome by CCS (Leitch et al., 2009). Notably, we found RTE1 dependent activation of ETR1 shares a couple of similarities as the activation of CCS dependent yeast SOD1. They both require copper and redox regulation for correct structure and function. They both are distinct from other homologs in terms of 'chaperone' dependence. They both have a unique proline near the conserved cysteine(s) within the end region of the protein. In both proteins, the unique proline is critical for the dependence of the chaperone protein. Therefore, it is highly possible that RTE1 functions like CCS. Probably similar to P144 in yeast SOD1, P9 in ETR1 may place a conformational restriction on the correct disulfide bonds formation and/or lead to incorrect inter- and/or intra-molecular disulfide bonds among C4, C6, C65 and C99, therefore resulting in a non-functional ETR1 EBD conformation. The small portion of the monomer ETR1 observed in the western blot may result from one kind of wrong disulfide bonds. RTE1 may act as a molecular chaperone, reducing the wrong disulfide bonds and aiding the correct disulfide bond formation. Those *RTE1*-independent ethylene receptors and *etr1* mutant alleles could form the functional conformation independently due to the lack of the structural

restriction on those correct disulfide bonds by P9. Thus they are independent of RTE1. Previous studies showed ETR1 carrying substitutions of C4 and C6 to Ala or Ser is still a functional receptor, suggesting the intermolecular disulfide bonds are not critical to ethylene receptor function (Chen et al., 2010; Xie et al., 2006). However, the wrong disulfide bonds as a result of P9 could be poisonous. RTE1's function could be to avoid the poisonous disulfide bonds. This model is consistent with the redox role of RTE1 proposed in the previous chapters. It is still unknown whether the specificity of AtCb5 for ETR1 is also related to P9.

Alternatively, P9 could affect the specific interaction of ethylene receptors with RTE1. The ETR1 receptor could interact with RTE1 because P9 causes a unique EBD conformation required for RTE1 interaction, whereas the ethylene receptors without P9 could not interact with RTE1 due to lack of the necessary conformation. The introduction of P9 to ERS1 (A102T) could convert it to a state which can be recognized by RTE1 and interact with RTE1. An example for the role of proline in the protein-protein interaction is the Rice GA receptor GIBBERELLIN-INSENSITIVE DWARF1 (GID1). The binding of GA to GID1 induces the formation of the GID1-GA-DELLA protein complex (Murase et al., 2008; Ueguchi-Tanaka et al., 2005). Wild type GID1 cannot interact with DELLA in the absence of GA. However, replacing proline at the 99^{th} residue in the loop region of GID1 to other amino acids causes GA-independent interaction of GID1 with the rice DELLA protein SLENDER RICE1 (SLR1), probably because the absence of proline places GID1 (P99A) in a molecular state mimicking that of wild-type GID binding the GA molecule (Yamamoto et al., 2010).

Another speculation is that RTE1 may function as peptidylprolyl isomerase (PPTase), which catalyzes the *cis-trans* isomerization of prolylpeptide bonds (Fischer and Schmid, 1990). Proline residues have the unique property of existing in both *cis* and *trans* isomers. Therefore the backbone switch in the polypeptide chain resulting from the prolypedptide bonds isomerization affects protein folding. Conceivably, the spontaneous folding of ETR1 EBD may favor a peptidyl-prolyl bond configuration at P9 that results in an inactive EBD conformation. RTE1 may convert the peptidyl-prolyl bond at P9 to a form required for the active EBD conformation.

Materials and Methods

Transgene Constructs and Plant Transformation

To introduce mutations into the *ERS1* and *ETR1* transgene constructs, *in vitro* site-directed mutagenesis was carried out using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). For *ERS1*, the missense mutations encoding T9P and the N12P were sequentially introduced into c-*ERS1* carrying the A102T mutation (the *ERS1* coding sequence driven by the *ETR1* native promoter and carrying the *ETR1* 3' UTR in the binary vector pPZP221, (Rivarola et al., 2009)). For *ETR1*, the missense mutation encoding P9T was introduced into c-*ETR1* carrying the A102T muation (the *ETR1* coding sequence driven by the *ETR1* native promoter and carrying the OCS terminator in the binary vector pMLBart, Dong et al. (2008)). All mutations were verified by nucleotide sequencing.

The sequences of the mutagenic primers are as follows:

ETR1 T9 For

5' GTCTGCAATTGTATTGAAACGCAATGGCCAGCG G 3'

ETR1 T9 Rev

5' CCGCTGGCCATTGCGTTTCAATACAATTGCAGAC 3'

ERS1 P9 For

5' CATGCGAT TGTTTTGAGCCGCATGTGAATCAAGATGAT G 3'

ERS1 P9 Rev

5' CATCATCTTGATTCACATGCGGCTCAAAACAATCGCA TG 3'

ERS1 P12 For

5' CGATTGTTTTGAGCCGCATGTGCCTCAAGATGATCTGTTAGTG 3'

ERS1 P12 Rev

5' CACTAACAGATCATCTTGAGGCACATGCGGCTCAAAACAATCG 3'

The mutated transgenes were transformed into wild-type plants and *etr1-7* mutant plants by the floral dip infiltration method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* strain GV3101. Transformed T_1 plants were selected on MS agar medium containing 90mg/L gentamycin for pPZP221 or were selected by spraying soil-grown plants with Basta (0.1% Finale^{TM}, Bayer Crop Science, Research Triangle Park, NC) for pMLBart. For each transgene, 10-20 independent transformed lines were examined in the T2 generation for segregation of ethylene insensitivity in the triple-response assay. For each transgene, measurements were made of 25-35 seedlings for two homozygous T3 lines.

To create the mutated transgenes in the *rte1-3* background, T2 lines were crossed with the *rte1-3* mutant and the F1 allowed to self-fertilize. In the resulting F_2

progeny, we identified plants that were homozygous for *rte1-3* (based on genotyping as described in Resnick et al. (2006)) and that carried at least one copy of the mutated *ERS1* or *ETR1* transgene (based on the gentamycin or Basta resistance marker). After self-fertilization of these F_2 individuals, the segregating homozygous transgene *rte1-3* double was identified in the resulting progeny on the basis of homozygous resistance to gentamycin or Basta.

Membrane protein isolation, SDS-PAGE and Western blotting

The ETR1-5xMyc transgenic lines in either *etr-7* null mutant or *etr1-7 rte1-2* double mutant background were described by Resnick et al. (Resnick et al., 2008). Membrane proteins isolation, SDS-PAGE and western blot analyses were performed as described by Dong et al. (Dong et al., 2008). Briefly, to isolate membrane proteins, 9 day-old light grown seedlings were homogenized in liquid nitrogen and then the extraction buffer (30mM Tris, pH 8.5, 150mM NaCl, 1mM EDTA and 20% v/v glycerol) containing 1% protease inhibitor cocktail (Sigma) , 10mM PMSF and 20mM NEM. The homogenate was strained through Miracloth and centrifuged at 8000g for 15min. Then the supernatant was centrifuged at 100000g for 1 hour. The membrane pellet was resuspended in 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% v/v glycerol and 1% Triton X-100 with protease inhibitors and 20mM NEM. Membrane proteins were mixed with 2x SDS loading buffer with or without 150 mM DTT, incubated in 37℃for 1 hour and then fractionated by SDS-PAGE on an 10% w/v polyacrylamide gel. After electrophoresis, proteins were electroblotted to a supported PVDF membrane (Bio-Rad). A 1:1000 dilution of the primary rabbit polyclonal anti-myc antibody (Sigma) was used, followed by a 1: 4000 dilution of the

goat anti-rabbit HRP secondary antibody (Pierce). Immunodecorated proteins were visualized by enhanced chemiluminescence detection using the SuperSignal West Femto maximum sensitivity kit (Pierce Chemical).

Chaper 6 : Conclusions and perspectives

The goal of this thesis has been to address how the novel protein RTE1 regulates ETR1 ethylene receptor signaling and investigate the basis for the specificity of RTE1 for ETR1, in order to advance our understanding of the plant hormone ethylene signaling pathway at the molecular level and provide a framework to understand the general role of RTE1 in other organisms.

Significance

This thesis adds a new player cytochrome *b*5 into the ethylene signaling pathway, advancing our understanding of the complexity of ethylene signaling. Prior to this work, we did not understand how RTE1 regulates ETR1 EBD conformation and how the specificity of RTE1 for ETR1 is achieved. The earlier models regarding the relationship between RTE1 and ETR1 were vague. This thesis provides a possible previously unknown mechanism for the regulation of the ETR1 ethylene receptor. The redox concept is introduced into the ethylene signaling pathway for the first time. This thesis also discovers the molecular basis for the difference between ETR1 and ERS1, therefore providing us a better understanding of the complex mechanism of ethylene receptor signaling between family members. In addition, this thesis suggests an important novel relationship between cytochrome b5 and RTE1. Of a broader significance, this connection advances our understanding of the conserved function of the RTE family in other organisms. Since animals do not possess ethylene receptor

homologs, the examination of the role of P9 in the RTE1 dependence may help to identify the targets of RTE1 action in animals.

