ABSTRACT

Title of Document: INVESTIGATION OF ETHYLENE SIGNAL TRANSDUCTION MECHANISMS: CHARACTERIZING THE NOVEL GENE *AWE1* AND TESTING HYPOTHESES OF RAF-LIKE CTR1'S FUNCTION IN VIVO

Mandy D. Kendrick, Ph.D., 2009

Directed By: Associate Professor Caren Chang, Associate Chair to the Department of Cell Biology & Molecular Genetics

Ethylene is a gaseous plant hormone affecting multiple plant processes. Sixteen years ago the first components of the ethylene signaling pathway, the receptor ETR1 and Raf-like kinase CTR1, were identified. Since then many additional components of the pathway have been elucidated through genetic screens. Recent discoveries suggest ethylene signaling, once thought to be a linear pathway from ethylene perception at the endoplasmic reticulum to transcriptional activation at the nucleus, is more complex with multiple auto-feedback loops and potential parallel kinase cascades downstream of the receptors. Although the genetic backbone of the pathway is well established, the signaling mechanisms of the components remain unclear. ETR1 displays histidine kinase activity *in vitro* and physically interacts with the next-known downstream component of the pathway, CTR1. However the histidine kinase activity of ETR1 is mostly dispensable for signaling to CTR1. How

then is CTR1 activated? I proposed that additional proteins, like AWE1, play a role in ETR1 to CTR1 signaling, and that the non-catalytic, amino-terminal region of CTR1 is required both for activation through direct interaction with the ETR1 receptor complex and for auto-inhibition of CTR1 kinase activity. ASSOCIATES-WITH-ETR1 (AWE1) was isolated in a yeast-two-hybrid screen for ETR1-interacting proteins and was of specific interest because the AWE1 clone also interacted with a portion of CTR1. Protein-protein interaction studies and genetic analysis of an *awe1* mutant support a role of AWE1 in repressing ethylene responses. However double mutant analysis, over-expression analysis, and protein sub-cellular localization studies suggest that AWE1's function in hypocotyl elongation and cell expansion is more general. AWE1's function may require ETR1 for proper regulation but is likely to lie outside of the direct step from ETR1 to CTR1.

To investigate a role of the CTR1 amino-terminal region in CTR1 regulation, I constructed transgenes consisting of truncated ETR1 receptors fused to truncated or full length CTR1 and examined how those transgenes carrying the truncated CTR1 (kinase domain only) affected *Arabidopsis* seedling growth compared to those transgenes expressing full length CTR1. I concluded that the CTR1 amino-terminal region may have a role in autoregulation, but additional components are required for regulation of CTR1 signaling.

# INVESTIGATION OF ETHYLENE SIGNAL TRANSDUCTION MECHANISMS: CHARACTERIZING THE NOVEL GENE *AWE1* AND TESTING HYPOTHESES OF RAF-LIKE CTR1'S FUNCTION IN VIVO

By

Mandy D. Kendrick

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2009

Advisory Committee:
Associate Professor Caren Chang, Chair
Associate Professor Gary Coleman
Assistant Professor June Kwak
Associate Professor Zhongchi Liu
Associate Professor Stephen Mount
Associate Professor Richard Stewart

# Dedication

This work is dedicated to my parents, Dane and Jill Kendrick, for their love and all of their sacrifices over the years to help their children grow and succeed. This work is dedicated to the memory of my Uncle J.W. Kendrick and my "Maryland Grandpa" Richard B. Owen; both of whom passed away during my tenure in graduate school. Uncle Jay had a huge heart and quietly spread peace to everyone he met. Grandpa Richard was not so quiet, but also big-hearted. He was a true American hero, left for dead on the beaches of Normandy, but miraculously living to tell about it for another 64 years.

# Acknowledgements

First I thank my advisor, Dr. Caren Chang, for sharing her knowledge, enthusiasm, patience, and time; for constantly encouraging me to take my work to the "next level;" and for her open-mindedness.

I also thank all of my committee members for seeing me through this process. Thank you to Dr. Chang and Dr. Steve Mount for teaching me all of the genetics that I know. Without their patience, I would not have made it to this point. I thank Dr. Zhongchi Liu for her role in developing my critical reading skills in her developmental biology course. Thanks to Dr. June Kwak for maintaining constant enthusiasm about my work, and to Dr. Richard Stewart for challenging me to rise to the next level of thinking through asking the question that leaves me thinking, "hadn't really thought about that…" Thank you to Dr. Gary Coleman for giving his time to serve as the essential Dean's representative for my committee.