A new hypothesis and the supporting evidence

The results in this thesis suggest several possible models for the specific regulation of ETR1 by RTE1. I favor that RTE1 could, together with AtCb5, play a role in the oxidative folding of the ETR1 EBD, promoting the active conformation of ETR1, since this model is the simplest and fits all the current data. In the absence of RTE1, the unique P9 in ETR1 conceivably places a conformational restriction on the formation of the intermolecular disulfide bonds at C4 and/or C6, leading to some incorrect inter- and/or intra-molecular disulfide bonds among the cysteine residues within the EBD such as C4, C6, C65 and C99, thereby resulting in a mis-folded EBD conformation that renders ETR1 inactive (**Figure 6-1 A**). In the presence of RTE1, RTE1 may promote an active EBD conformation by overcoming the conformation block by P9 and facilitating the formation of the correct disulfide bonds (**Figure 6-1 B**). AtCb5 may act upstream of RTE1 and activate RTE1 through giving the redox potential to RTE1 (**Figure 6-1 B**).

I propose the above model based on the following findings in this thesis.

1) The *rte1* loss-of-function mutation can be partially rescued by silver ions and cold temperature, which override the dependence of wild-type ETR1 and certain dominant ETR1 mutant alleles on RTE1. These findings support the hypothesis proposed by Resnick et al. (2008) that RTE1 is probably involved in regulating the conformational change of the ETR1 ethylene binding domain to promote the ETR1 receptor signaling. The silver ions are assumed to replace the copper cofactor and to

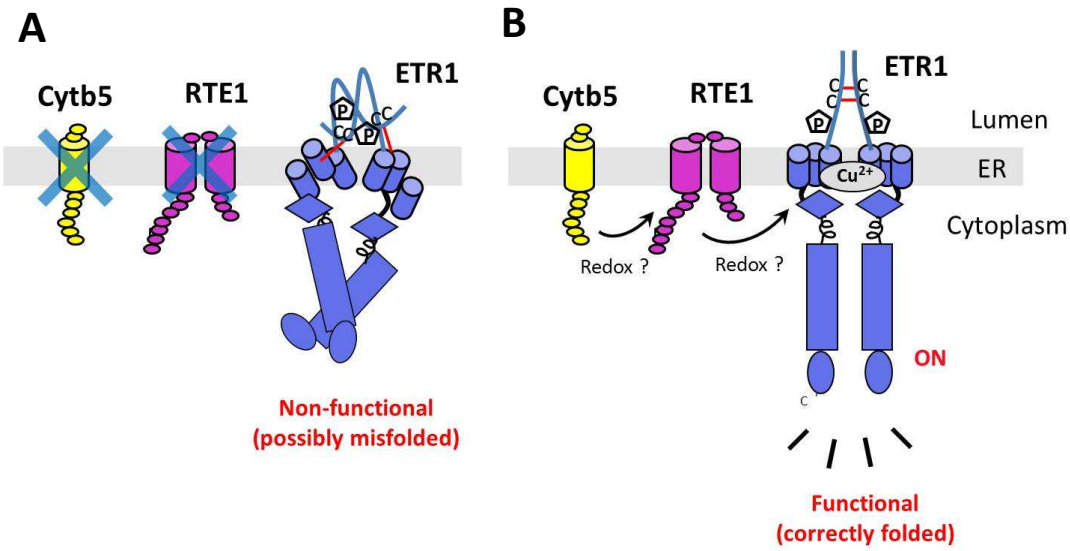


Figure 6-1 Model of regulating the oxidative folding of the ETR1 EBD by RTE1 and AtCb5

(A) In the absence of RTE1 and/or cytochrome b5 (Cytb5), proline 9 (indicated as a pentagon with "P") in the ETR1 EBD may place a conformational restriction on the nearby intermolecular disulfides formed at C4 and C6 of ETR1 homo-dimers, therefore leading to incorrect inter- and/or intra molecular disulfide bonds formed among C4, C6, C65 and C99, consequently resulting in a mis-folded ETR1 EBD which makes the ETR1 receptor non-functional. (B) RTE1 interacts with ETR1 to overcome the conformational block by proline 9 and allow oxidation of correct disulfide bonds, thus forming a correctly folded ETR1 EBD that can be capable of signaling. Cytb5 may act upstream of RTE1 and activate RTE1 by providing the redox potential to RTE1. The possibility that RTE1 acts upstream of cytb5 cannot be ruled out.

cause the EBD to be stuck in a conformational state that inhibits the conformational transition needed to shut ethylene receptor signaling off. Temperature also could affect protein folding and stability and the ethylene receptor EBD conformation could be sensitive to temperature change. Therefore, RTE1's function may be related to the conformation of ETR1 EBD.

2) Cytochrome b5, which is well known as an electron transfer hemoprotein involved in numerous oxidation/reduction reactions, appears to have functional parallels with RTE1 in regulating ETR1 receptor signaling. An ER membrane-localized Arabidopsis cytochrome b5 (AtCb5) isoform D was isolated from the screen for RTE1-interacting proteins using the yeast split-ubiquitin assay. The elaborate genetic analyses on mutants of three *AtCb5* isoforms revealed that these mutants have striking similarities with *rte1* in ethylene response defects and ethylene receptor allele specificity. In addition, genetic evidence showed that AtCb5 and RTE1 might act in the same pathway in regulating ETR1 signaling. Since AtCb5 can carry out a variety of redox reactions and RTE1 may function similarly as AtCb5, RTE1 might also be involved in redox reactions based on genetic suggestions. The ETR1 EBD conformation could be regulated through redox modification by AtCb5 and RTE1.

3) Interestingly, RTE1 homologs can bind heme in vitro, which supports the above hypothesis that RTE1 may carry out redox with AtCb5. It is still unclear which protein is upstream in the pathway that regulates ETR1. But I favor the model in which AtCb5s act upstream of RTE1 for a couple of reasons. Firstly, the *atcb5-d* loss-of-function mutation cannot block the reduced ethylene sensitivity conferred by overexpression of RTE1. This, however, does not rule out the possibility that RTE1

can be upstream regulator of several downstream cytb5 isoforms, since at least two other cytb5 family members also affect ethylene signaling. Secondly, RTE1 physically associates with ETR1, so I speculate that ETR1 could be the direct target of RTE1 action. Thirdly, cytb5 transfers electrons to numerous electron acceptor proteins such as the cytochrome P450 superfamily, thus regulating various redox reactions, whereas RTE1 has only one known target, ETR1. Given this known function of cytb5, it is conceivable that RTE1 accepts electrons from Atcb5.

4) The specificity of RTE1 for ETR1 had been a mystery since the identification of *RTE1*. The finding that a unique proline residue (P9) is largely responsible for the specificity of RTE1 for ETR1 has started to unlock this mystery. Introducing P9 into the *RTE1*-independent receptor ERS1 is sufficient to convert it into an *RTE1*-dependent receptor. Although why a proline residue can make ETR1 distinct from other ethylene receptors remains unclear, the observation that a small portion of ETR1 dimers are affected by the *rte1* mutation and the striking similarity between ETR1 activation by RTE1 and SOD1 activation by CCS raise the possibility that the presence of P9 may affect disulfide bonds that may be important for functional conformation of ETR1, just like the role of proline 144 in the CCS dependent SOD1 homolog. This hypothesis fits the above model that RTE1 and AtCb5 may regulate ETR1 EBD through redox modification, because redox affects disulfide bonds. Since P9 is only conserved in ETR1 orthologs, this suggests that all ETR1 orthologs may depend on RTE1.

Consistent with our theory that redox is important for proper folding of ETR1, Bueso *et al.*(2007) reported that the *catalase2* (*cat2*) mutant, in which H_2O_2 is

accumulated, displays ethylene insensitivity in seedlings ethylene triple response. The first 128 amino acids of ETR1 that are defined as EBD and C65 that is required for the copper cofactor binding are required for H_2O_2 sensing and/or signaling in H_2O_2 induced stomatal closure (Desikan et al., 2005). These results suggest that H_2O_2 sensing and/or signaling most likely involve oxidation of the cysteine 65 residue (as well as the other conserved N-terminal cysteines in ETR1) and oxidation of ETR1 may promote it to be more functional, thus leading to ethylene insensitivity. Based on this hypothesis, it will be interesting to test whether *cat2* can rescue the nonfunctional *ETR1* and *ETR1-2* receptor in the *rte1* null mutant. Since cytochrome b5 receives the redox potential from either NAPDH-cytochrome P450 reductase or NADH-cytochrome b5 reductase, we suspect that these reductases may be also involved in AtCb5 and RTE1 dependent ETR1 signaling. A question is whether mutants in these reductases have similar ethylene phenotypes as the *atcb5* and *rte1* mutants.

Questions, alternative models and future work

Although the above model is the simplest and fits all the current data, there are many questions remaining and several other possibilities cannot be ruled out. More investigations about whether ETR1 is subject to the redox modification by AtCb5 and RTE1 are needed. For example, to test whether the conserved Cys residues in ETR1 are subjected to redox regulation by RTE1, the redox status of ETR1 in the presence or absence of RTE1 can be examined by labeling and distinguishing between protein sulfhydryls (reduced Cys residues) and disulfides (oxidized Cys residues). The strategy of in vivo determination of the Cys redox status can be used as described by Despres et al. (2003). As I discussed in Chapter 3, it is highly possible that AtCb5 and

RTE1 affect the conformation of the ETR1 EBD by exerting effects on the membrane lipids. Cytochrome b5 is well known to play a role in lipid biosynthesis and metabolism by transferring electrons to and thus activating a variety of oxidases like fatty acid desaturase and hydroxylase (Schenkman and Jansson, 2003; Vergeres and Waskell, 1995). The ETR1 EBD conformation may be highly sensitive to membrane composition and fluidity. Notably, I isolated a non-specific lipid transfer protein (ns-LTP) (At1g48750), which could affect membrane lipid composition, from cDNA library screening for putative RTE1 interacting proteins using the yeast split-ubiquitin assay. In addition, cold temperature partially rescued the *rte1* mutation in two *etr1* dominant mutant alleles (*E38A* and *F58A*). Since cold temperature could cause lower membrane fluidity, it is speculated that a rigid membrane environment may prevent E38A and F58A receptors from switching towards the off state independently of RTE1. These results are consistent with the possibility that RTE1, together with AtCb5 and ns-LTP, affects ETR1 EBD conformation through exerting effects on the membrane environment where the ETR1 EBD resides. Another possibility is that the copper cofactor that is required for ethylene binding could be the target of redox regulation. The different redox states of copper may affect ETR1 EBD conformation. However, the evidence showed that RTE1 function seems to be unrelated to copper (Resnick et al., 2008).

It is still not known whether RTE1 can carry out redox reactions, although RTE1 may play a similar role as AtCb5 based on genetic analysis and the finding that RTE1 binds heme in vitro. A couple of specific questions need to be further investigated to help us to evaluate whether RTE1 could carry out redox communication with AtCb5.

For example, the physical interactions between AtCb5s and RTE1 as well as AtCb5s and ETR1 in planta need to be tested using BiFC. In addition, the same strategy proposed for testing in vivo redox states of ETR1in the last paragraph can be applied to measure in vivo redox status of Cys residues of RTE1 in *atcb5* mutants. Another strategy to measure whether RTE1 can carry out redox is to test redox changes in the *rte1 rth* double mutant using redox sensitive GFP (roGFP). We still don't know whether AtCb5 acts upstream or downstream of RTE1.The clear genetic epistasis of *AtCb5* and *RTE1* needs to be obtained by the test of whether the *AtCb5-D* over-expression phenotype (reduced ethylene sensitivity) can be blocked by the *rte1-3* null mutation.

Another question that needs to be validated is whether RTE1 binds heme in vivo. It is possible that RTE1 binds some other porphyrins in plants such as chlorophylls, bilins and corrins that are structurally similar to heme. A heme specificity test and an in vivo heme binding test are needed. If RTE1 were truly a hemoprotein, what is the possible molecular function of RTE1 as a hemoprotein? RTE1 could be involved in redox regulation of ETR1 together with AtCb5. Another speculation is that RTE1 may provide AtCb5 with heme, which exerts a redox effect on ETR1, or deliver heme to ETR1 (if ETR1 binds heme). To gain more insights into the question of whether AtCb5, RTE1 and ETR1 form a protein complex, tandem affinity purification (TAP) tagging and mass spectrometry can be used to identify proteins and compounds that interact with RTE1 and ETR1. It will not only verify whether cytochrome b5 interacts with RTE1 and ETR1 and whether RTE1 binds heme, but also uncover unknown protein posttranslational modification in RTE1 and ETR1 and identify proteins in the

RTE1 and ETR1 complex not discovered through the yeast-based screens. Our lab has already generated TAP-tagged RTE1 and ETR1 transgene plants. Both constructs have been shown to be able to rescue the corresponding loss-of-function mutant phenotype.

It is an exciting finding that proline at position 9, conserved only in ETR1 orthologs, plays a role in the specificity of RTE1 for ETR1. However, the underlying mechanism still remains unclear. It is speculated that P9 may cause a certain conformation in the EBD that requires RTE1. Is the ethylene insensitivity conferred by the introduction of P9 into the *ERS1* (*A102T*) transgene dependent on AtCb5s? If so, it will not only provide another parallel between AtCb5 and RTE1, but also suggest that P9 is related to redox. If P9 blocks the formation of intermolecular disulfide bonds at C4 and C6, thus leading to wrong disulfide bonds with other cysteines (in the absence of RTE1), I predict that eliminating C4 and C6 would make ETR1 and ETR1-2 independent of RTE1 since the poisonous disulfides would not be formed.

In addition to the possible redox role of RTE1, an alternative hypothesis is that RTE1 might have a function similar to peptidylprolyl isomerase (PPIase), which catalyzes the *cis-trans* isomerization of the peptidyl-prolyl bond (Schmid, 1993) (**Figure 6-2 A**). The nascent prolyl peptide bond between E8 and P9 of ETR1 might not be in the correct conformation in the native state during the folding of ETR1, resulting in blockage of the correct disulfide bonds and/or the formation of wrong disulfide bonds in the absence of RTE1 (**Figure 6-2 B**). Conceivably, RTE1 could convert the incorrect prolyl isomer of P9 to the correct one and thereby indirectly

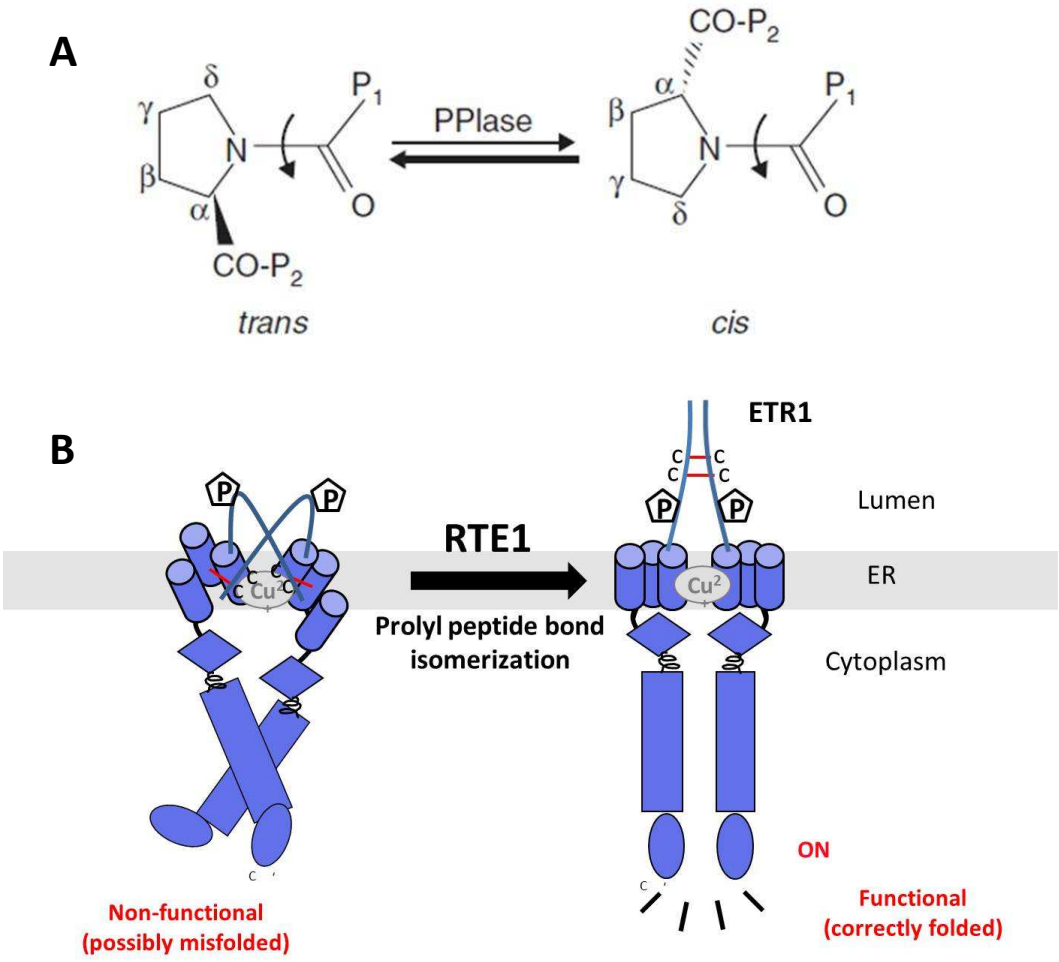


Figure 6-2 Model of regulation of the ETR1 EBD through peptidylprolyl isomerization by RTE1

(A) The cis–trans isomerization reaction catalyzed by PPIases. The illustration is taken from (Wang and Heitman, 2005). P1 and P2 indicate the amino acids on the both side of the proline. (B) In the absence of RTE1, the nascent prolyl peptide bond between E8 and P9 of ETR1 may not be the correct isomer required for a functional ETR1 EBD conformation, resulting in blockage of the correct disulfide bonds and/or the formation of wrong disulfide bonds during the folding. RTE1 could isomerize the

incorrect prolyl peptide bond at P9 to the correct one and thereby indirectly promote the formation of the correct disulfide bonds, causing the folding of a functional ETR1 EBD.

facilitate the formation of the correct disulfide bonds (**Figure 6-2 B**). Therefore, the isomerization of the prolyl bond could help oxidative folding of ETR1, similar to how prolyl isomerase accelerates the oxidative folding of reduced RNase T1, coupled with formation of the disulfide bonds (Schonbrunner and Schmid, 1992). Such a proposed PPIase-like activity of RTE1 would be consistent with the model that RTE1 is involved in the oxidative folding of ETR1. I speculate that RTE1 may have either redox ability or PPIase-like activity or both. However, there has been no evidence so far for the speculation that RTE1 has PPIase-like function, e.g. no sequence similarity is found between RTE1 and PPIases. In addition, PPIase-like function would not explain all the findings, because P9 is critical to, but not sufficient for, complete *RTE1* dependence (i.e., the absence of P9 cannot cause the complete loss of *RTE1*-dependent ethylene insensitivity conferred by the ETR1 (A102T) allele and none of the ERS1 (A102T, T9P) transgene lines exhibit ethylene insensitivity at the same strength as *etr1-2*). Besides P9, there is only one another proline (P12) that is unique to ETR1 (absent in ERS1) across the EBD. However, my results showed that introducing P12 does not enhance the *RTE1*-dependent ethylene insensitive phenotype conferred by the ERS1 (A102T, T9P) transgene line, indicating that P12 does not play a role in RTE1 dependence. It is conceivable that other residues cooperating with P9 are responsible for the *RTE1* dependence. The next step will be identifying such residues that are unique in ETR1. Another question is whether the ethylene receptors that have conserved P9 in other plants are also *RTE1*-dependent, including all ETR1 orthologs as well as OsERS, PaERS1 and PhaERS.