I also thank the members (past and present) of the Chang lab for their friendships and help over the years. Thanks to Jason, Jo, Maximo, and An for their friendships and interesting debates, and to Chris, Chunhai, Jianhong, and Ray for continued their friendship and making the lab a good working environment. Thank you to Chunhai Dong for continually growing the tobacco for lab research and for sharing his time and knowledge. Thank you to Bradley Mechak for helping me to clone the *AWE1* cDNA, and to Ray Chen for constructing the AWE1-RFP localization transgene. Thank you to the multiple undergraduate students who have fun to work with, especially Lien, Belen, Stephanie, Mary, Joanna, Yeojin, Maia, Jeff, Jeff, Marko, Franklin, Horatiu, and Hunter. Thank you to the others within our

department who have made graduate school so much easier. Specifically thank you to Raju Shah for saving data after my computer crashed, to Amy Beaven for teaching me how to use the microscope, to Kathleen Stauder for making sure I received my fellowship stipends, to Ruth, Nicole, and Sarah for their willingness to help and all the football conversations that we have had. Thank you to Claudia for being so eager to help and for keeping me up-to-speed with the latest St. Louis Cardinals news. Thank you to Dr. Todd Cooke for helping me with the application process for the CBMG program and to Dr. Heven Sze for her genuine care for the students in the department.

Thanks to my family, because I would be nowhere without their love and support! Thank you to my parents, Dane and Jill Kendrick. Specifically thanks to my father for his big heart, and my mother for her enthusiasm and for constantly reinforcing the concept "it is okay to be different" through out my life. Thank you to my siblings and nieces and nephews for all of the smiles that they send my way: Chad, Annie, Clara, Ben, Kacy, Emma, Laina, Jared, Ashley, Colton, Chase, Sam and Ashley. Thank you to Annie for our frequent conversations trying to keep up on what is going on in each other's life, to Kacy for teaching me about endurance, to Jared for listening to more "girl stories" than any brother should be subject to, and to Sam for telling me to chill out because "it's all good." Thank you to the entire Kendrick and Deaver clans back home in Missouri who I miss dearly and to Georgia and Becky for our friendships that keep going over the miles. Thanks to my friends that I have met while in graduate school for all of the fun times we have shared and hopefully will continue to share: Ellen, Adrianna, John, Rosemary, Anni, Jo, Courtney, and my

volleyball team Scared Hitless. Thank you to my friends outside of school who have taught me valuable life lessons: LaRue, Joel, Carol, Andy, Gwen, Gaylen, Virginia, Laura, Jason, Donna, Dina, Kate, Jim, Vicki, Joyce, and Hussein Barre. Thank you to Mike and Carol Barr for adopting me like one of their own children. Thank you to Brittany "Champ" Barr for being a great friend and another female fan of major league baseball!

I especially want to thank Sam for his unconditional support and his constant desire to make my life perfect, often at his own sacrifice. Thank you, Nut. And I cannot forget my dog, Walker. Any long days at work usually end with a smile when I step through the door at home to be greeted by a very excited dog wagging his tail, in delight to see me. Finally, I want to thank the 2009 Mizzou Tigers basketball team for being so much fun to watch this season and providing entertaining breaks during this thesis writing time.

# Table of Contents

# List of Figures

# List of Abbreviations

**2D-DIGE** 2-dimensional gel electrophoresis
**5'-FOA** 5-fluoroorotic acid
**ABA** abscisic acid
**ACC** 1-Aminocyclopropane-1-carboxlic acid
**AVG** aminoethoxyvinylglycine
**AWE** ASSOCIATES-WITH-ETR1
**BLAST** basic local alignment search tool
**BiFC** Bimolecular Fluorescence Complementation Analysis
**CaMV** Cauliflower Mosaic Virus
**COI** CORONATINE INSENSITIVE
**CSN** COP9 Signalosome
**CTR** CONSTITUTIVE TRIPLE RESPONSE
**CTR1 KD** CONSTITUTIVE-TRIPLE-RESPONSE1 KINASE DOMAIN
**CTR1 FL** CONSTITUTIVE-TRIPLE-RESPONSE1 FULL LENGTH
**dCAPS** derived cleaved amplified polymorphic sequences
**EBD** ethylene binding domain
**EBF** EIN3-BINDING F-BOX PROTEIN
**EBS** ethylene binding sequence
**EDR** ENHANCED DISEASE RESISTANCE
**EER** ENHANCED-ETHYLENE RESPONSE
**EIL** ETHYLENE-INSENSITIVE3-LIKE
**EIN** ETHYLENE-INSENSITIVE
**EMS** ethyl methanesulfonate
**ER** endoplasmic reticulum
**ERF** ETHYLENE RESPONSE FACTOR
**ERS** ETHYLENE RESPONSE SENSOR
**EST** expressed sequence tag
**ETP** EIN2 TARGETING PROTEIN
**ETR** ETHYLENE RESPONSE
**FFC** FIFTY-FOUR CHLOROPLASTS
**Fts** FILAMENTOUS TEMPERATURE SENSITIVE
**GA** gibberrelic acid
**GADPH** GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT 1
**GAF** cGMP-regulated mammalian phosphodiesterases, cyanobacterial adenyl cyclases, formate-hydrogen lyase transcriptional activator
**GCR** G-PROTEIN COUPLED RECEPTOR
**GID** GIBBERELIN ACID INSWENSITIVE DWARF
**GFP** GREEN FLUORESCENT PROTEIN
**GLB** GLABROUS
**JA** jasmonic acid
**JAZ** JASMONATE-ZIM-DOMAIN
**Lba** left border A