In the long term, although membrane proteins remain challenging to crystallize, the crystal structure of ETR1 EBD would finally give us a better understanding of the mechanism by which how ETR1 EBD is regulated. Analyzing the 3D structure and where each amino acid resides within the structure, especially in the EBD, may shed some light into questions such as how the various dominant *etr1* missense mutations affect the receptor structure resulting in ethylene insensitivity, how the proline 9 residue affects ETR1 EBD conformation, and how RTE1 and ETR1 interact.

Since my work suggests a possible connection between cytochrome b5 and RTE, carrying out redox could be the conserved function of RTE1 in other organisms. Animals have no ethylene receptor homologs, so the targets of RTE1 in animals remain to be discovered. The target proteins of RTE1 action in animals might also have a key proline that affects the disulfides important for their proper conformation. RTE1 might, together with cytb5, facilitate the oxidative folding of their target proteins in animals. The relationship I discovered between cytochrome b5 and RTE1 can now be investigated in animal systems.

Another interesting question is how RTE1 and ETR1 co-evolved. Was the original function of RTE1 to activate ethylene receptors or did RTE1 have another function that was co-opted by plant ethylene receptors? RTE1 is highly conserved throughout eukaryotes except fungi, and is absent in prokaryotes (Klee, 2006). Ethylene receptor sequences are found in plants and the cyanobacteria *Synechocystis* and *Anabaena*, which suggests that eukaryotic ethylene receptors may have evolved from a plastid origin (Bleecker, 1999; Mount and Chang, 2002; Rodriguez et al., 1999). The ethylene receptor appears to be more ancient than RTE1, due to its

presence in cyanobacteria and RTE1's absence in prokaryotes. *Chlamydomonas* has one copy of *RTE*, but no ethylene receptor genes. One *RTE1* gene and three ethylene receptors genes can be found in *Marchantia* (liverwort), the most distantly related plant species for which there is a nearly complete genome sequence (personal communication, Dr. John Bowman at Monash University, Australia). In *Physcomitrella* (moss), there are two *RTE* genes and seven ethylene receptors genes (*PpETR1- PpETR7*). Since *RTE1* is found in a wider range of organisms than the ethylene receptor genes, most likely emerged separately from ethylene receptor genes during the evolution of life, suggesting that the ancestral RTE1 function is unlikely to be related to ethylene. In some early ancestor of land plants, the ancestral ethylene receptor and RTE1 were together in the same organism. In that early ancestor, it is unlikely that the ethylene receptor genes had already duplicated. It is possible that the ETR1 receptor and RTE1 co-evolved together, prior to the appearance of ethylene receptor family members, and subsequently ETR1 duplicated and evolved into other ethylene receptors, some of which evolved to be free from RTE1 dependence. Alternatively, after there were already multiple ethylene receptors, RTE1 either evolved to activate certain ones, or certain ethylene receptors evolved to become dependent on RTE1. Based on my results from chapter 5, P9 plays a substantial role in the RTE1 dependence of ethylene receptors, raising the question of whether the ancestral ETR1 protein had the proline 9 or not. Among the three *Marchantia* ethylene receptor genes, one has the proline residue corresponding to P9 of *Arabidopsis* ETR1, one does not have it, and the third one is unclear due to unfinished gene assembly (personal communication, Dr. John Bowman). In

Physcomitrella, all PpETRs have the conserved proline residue in the corresponding position of proline 9 in Arabidopsis ETR1, with the exception of PpETR2. The PpETRs and MpETR with the conserved proline resemble Arabidopsis subfamily I ethylene receptors, whereas the PpETR and MpETR without the conserved proline resemble Arabidopsis subfamily II ethylene receptors (**Figure 6-3**). Based on current data, it is unknown whether P9 is gained or lost during the evolution of ethylene receptors, because we lack an organism that carries only one ethylene receptor. One possibility is that P9 was present originally and then lost by some ethylene receptors. If this is the case, P9 could have been lost at divergent points B and D (**Figure 6-3**). I speculate that the ancestral ethylene receptors had P9 and required RTE1 for activation, and subsequently the ancestral ethylene receptor gene duplicated and evolved into other ethylene receptors that lost P9 during evolution, thereby freeing them from their dependence on RTE1. Interestingly, Desikan *et al*.(2005) reported that the Arabidopsis ethylene receptor ETR1 can mediate H_2O_2 signaling in stomatal guard cells. I speculate that the ethylene receptor ETR1 might have evolved with RTE1 to sense and respond to the redox signal. The other ethylene receptors may have lost the ability to mediate redox signals due to loss of P9, and there was no selection pressure to keep P9 in these ethylene receptors to depend on RTE1. ETR1 still can mediate both ethylene signaling and redox signaling; therefore P9 was necessary to interact with RTE1. The alternate possibility is that P9 arose in some of the ancestral ethylene receptors, such as at the divergent point C and then lost P9 again at the divergent point D (**Figure 6-3**). If this is the case, P9 might have been

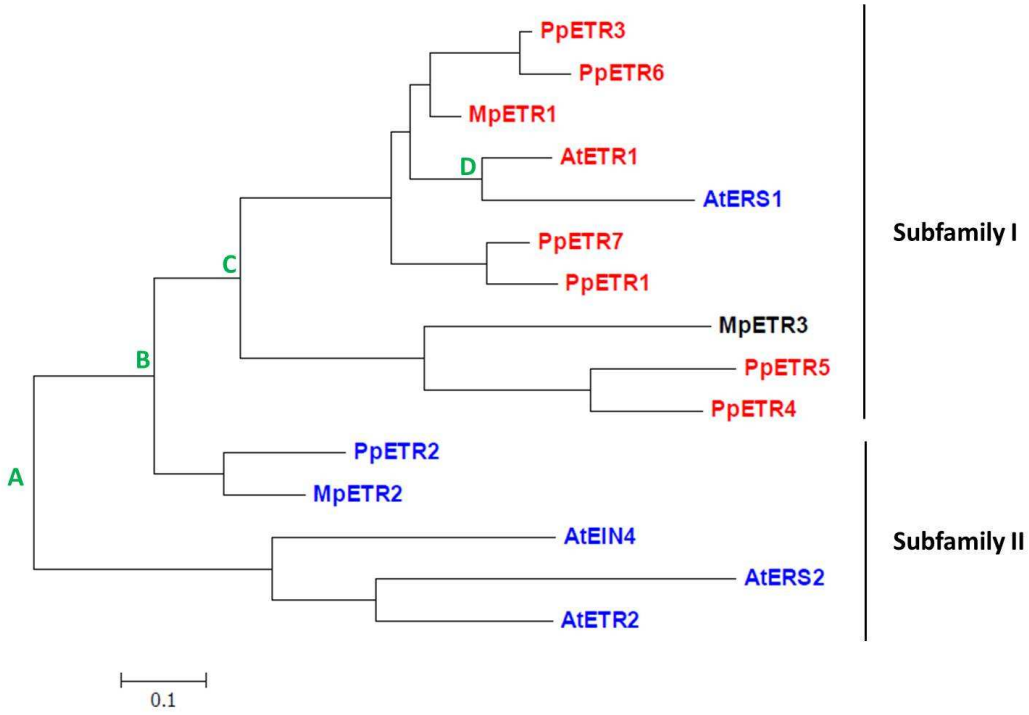


Figure 6-3 Phylogenic tree of *Marchantia*, *Physcomitrella* and *Arabidopsis* ethylene receptors

Marchantia polymorpha (Mp), *Physcomitrella patens subsp. patens* (Pp) and *Arabidopsis thaliana* (At) ethylene receptors are compared. The following sequences are shown: AtETR1 (NP_176808); AtERS1 (NP_181626); AtETR2 (NP_188956); AtERS2 (NP_171927); AtEIN4 (NP_187108); PpETR1 (XP_001751520); PpETR2 (XP_001769490); PpETR3 (XP_001762445); PpETR4 (XP_001772050); PpETR5 (XP_001756149); PpETR6 (XP_001754898); PpETR6 (XP_001774409). The *Marchantia* genome is unfinished and the three *Marchantia* ETR sequences are provided by Dr. John Bowman at Monash University, Australia. A multi-sequence alignment of the above ethylene receptor protein sequences were generated using *MEGA* version 5 (Tamura et al., 2011). Then the alignment was used to build the phylogenetic tree using the maximum likelihood clustering method of *MEGA* version

5 (Tamura et al., 2011). Some ethylene receptors have the proline residue corresponding to P9 of ETR1 (red), whereas some others don’t have it (blue). It is unclear whether MpETR3 have the proline residue corresponding to P9 of ETR1 since the MpETR3 sequence is incomplete.

accidentally gained and recognized by RTE1. Since RTE1 can help these receptors with P9 to be functional, they co-evolved together as a functional unit. The answer may become clearer once the genomes or transcriptomes of Charophyta (green algae) are available. Charophytes have an earlier common ancestor with higher plants than liverworts, and if the Charophytes have ethylene receptor genes, we will be able to see whether they carry P9 or not.