**MAPK** mitogen-activated protein kinase
**MAPKK** mitogen-activated protein kinase kinase
**MAPKKK** mitogen-activated protein kinase kinase kinase
**MES** 2-(N-morpholino)ethanesulfonic acid
**MKK** mitogen-activated protein kinase kinase
**MPK** mitogen-activated protein kinase
**MS** Murashige and Shookg
**PA** phosphatidic acid
**PAM** (PCI/PINT)-associated module
**PCR** polymerase chain reaction
**PERE** Primary ethylene response element
**PHYRE** Protein Homology-analogY Recognition Engine
**PP1** PROTEIN PHOSPHATASE 1
**PP2A** PROTEIN PHOSPHATASE 2A
**PPM** parts per million
**PS** phosphatidylserine
**RAN** RESPONSIVE-TO-ANTAGONIST
**Rat** *S. cereviseae* 5'→3' exoribonuclease involved in RNA transcription termination
**RCN** altered response to NPA
**RFP** RED FLUORESCENT PROTEIN
**RTE** REVERSION-TO-ETHYLENE SENSITIVITY
**RT-PCR** reverse transcription polymerase chain reaction
**SIMK** SALT STRESS INDUCED MAP KINASE
**SIMKK** SALT STRESS INDUCED MAP KINASE KINASE
**TAIR** The *Arabidopsis* Information Resource
**T-DNA** transferred DNA
**TFIID** TATA BOX BINDING FACTOR (TFIID)-interacting domain
**TIC** translocon at inner membrane of chloroplasts
**TIR** transport inhibitor response
**TM** transmembrane domain
**USDA** United States Department of Agriculture
**UTR** untranslated region
**WT** wild type
**XRN** exoribonuclease
**YFP** YELLOW FLUORESCENT PROTEIN

# Chapter 1: Introduction

*Historical Perspective*

The simple hydrocarbon gas ethylene is a phytohormone that has profound effects on many aspects of life. The first known recording of ethylene was by Dutch chemists who synthetically produced the gas in 1794 [1]. Since that time, ethylene has become the most produced organic compound in the world [2]. However, the significance of this gas dates back to a much earlier time and is much broader than its synthetic use in the thermoplastic industry [2].

In ancient Egypt workers slashed open sycomore fruits during the harvesting, because the practice of wounding the fruit hastened the onset of ripening [3]. Early Chinese farmers burned incense in closed rooms where their pear crops were stored to stimulate ripening [4]. There is no evidence that either the Egyptians or the Chinese understood the mechanisms underlying these techniques that triggered fruit ripening, however, observations made in the late 1800's and early 1900's revealed that ethylene was the likely stimulant in both cases. Likewise, it was not until ~1700 years after the last Oracle of Delphi, when geologists identified ethylene as the probable gaseous vapor responsible for the hallucination of the Pythia, or oracle women, leading to their ability to converse with Apollo and predict the future [5]. The hypohtesis is that ethylene escaped from a fissure in the Earth's surface and rising into the oracle's chamber [5].

In 1924 F. Denny evaluated the affects of multiple gasses such as car exhaust, stove gas, and sweatshop atmosphere on fruit ripening. He concluded that ethylene was the active ingredient in each of the tested gasses that triggered fruit ripening [6]. It had been documented even earlier (1901) by D. Neljubow that ethylene was the responsible agent in illuminating gas, which had leaked out of a gas main, that induced premature defoliation of the surrounding trees [7]. But ethylene was not known to be an endogenously produced hormone until 1934 when Gane found that apples could produce ethylene [8]. Thirty years later, McGlasson showed that endogenous ethylene production increased upon fruit wounding [9], a plausible explanation for why the Egyptians observed that cutting open the sycomore fruit hastened ripening.

In addition to fruit ripening and senescence, ethylene has been associated with many other plant developmental processes such as signaling for seed germination, inhibiting cell expansion and division, and promoting flowering and petal abscission. Besides wounding, abiotic and biotic stresses can trigger ethylene production in the plant [10]. Each of these ethylene-related processes can affect a plant's ability to survive and reproduce. It is these attributes that make the study of ethylene agronomically significant, because ethylene can impact processes that in turn impact crop yield. With a growing world population, less available suitable land for growing crops, and the increasing utilization of crops for non-food purposes, it is essential that we understand the complex processes of a plant, such as ethylene perception and signaling, if we are to achieve maximum crop yields in the future.

The ethylene biosynthesis pathway was completed in 1979 after 13 years of work by the biochemist Shang Fa Wang, when Wang and his graduate student, Douglas Adams, in a race with other labs, discovered that the intermediate between S-AdoMet and ethylene production in the plant was 1-Aminocyclopropane-1-carboxlic acid (ACC) (www.universityofcalifornia.edu). In the plant, ethylene is produced from the conversion of S-AdoMet into ACC by a large family of tightly regulated ACC synthase genes. Once produced, ACC is immediately converted to ethylene by ACC oxidase [11]. The ACC oxidase enzymes are believed to be localized to the cytosol [12] and to convert ACC to ethylene as soon as ACC is produced [13]. Ethylene is then perceived by a family of endomembrane receptors.

The relatively quick dissection of the essential components of the signal transduction pathway, downstream of ethylene biosynthesis, was largely due to the ease of genetic screens in the model plant *Arabidopsis*, a good model for molecular genetics due to its short life cycle, small stature, and small genome. *Arabidopsis* seedlings, when grown in the dark in the presence of exogenous ethylene, have a distinct and well documented phenotype termed the "triple response phenotype," which consist of a shortened and thickened hypocotyl, shortened primary root with an increased number of root hairs, and an exaggerated apical hook (Figure 1-1) [14]. Initial genetic screens led to the isolation of two classes of mutants: those that were insensitive to exogenous ethylene and those that constitutively exhibited the triple response, even in the absence of ethylene. Map-based cloning of the isolated mutants resulted in the identification of many components of the pathway, like *ETHYLENE*

*RESPONSE1* (*ETR1*), the first plant hormone receptor (and ethylene receptor) to be cloned [15].

*Ethylene Signal Transduction*

**The Ethylene Receptors Negatively Regulate Ethylene Responses**

ETR1 is one member of a family of five ethylene receptors in *Arabidopsis*. This family can be further divided into two subfamilies. Subfamily I consists of ETR1 and ERS1, and subfamily II consist of ETR2, ERS2, and EIN4 [16]. Gain-of-function mutations within any of the receptors render plants insensitive to ethylene. Loss-of-function mutations in the subfamily 1 receptors confer ethylene-hypersensitivity while subfamily II loss-of-function mutants behave similar to wild type phenotypically (likely due to receptor redundancy). Combinatorial loss-of-function receptor mutants display a constitutive triple response phenotype in the absence of ethylene [16]. Thus, the receptors are essential for repressing ethylene responses and behave as negative regulators of ethylene responses: actively signaling to repress ethylene responses in the absence of ethylene and turning off this signal upon ethylene binding [15-17] (Figure 1-1**)**.

Similar to other gasses such as nitric oxide and oxygen, ethylene requires a transition metal cofactor to achieve high affinity binding to the receptors. In the case of the ethylene receptors, a $Cu^{2+}$ co-factor, delivered by the copper transporter RAN1 [18], is required for receptor function [19]. The receptors form homodimers through disulfide linkage of two cysteine residues (Cys4 and Cys6 in ETR1) within their amino-terminal transmembrane spanning region (which also functions to target the