Appendices

Appendix A. The ethylene receptor GAF domains are capable of mediating higher-order heteromeric receptor interaction

Dr. G. Eric Schaller's lab (Dartmouth College) showed there are heteromeric interactions among Arabidopsis ethylene receptors. I contributed to this work by performing yeast two-hybrid analysis to determine which regions of the receptors were capable of mediating heteromeric interactions, examining interactions between the subfamily 1 receptor ETR1 and the subfamily 2 receptor ETR2 (**Fig. A-1**). Constructs containing the entire soluble domains but lacking the N-terminal transmembrane domains were tested and determined to interact based on two different reporters (HIS3 and LacZ). Similar results were also obtained when we examined interaction between the soluble domains of ETR1 and ERS2 (data not shown). The soluble region of greatest sequence similarity between the subfamily 1 and 2 receptors is the GAF domain. GAF domains have been shown to mediate cGMP binding and light regulation in some proteins, but their function in the ethylene receptors is unknown (Aravind and Ponting, 1997). We found through the examination of additional truncated versions of the ETR1 and ETR2 receptors that the GAF domain was sufficient to mediate their interaction, although based on the LacZ reporter analysis the strength of this interaction was reduced compared with that observed with the entire soluble domains (**Fig. A-1**). Constructs were made by Dr. Chi-kuang Wen, a previous postdoc in our lab. This result has been published (Gao et al., 2008).

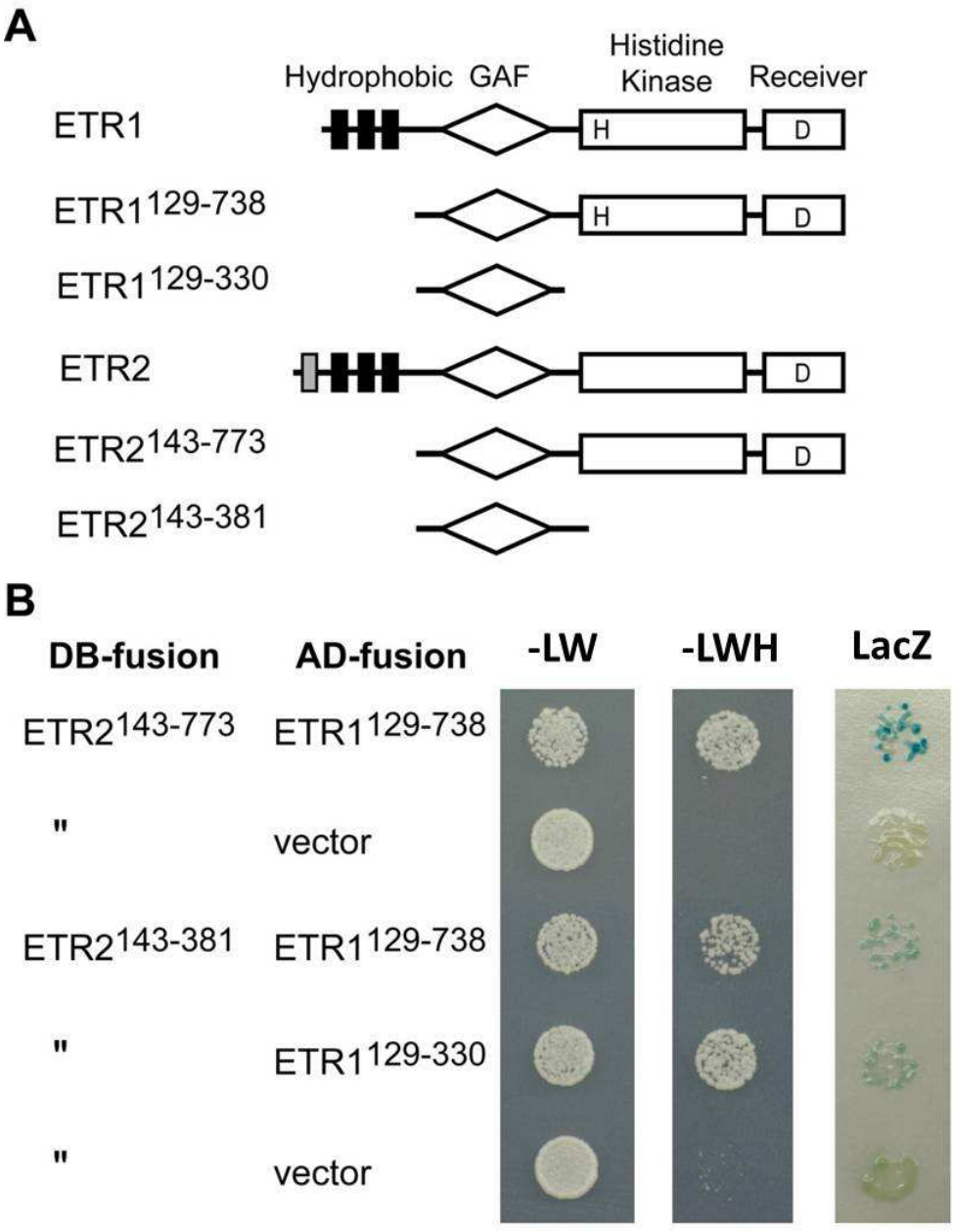


Figure A-1 Yeast two-hybrid analysis of ethylene receptor interactions

Structure of ETR1, ETR2, and constructs used for analysis. The hydrophobic ethylene sensing domain (hydrophobic segments indicated by *black* and *gray bars*), the GAF domain, the His kinase domain, and the receiver domain are indicated. *H* and *D*

indicate putative phosphorylation sites. The ETR1 and ETR2 full-length receptors consist of 738 and 773 residues, respectively. (B) Results of yeast two-hybrid analysis. The portions of the ethylene receptors fused with the DNA binding domain (*DB-fusion*) and transcriptional activation domain (*AD-fusion*) are indicated. Cells were spotted onto agar medium from 1/100 dilutions of liquid overnight cultures and grown for 3 days. Medium lacking leucine and tryptophan (-*LW*) selects for the DB-fusion and AD-fusion plasmids, respectively. Protein interactions are shown by growth on medium lacking histidine (-*LTW*) and by the X-gal filter assay (*lacZ*). The X-gal filter assay was performed on the same overnight cultures grown on -LW medium, but spotted at a dilution of 1/1000, with staining shown after a 4-h, 22 °C incubation. As a negative control, no activation of reporter genes was detected when the ETR1 AD-fusions were tested with a lamin DB-fusion (data not shown).

Appendix B. Isolate and clone a new suppressor of *etr1-2*: *REVERSION TO ETHYLENE-SENSITIVITY6* (*RTE6*)

The *rte6* mutation partially suppresses *etr1-2* ethylene insensitivity

To uncover regulators of ethylene signaling, our lab carried out genetic screens to search for suppressors of the dominant ethylene-insensitive mutant *etr1-2*. *reversion to ethylene-sensitivity6* (*rte6)* was one of the mutants isolated from such suppressor screen. Dr. Chunhai Dong, a former postdoc in our lab, performed some early genetic analyses and revealed that *rte6* is a recessive, extragenic suppressor mutant of *etr1-2* (data not shown). I did phenotypic analyses of the *rte6* mutant in greater detail to verify the suppression. The *rte6* mutation only shows partial suppression of the *etr1-2* allele in the ethylene triple response. The *etr1-2 rte6* mutant line exhibits a partial ethylene triple response in the presence of ACC including the shortening and thickening of hypocotyl, a slight curvature of the apical hook and a slight inhibition of the root elongation (**Figure B-1 A, B**). *ETHYLENE-RESPONSE-FACTOR1* (*ERF1*) is a positive regulator of downstream ethylene signaling and considered a marker gene of the ethylene response. To test whether ethylene signaling in *etr1-2 rte6* is upregulated compared to the ethylene insensitive mutant *etr1-2* in response to ethylene, I performed PT-PCR to examine marker gene *ERF1* expression. Consistent with the ethylene triple response phenotype, the *etr1-2 rte6* mutant line has an increased level of *ERF1* mRNA compared to that in *etr1-2*, but does not have a level as high as the wild type and *etr1-2 rte1-3* (**Figure B-1 C**). The *etr1-2 rte6* mutant was also tested for another ethylene phenotype, adult leaf senescence. When the *etr1-2 rte6* adult plants were treated with 100 ppm for four days, their leaves