# FIGURE 1-1

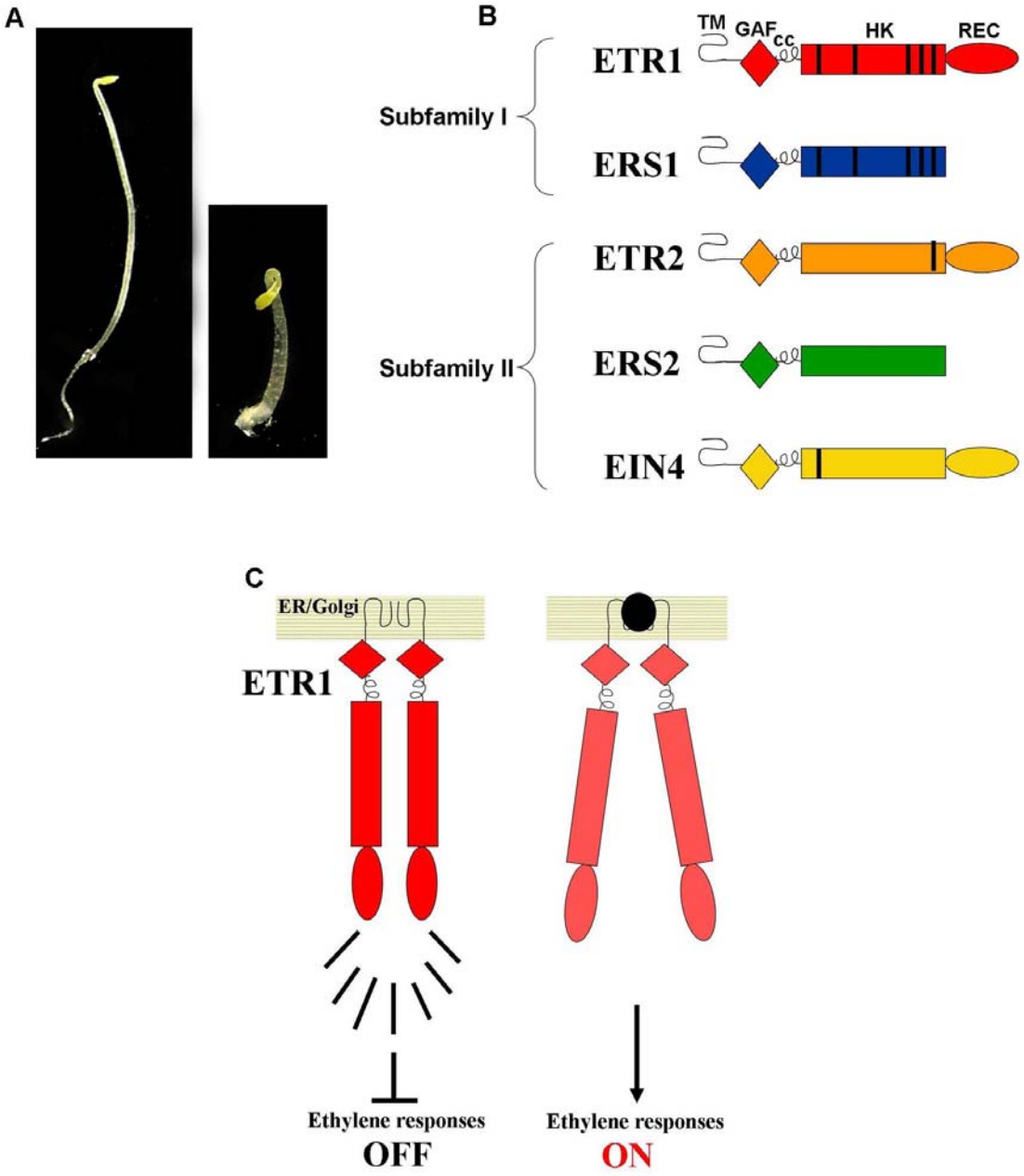

A
B
TM
GAF
CC
HK
REC
ETR1
ERS1
ETR2
ERS2
EIN4
Subfamily I
Subfamily II
C
ER/Golgi
ETR1
Ethylene responses
OFF
Ethylene responses
ON

**Figure 1-1:** Introduction to ethylene signaling. **A.** The triple response phenotype exhibited by etiolated *Arabidopsis* seedlings when grown in the dark in the presence of ethylene (right seedling) consists of a shortened and thickened hypocotyl region, an exaggerated apical hook, and short roots with lateral root hair proliferation. **B.** *Arabidopsis* has a family of five ethylene receptors which can be further subdivided into two subfamilies. **TM** = transmembrane domain, **GAF** = The GAF domain, **CC**= coiled coil domain, **HK** = histidine kinase domain, and **REC** = receiver domain. The subfamily I receptors have the conserved motifs required for proper histidine kinase activity (black bars on the histidine kinase domain indicate the position of these motifs), while the subfamily II receptors lack some or all of these motifs. **C.** The ethylene receptors, including ETR1, are negative regulators of signaling and signal in the absence of ethylene (receptor on the left) to repress downstream ethylene responses. Once ethylene binds (black circle) to the receptor, the receptor stops signaling, allowing ethylene responses (similar to the triple response seen in A, to be carried out.)

receptors to the endomembrane systems [20, 21]. Homodimers are the functional unit of the receptors, and each homodimer binds one molecule of $Cu^{2+}$and one molecule of ethylene within the amino-terminal transmembrane spanning pocket of the receptors (Figure 1-2) [19, 20]. Interestingly, Cys to Ala mutations that disrupt these disulfide linkages do not fully disrupt ETR1's ability to signal, implying that there are additional, unidentified non-covalent mechanism(s) that aid in homodimer formation [22, 23]. The ethylene-binding region of the receptors is comprised of three transmembrane domains in the subfamily I receptors and four in the subfamily II receptors. (The significance of the fourth transmembrane domain of the subfamily II receptors remains unknown.) In addition to binding ethylene and localizing the receptors to the endomembranes, the transmembrane domains also function in turning off receptor signal output once ethylene binds [24].

Adjacent to the receptor transmembrane domains is the GAF domain. GAF domains (GAF standing for *c**G**MP*-regulated mammalian phosphodiesterases, cyanobacterial **a**denyl cyclases, and a **f**ormate-hydrogen lyase transcriptional activator) are a subset of a larger family of small-molecule-binding domains (SMBD's), present in over 1600 proteins, and found in all three known domains of life [25]. GAF domains are commonly found in proteins involved in signal transduction or transcriptional activation and can bind nucleotides or tetrapyrroles like chlorophyll or phycobillins [25]. It is currently unknown whether the receptors' GAF domains bind a small molecule or what that small molecule might be. One function of the receptor GAF domains that was recently deduced is their role in the non-covalent heteromeric interactions among receptors. Truncated ETR1 and ETR2

receptors with only the GAF domain can physically associate with other receptors [23, 26], suggesting that higher order, heteromeric receptor interactions may play a role in transmitting the ethylene signal (in addition to receptor homodimers) formation [22, 23].

Sequence analysis of the receptors reveals that they share similarity to prokaryotic two-component systems which use histidine kinase activity as a signaling mechanism. The GAF and coiled coil domains of each receptor link the amino-terminal input (ethylene binding domain) to a carboxy-terminal histidine kinase domain [15]. While all five receptors have the histidine kinase-like domain, only the subfamily 1 receptors, ETR1 and ERS1, have the five essential motifs for histidine kinase activity and display histidine kinase activity *in vitro* (Figure 1-1)[27]. Extensive analysis of the subfamily I receptors' histidine kinase domains reveals that the histidine kinase activity is at least mostly dispensable for ethylene signal transduction. However the subfamily I receptors are essential for proper transmission, as the subfamily I double null mutant has severe phenotypic affects including sterility [28]. Interestingly, three of the receptors, ETR1, ETR2, and EIN4 have a response regulator-like receiver domain fused to the carboxy-terminal of the histidine kinase domain (Figure 1-1). In the prokaryotic counterparts, this domain is the acceptor of the phosphate generated by the histidine kinase activity. The function of this receiver domain in ethylene signaling also remains unclear.

Recently it was found that the *Arabidopsis* ETR2 receptor is degraded by an ER-associated 26S Proteosome complex upon ethylene binding [29]. Two tomato subfamily II receptors, ETR4 and ETR6 are also degraded upon ethylene binding

[30]. Degradation of the receptors could perhaps be a mechanism for signal transmission as receptor degradation (upon ethylene binding) should, in theory, lead to the alleviation of downstream ethylene responses, although it is unknown which receptors (or if all receptors) are targeted for degradation upon ethylene-binding. To date there is no evidence supporting ETR1 receptor degradation through the 26S Proteosome complex. ETR1 is a unique receptor in that it is the only receptor that has been shown to localize to the Golgi as well as the endoplasmic reticulum [21]. (Currently there is no evidence that the other receptors localize to the Golgi.) Additionally ETR1 is the only receptor known to require the novel, integral membrane protein, RTE1, for proper function [31]. *REVERSION-TO-ETHYLENE SENSITIVITY1* was identified based on two *rte1* alleles ability to suppress the ethylene insensitivity conferred by the *etr1-2* mutation [31]. RTE1's function seems to be specific to ETR1, as the *rte1* alleles cannot suppress other receptor gain-of-function mutations that confer ethylene insensitivity [31]. Additionally, *rte1* alleles can only suppress a subset of *etr1* ethylene-insensitive alleles [31], suggesting that RTE1's genetic interaction with ETR1 is very specific, possibly based on confirmation of the ETR1 receptor. Little is known about RTE1's role in negatively repressing ethylene responses through ETR1 [17]. However, *RTE1* expression is ethylene-inducible, suggesting that it may function in a negative feedback loop acting to reset the repression of the ethylene signal once ethylene has dissociated from the ETR1 receptor.

**CTR1 is a Raf-like Kinase that Represses Ethylene Responses**

Downstream of the receptors and RTE1 is another negative regulator of ethylene signaling, the Raf-like serine/threonine kinase CTR1 [32]. CTR1 is a putative mitogen-activated protein kinase kinase kinase (MAPKKK) with a 550 residue amino-terminal, non-catalytic region and a carboxy-terminal kinase domain. The *ctr1-1* loss-of-function allele, which disrupts CTR1 kinase activity *in vitro*, was isolated in a genetic screen for mutants that display a constitutive ethylene response [32]. Therefore, CTR1, similarly to the receptors, is a negative regulator of ethylene signaling, and the CTR1 kinase activity is essential for repressing ethylene responses [32, 33].

CTR1 homologs are found only in eukaryotes from plants to mammals. Consequently a long standing question in the field is "how does a prokaryotic-like receptor regulate a conserved eukaryotic kinase?" The histidine kinase domains of the two subfamily I receptors physically associate with the amino-terminal region of CTR1 [34, 35]. This physical association is essential as a missense point mutation in the CTR1 amino-terminal region, *ctr1-8*, that does not affect CTR1 kinase activity *in vitro*, disrupts association with the subfamily I receptors and confers a constitutive ethylene response phenotype *in planta* [33] (Shockey, 2004 dissertation.) CTR1 peripherally associates with the ER, and immunoprecipitation studies indicate that CTR1 is a member of the ETR1 receptor complex *in planta* [36]. To our knowledge this is the only known example of a direct and essential interaction between a prokaryotic-like two component system and a Raf-like kinase.

The mechanisms by which CTR1 signaling is deactivated and activated remain unknown, and most current hypotheses are based on the mammalian Raf homologs. The Raf kinase is recruited to the plasma membrane where it associates with the plasma-membrane bound Ras for activation. While experimental data suggest that CTR1 is not translocated to/from the ER for activation/deactivation [36], genetic data suggest that CTR1 directly interacts with the receptors for activation. This interaction could be lost upon ethylene binding, mimicking the *ctr1-8* mutation, leading to CTR1 deactivation.