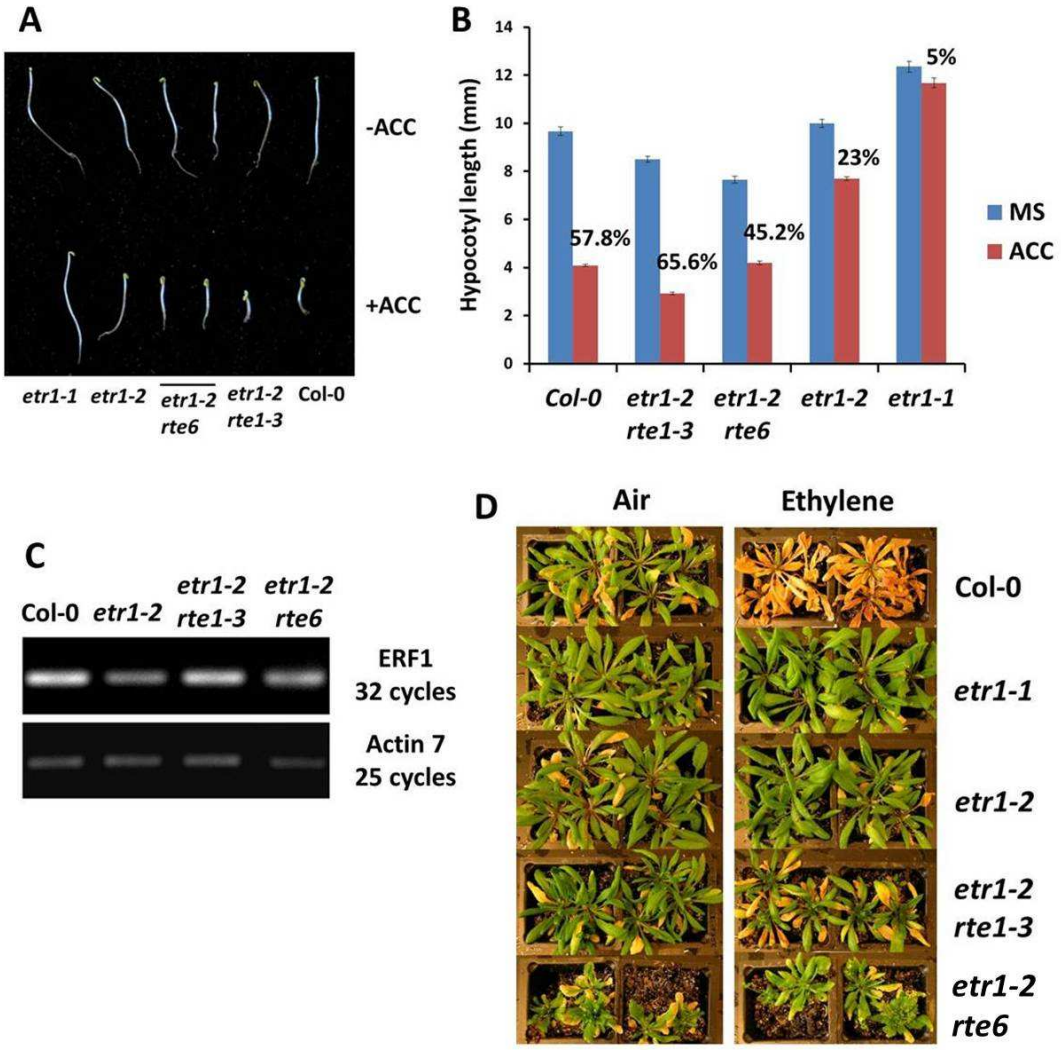


Figure B-1 Ethylene responses of *etr1-2 rte6*

Analysis of 4-day-old dark-grown etiolated seedlings in the absence or presence of 100μM ACC. Representative seedlings of the wild type (Col-0), *etr1-1*, *etr1-2*, *etr1-2 rte1-3* and *etr1-2 rte6* were shown. (B) Measurements of hypocotyl length for etiolated seedlings of indicated genotypes shown in (A). About 20-48 seedlings were measured and mean ± standard error is shown per genotype at MS and 100μM ACC. The values above the columns represent the percentage of reduced hypocotyl length

on ACC relative to that on MS. (C) RT-PCR showing the *ERF1* (*ETHYLENE-RESPONSE-FACTOR1*) expression level of the indicated genotype. Top panel is RT-PCR using ERF1-specific primers while the bottom panel is product using Actin 7-specific primers showing that all samples had similar levels of RNA. The RNA was prepared from 4-day-old etiolated seedlings grown on 100µM ACC. (D) Ethylene-induced leaf senescence in ~6-week old plants with or without 100pm ethylene for 4 days. Senescence is shown in the wild type, *etr1-2 rte1-3*, but not in *etr1-1, etr1-2* and *etr1-2 rte6*.

almost did not senesce in contrast to the wild type and *etr1-2 rte1-3* (**Figure B-1 D**). These results suggest that *rte6* might affect ethylene signaling, but is probably acting on downstream players of the ethylene signaling pathway since it doesn't show suppression of *etr1-2* in all ethylene phenotypes.

Molecular cloning of the *RTE6* gene

To uncover the *RTE6* product, I carried out map-based cloning to isolate the *RTE6* gene. The *rte6 etr1-2* mutant, which was generated in the Columbia (Col-0) ecotype, was backcrossed to *etr1-2* twice and then crossed to an *etr1* mutant in the Landsberg-*erecta* (L*er*) background. These crosses were done by former postdoc Dr. Chunhai Dong, and I subsequently performed the map-based cloning of the *RTE6* gene. Plants from the F2 population of this cross were screened for the triple response phenotype on 100μM ACC, and the seedlings that exhibit the triple response were isolated as the mapping population. All mapping population individuals had their progeny's triple response assayed to confirm the phenotype in the parent. The mapping population was genotyped throughout the Arabidopsis genome using markers designed from Monsanto's L*er* polymorphism collection (http://www.arabidopsis.org/browse/Cereon/index.jsp). The *rte6* mutation was roughly positioned around the centromere of chromosome 2. After identifying the markers tightly linked to *rte6* in a mapping population of 580, the *rte6* mutation was found to lie within an approximate27 kb region carried by the bacterial artificial chromosome (BAC) T10F5 (**Figure B-2**). Sequencing the genes within this window revealed a C to T nonsense mutation at nucleotide 1492 of At2g13540, resulting in the conversion of a glutamine at the 498th residue to a stop codon (**Figure B-2**).

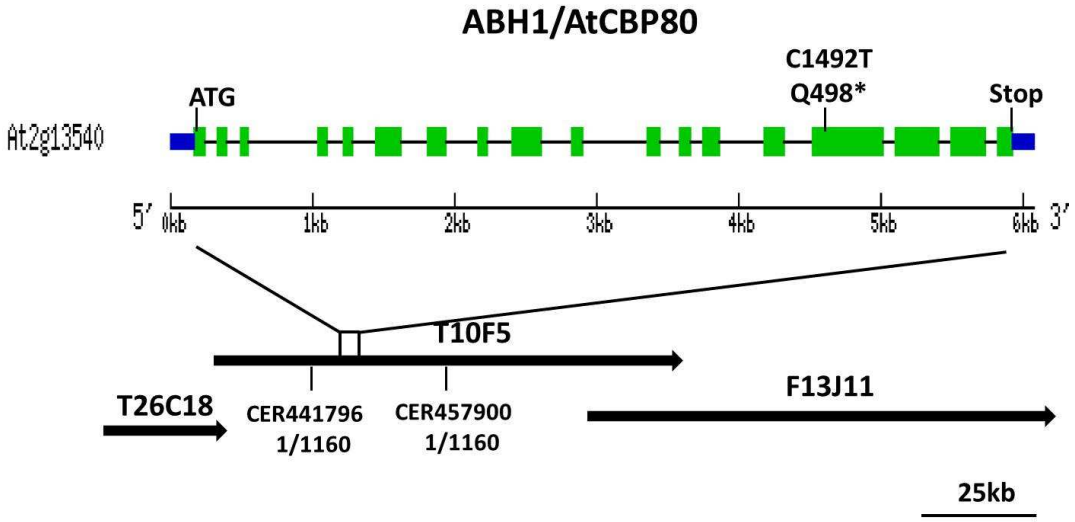


Figure B-2 Map based cloning of the *RTE6* gene

The *rte6* mutation was found to lie within a region of 27kb on BAC T10F5 using the map-based cloning technique. The *rte6* mutation is a C to T nonsense mutation at nucleotide 1492 of At2g13540, which is called *ABH1* or *AtCBP80*, resulting in the conversion of a glutamine at the 498th residue to a stop codon. ABH1/AtCBP80 is about 6kb, containing 18 exons (green boxes) and 17 introns. The 5'and 3' UTR are indicated in blue. In the lower portion of the figure, the BAC clones carrying the 120 kb region are shown. The genotyping markers used for mapping the *rte6* mutation in these BAC clones are labeled. Numbers below the genotyping marker names are number of recombinant chromosomes found out of the number of chromosome tested.

At2g13540, which is known as *ABA Hypersensitive 1* (*ABH1*), encodes the Arabidopsis homolog of a nuclear mRNA cap binding protein CBP80 that forms a heterodimeric complex with CBP20 and function in binding the mRNA cap structure (Hugouvieux et al., 2001). The *abh1* mutant shows ABA-hypersensitive seed germination inhibition, stomatal closing and guard cell cytosolic calcium increases as well as reduced wilting during drought (Hugouvieux et al., 2001). To assess whether *RTE6* is *ABH1*, I tested *etr1-2 rte6* for the ABA phenotype and compared it with the existing *abh1-1* loss-of-function mutant. Both *etr1-2 rte6* and *abh1-1* exhibit germination inhibition at low dose of ABA (0.5µM) compared to the wild type and *etr1-2* (data not shown). Moreover, the *etr1-2 rte6* adult plant is smaller than the wild type and has a serrated leaf phenotype, which are same as the phenotypes reported for the *abh1-1* mutant in the adult stage (Hugouvieux et al., 2001) (data not shown). The similarity in the ABA phenotype exhibited by *etr1-2 rte6* and *abh1* suggests that the *rte6* mutation affects the *ABH1* gene.

Appendix C. Two proteins containing tetratricopeptide repeat (TPR) motifs interact with ETR1 and ERS1

Background

A complete understanding of the ethylene receptor signaling mechanism could be difficult to achieve if additional unknown components are involved. Although most of the components in the ethylene signaling pathway were isolated through genetic screens, these screens appear to be saturated. An alternate pathway that bypasses CTR1 is thought to exist, since the *ctr1* null mutant can still respond further

to ethylene (Larsen and Chang, 2001). In addition, the subfamily I receptor null mutant has severe defects not seen in the *ctr1* null, indicating that the receptors have downstream targets besides CTR1. To date, there are no known components acting between ETR1 and CTR1 from genetic screens. We know that protein-protein interactions are an integral aspect of signal transduction. Yeast two-hybrid assay has been shown a powerful tool to probe protein-protein interaction. Our lab screened for potential ETR1 and ERS1 interacting proteins by using the yeast-two hybrid assay, thus *AWE1*, *AWE2* and *AtTRP1* were isolated. These proteins that potentially interact with the ethylene receptors could regulate the receptor activity, or even could anchor the receptors and CTR1 together, or bring CTR1 to the receptors or serve as a scaffold for the receptors and CTR1 substrates. The primary objective of this project is to determine whether the two proteins (AWE2 and AtTRP1) are involved in the ethylene signaling pathway.

The tetratricopeptide repeat (TPR) is a degenerate 34 amino acid sequence identified in a wide variety of proteins, present in tandem arrays of 3–16 motifs, which form scaffolds to mediate protein–protein interactions and often the assembly of multiprotein complexes. Individual TPR domains are composed of two anti-parallel alpha helices separated by a turn. Multiple TPR domains are often arranged to form a large surface area available for ligand binding (Das et al., 1998). Within TPR-containing proteins, the TPRs are usually arranged in tandem arrays of 3–16 motifs, although individual, or blocks, of TPR motifs may be dispersed throughout the protein sequence. They are involved in cell cycle regulation, interaction with

chaperones, transcription control, and protein degradation (Das et al., 1998; Goebl and Yanagida, 1991).

A former graduate student in the Chang lab identified the gene At4g10840 in the yeast two-hybrid assay by screening for ETR1 histidine kinase domain and receiver domain (ETR1 HK+R) interacting proteins. We name this gene *Associates With ETR1 2* (*AWE2*). *AWE2* has two splicing variants (**Figure C-1 A**). *AWE2.1* encodes a putative protein of 609 amino acids and *AWE2.2* encodes a putative protein of 531 amino acids. AWE2.1 contains 9 tetratricopeptide repeat (TPR) motifs from the amino acid 140 to 549 and a novel N terminal sequence of 139aa. AWE2.2 has the same N-terminal part of AWE2 and misses the C-terminal 78 amino acids of AWE2.1. In the Arabidopsis genome, two other genes (At3g27960 and At1g27500) share the highest sequence similarity to AWE2, with 55% identity and 73% similarity over 571 amino acids and 57% identity and 75% similarity over 516 amino acids respectively. AWE2 orthologs are found in plants but not in animals.

Another former student in the Chang lab identified another protein containing TPR motifs (Arabidopsis Tetratricopeptide Repeat Protein, AtTRP1) by screening for interactions with the ERS1 histidine kinase domain in the yeast two-hybrid assay. AtTRP1 is encoded by At4g30480 which has three splicing variants (**Figure C-1 B**). *AtTRP1.1* encodes a putative protein of 208 amino acid residues with a molecular weight of 23KDa. *AtTRP1.2* encodes a putative protein of 277 amino acids residues with a molecular weight of 30.9KDa. *AtTRP1.3* encodes a putative protein of 161 amino acid residues with a molecular weight of 17.8KDa. The protein At4g30480.2 (AtTRP1) contains three TPR motifs from amino acid 105 to 213. *AtTRP1* is a single

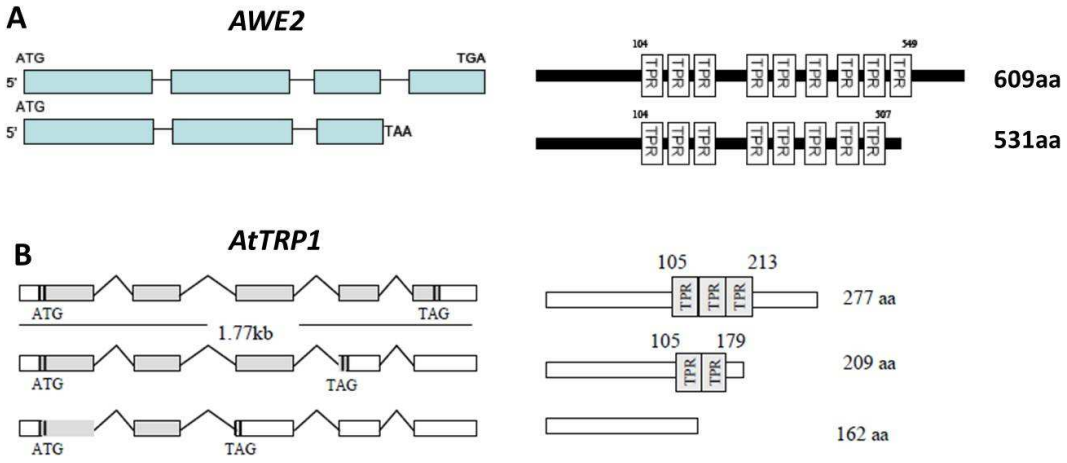


Figure C-1 Both *AWE2* and *AtTRP1* have splicing variants

The *AWE2* gene has two predicted splicing variants (left) (A), whereas the *AtTRP1* gene has three predicted splicing variants (left) (B). The corresponding putative proteins with TPR motifs are indicated on the right. The figure (B) is taken from (Lin et al., 2009).

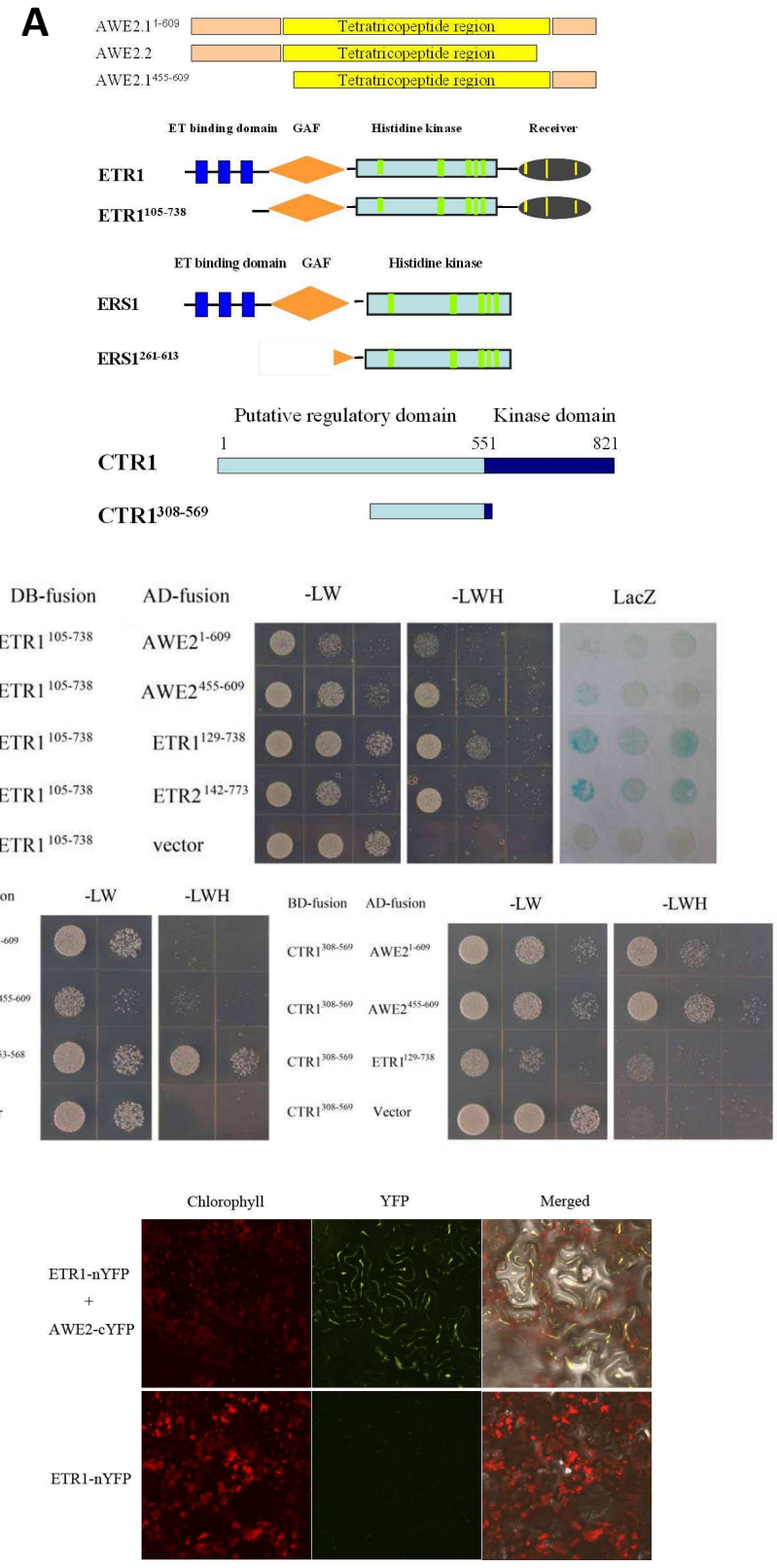
copy gene in the *Arabidopsis* genome although a large number of genes encode proteins containing TPR1 motifs. A phylogenetic tree analysis using the full-length protein sequences of 91 TPR genes from the A*rabidopsis* genome as entry indicated AtTRP1 is distantly related to AWE2 (data not shown). Blast search revealed a family of highly conserved TPR1 homologs widely distributed in eukaryotes, including human, mouse, fly, tomatoes, maize, etc. The human TPR1 sequence has 43% identity and 63% similarity with AtTRP1 over 179 amino acids. AtTRP1 shares high similarity to tomato SlTPR1 with 72% similarity over the entire sequence. However, there are no mutant phenotypes documented or functional studies on these genes except human TPR1. Both AWE2 and AtTRP1 are predicted to be soluble proteins.