Another potential parallel between Raf and CTR1 may be a role of phosphatidic acid (PA) in kinase regulation. One of the factors involved in Raf translocation is its binding to PA [37]. PA is a phospholipid, and in plants, PA levels increase rapidly in response to abiotic and biotic stresses [38]. Recently it was found that the CTR1 kinase domain can bind to PA *in vitro* [39]. However, unlike in the case of Raf, where PA binding aided in activation, PA binding to the CTR1 kinase domain inhibited CTR1 *in vitro* kinase activity [39]. Genetic support for a model in which PA can inactivate CTR1 is unavailable and will be difficult to obtain as PA is synthesized by a large family of proteins and loss-of-function mutants may have pleiotropic phenotypes, because of PA's role in multiple pathways.

Another question that will be challenging to elucidate genetically is the substrate of CTR1. Because CTR1 is a putative MAPKKK, one attractive hypothesis is that CTR1 signals through a MAP kinase cascade. However there is minimal work to support this hypothesis, and interestingly, all biochemical results to date suggest that if CTR1 signals through a MAP kinase cascade, the cascade would be unique in

that CTR1, the MAPKKK, would act to repress the MAPKK and MAPK instead of activating them [40, 41].

**EIN2, a Mysterious Protein, Promotes Ethylene Responses**

The next-known downstream component from CTR1 is the positive regulator EIN2. EIN2, a large integral membrane protein of 1294 residues, has twelve transmembrane domains in the amino terminal region that share similarity to the Nramp family of metal ion transporters (although metal transporting activity of EIN2 has not been detected). This is followed by a large (roughly 700 amino acid) carboxy-terminal soluble region with a coiled coil domain but no other conserved motifs [42]. Various point mutations in either the hydrophobic amino-terminal region or in the carboxy-terminal of EIN2 render *Arabidopsis* seedlings completely insensitive to ethylene [42]. Because of the complete ethylene-insensitivity in the *ein2* mutant background, many labs have utilized *ein2* mutants to begin dissecting the cross talk between ethylene and other signaling pathways. *ein2* mutants are hypersensitive to ABA [43] as well as glucose [44]. Interestingly ABA is required for the *ein2* mutant's hypersensitive response to glucose [44]. The exact mechanisms of EIN2 function within the ethylene signaling pathway, as well as any specific role EIN2 may play in the other hormone pathway, remain unknown. Recently it was shown that EIN2 is degraded rapidly by two F-box proteins, ETP1/ETP2 in the absence of ethylene [45].

**EIN3 and EIL1 Transcription Factors Are Regulated by the EBF1/2 F-Box Proteins**

EIN3 and a class of EIN3-like positive transcription factors act downstream of EIN2 and bind as homodimers to the "**p**rimary **e**thylene **r**esponse **e**lement" (PERE)