Human TPR1 (also named TTC1, accession number: NM_003314), a 292-amino-acid protein with three TPR motifs from amino acid 116 to 222, interacts with several Gα proteins and Ha-Ras preferentially in its active form. Overexpression of TPR1 promotes accumulation of active Ras. TPR1 was found to compete with the Ras-binding domain (RBD) of Raf-1 for binding to the active Ras, suggesting that it may also compete with Ras GTPase-activating protein, thus contributing to the accumulation of GTP-bound Ras (Marty et al., 2003). TPR1 was also reported to be involved in regulating Hsp70 dependent folding of chemically denatured substrates. Hsp40 and TPR1 are chaperone adaptors that interact with the amino terminal and carboxy terminal domains of Hsp70, respectively. The ternary complex of Hsp70/Hsp40/TPR1 enhances Hsp70's chaperonin capacity. HspBp1, a negative modulator of Hsp70, completely inhibited Hsp70-dependent folding. However, the

inhibitory effect of HspBp1 was reversed in the presence of Hsp40 and TPR1 (Oh and Song, 2003).

AWE2 and AtTRP1may specifically interact with ETR1 and ERS1 respectively

The yeast two-hybrid assay revealed that AWE2 can interact with the ETR1 soluble domain and a portion of CTR1 (308-569), but not with the ERS1 HK domain (**Figure C-2 A, B**). AWE2 could physically associate with ETR1 in *planta*, since a positive BiFC signal was observed for ETR1 and AWE2 in Agrobacterium infiltrated tobacco leaves (**Figure C-2 C**). Preliminary results show that the product of one *AtTRP1* splicing variant (*AtTRP1.2*) interacts with the ERS1 HK domain (**Figure C-3**) in the yeast two-hybrid assay. Lin et al. (2008) reported that SlTPR1, the tomato homolog of AtTRP1, interacts with the ethylene receptors NR (which is an ortholog of Arabidopsis ERS1) and LeETR1 in the yeast two-hybrid and in vitro. Later they reported that AtTRP1 interacted preferentially with Arabidopsis ERS1 in yeast two-hybrid assays and the interaction of AtTRP1 and ERS1 was confirmed by co-immunoprecipitation (Lin et al., 2009). These data implied that AWE2 could specifically interact with ETR1 while AtTRP1 may specifically interact with ERS1. Since AWE2 interacts with both ETR1 and CTR1, it may be one of the components of the protein complex involving ETR1 and CTR1, and may play a role in ethylene signal transmission from ETR1 to CTR1. I obtained a T-DNA insertion line for *AWE2* (from the Salk institute). *awe2* (Salk_121703) carries a T-DNA in the second exon of the *AWE2* coding sequence. An RT-PCR analysis of the *awe2* T-DNA insertion mutant showed that the *AWE2* transcript is eliminated. When tested on different doses of ACC, it shows the same ethylene phenotype as the wild type. Since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Tetratricopeptide region
AWE2.2
Tetratricopeptide region
AWE2.1 455-609
Tetratricopeptide region
ET binding domain
GAF
Histidine kinase
Receiver
ETR1
ETR1 105-738
ET binding domain
GAF
Histidine kinase
ERS1
ERS1 261-613
Putative regulatory domain
Kinase domain
1
551
821
CTR1
CTR1 308-569
B
DB-fusion
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ETR1 105-738
AWE2 1-609
ETR1 105-738
AWE2 455-609
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CTR1 308-569
AWE2 455-609
CTR1 308-569
ETR1 129-738
CTR1 308-569
Vector
C
Chlorophyll
YFP
Merged
ETR1-nYFP
+
AWE2-cYFP
ETR1-nYFP

Figure C-2 AWE2 is able to interact with ETR1 and CTR1, but not with ERS1

A truncated form of AWE2 (455-609) was isolated as an AD fusion protein from an AD-cDNA library. Both full-length and truncated AWE2 were tested against various truncations of ETR1, ERS1 and CTR1 (A). Interaction is indicated by growth on medium without histidine (-LWH) and LacZ activity (blue cells) (B). AWE2 is expressed as an AD fusion using pACTII, while the receptors and CTR1 are expressed as DB fusions using pLexA. Yeast two-hybrid assays were carried out in yeast strain L40. Growth on medium containing histidine (-LW) is included to demonstrate loading of transformants. (C) BiFC visualization of interaction of ETR1 with AWE2 in Agrobacterium infiltrated tobacco (*Nicotiana benthamiana*) leaves.

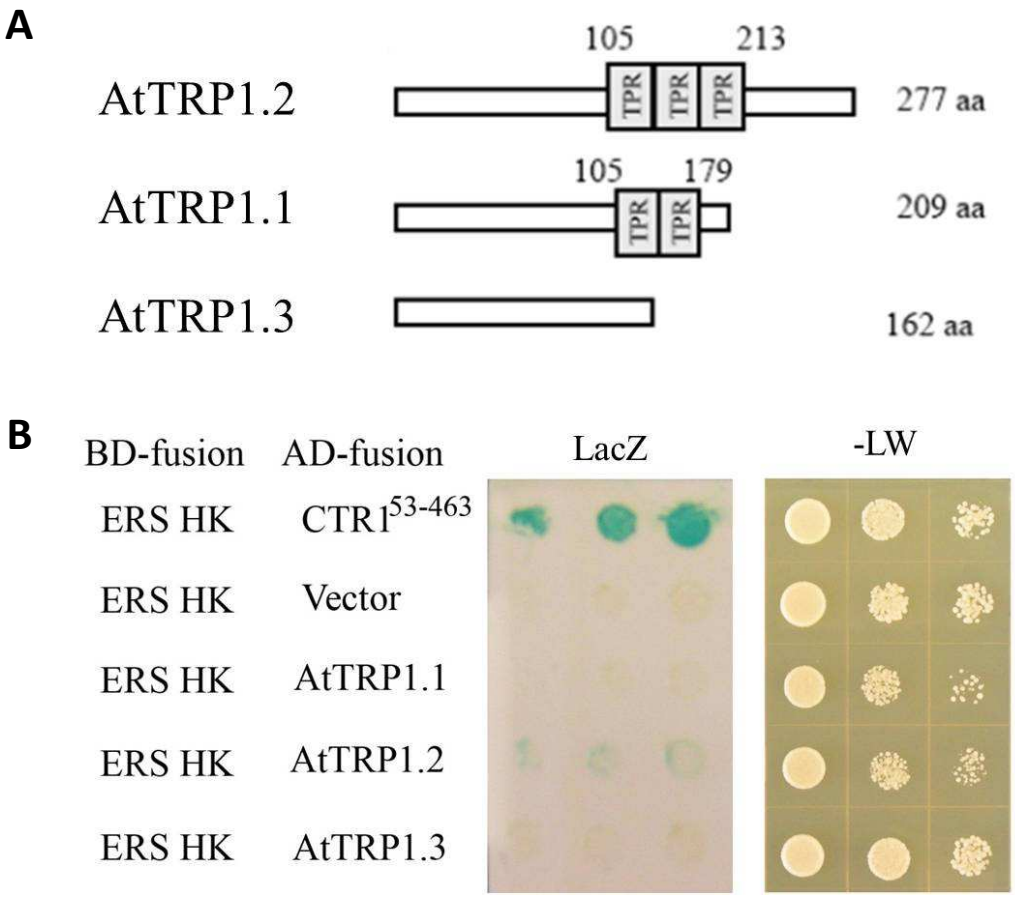

Figure C-3 AtTRP1.2 interacts with ERS1 in the yeast two-hybrid assay.

(A) Diagram of proteins encoded by three splicing isoforms of the *AtTRP* gene. (B) The yeast two-hybrid assay of the interaction of the ERS1 HK domain with the three AtTRP1 isoforms and CTR1 (53-463), which is used as the positive control. Interaction is indicated by LacZ activity (blue color). Growth on medium without leucine and tryptophan (-LW) is included to demonstrate loading of transformants.

AWE2 has two close homologs in Arabidopsis, there might be functional redundancy among *AWE2* homologs or *AWE2* may not the correct member that plays a role in ethylene signaling. I may need to knock down additional homologs in order to detect a phenotype. AtTRP1 may play a similar role with AWE2, but specific to ERS1. High sequence similarity (63%) between AtTRP1 and human TPR1 suggested that they may possess similar function. It has been reported that human TPR1 interacts with several Gα proteins and Ras and competes with Raf-1 for Ras binding (Marty et al., 2003). One speculation is that the interaction of AtTRP1 with the ethylene receptors may compete for ethylene receptor binding with CTR1.

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