# FIGURE 1-2

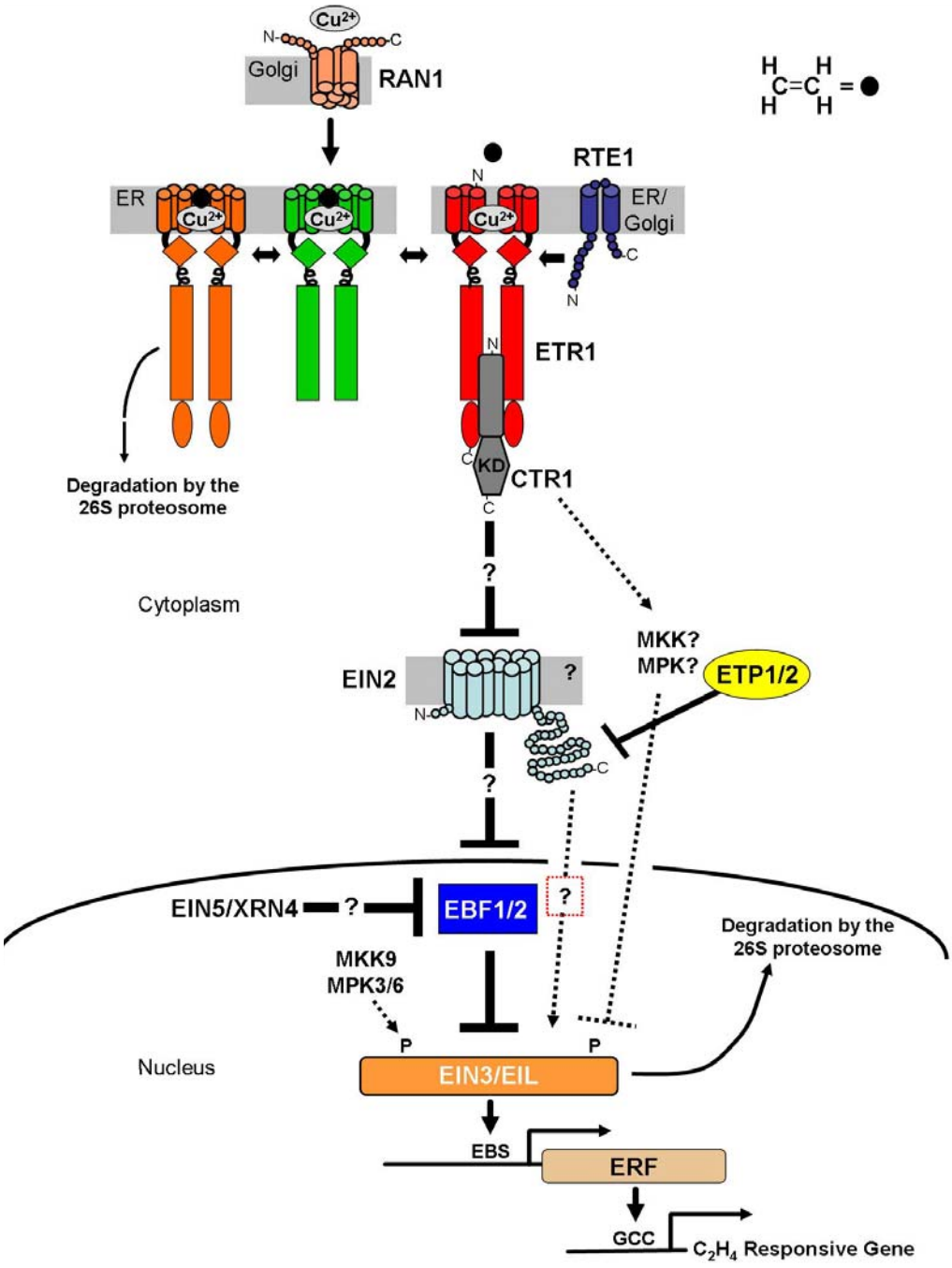

Cu2+
N-
-C
Golgi
RAN1
H H
C=C
H H
=
RTE1
N
ER
Cu2+
Cu2+
Cu2+
ER/
Golgi
C
N
N
ETR1
C
KD
CTR1
C
Degradation by the
26S proteosome
?
Cytoplasm
MKK?
MPK?
ETP1/2
EIN2
?
N-
-C
?
EIN5/XRN4
?
EBF1/2
?
Degradation by the
26S proteosome
MKK9
MPK3/6
P
P
Nucleus
EIN3/EIL
EBS
ERF
GCC
C2H4 Responsive Gene

**FIGURE 1-2:** The Ethylene Signal Transduction Pathway. (Figure is modified from Kendrick and Chang, 2008.) Ethylene is perceived at the endomembranes by a family of receptors that share similarity to prokaryotic two-component signaling systems. RTE1 and the copper transporter RAN1 act at or upstream of the receptors. The receptors and CTR1 signal to repress ethylene responses. Once ethylene binds to the receptors the signal is inactivated, causing CTR1 to be inactivated, allowing downstream ethylene responses to be carried out. EIN2 is a positive regulator downstream of CTR1 and is thought to be required for all ethylene responses, as loss-of-function mutations in *EIN2* seem to render *Arabidopsis* seedlings completely insensitive to ethylene. ETP1/2 are F-box proteins that target EIN2 for degradation in the absence of ethylene. Downstream of EIN2, *EIN3* and the *EIL*'s encode transcription factors that activate ethylene responses. EIN3 and EIL1 are regulated by two F-Box proteins, EBF1 and EBF2. *EIN5/XRN4* encodes an exoribonuclease that seems to negatively affect the transcription of *EBF1* and *EBF2*.

within the promoters of other transcription factors, such as the **E**THYLENE **R**ESPONSE **F**ACTOR's (ERF's), which in turn bind to GCC boxes in the promoters of several pathogen response genes [46]. The EIN3 and EIL1 transcription factors are tightly regulated by two F-box proteins, EBF1 and EBF2, which target the transcription factors for degradation through the 26S Proteosome in the absence of ethylene. EBF1/2 appear to have overlapping but non-redundant roles in targeting EIN3 and EIL1 for degradation as the double *ebf1 ebf2* mutant has a more severe, additive ethylene response phenotype than either of the single mutants [47-49]. Additionally, kinetic analysis of the hypocotyl growth rate of *ebf1* and *ebf2* single mutants reveals that the two F-box proteins act at different time points within the signaling pathway. EBF1 acts to degrade EIN3/EIL1 prior to/at the onset of ethylene responses, while EBF2 plays a larger role in degrading EIN3/EIL1 after the ethylene responses have been activated [50], perhaps functioning to reset the repression of ethylene responses. Such tight regulation of the EIN3 and EIL1 transcription factors, which promote ethylene responses, may be critical for a sessile organism's ability to quickly respond to stresses.

Similarly to EIN2, there have been multiple studies investigating EIN3 as a point of cross talk. These finding suggest that EIN3 may be a point of convergence of at least the ethylene, glucose and light signaling pathways [51-53].

**The Exoribonuclease EIN5 Promotes Ethylene Responses**

EIN3 regulation might occur through the regulation of the EBF1/2 F-box proteins themselves. EBF1/2 are indirectly regulated by ETHYLENE-INSENSITIVE5, which is the previously characterized 5'→3' exoribonuclease XRN4

[54, 55]. EIN5/XRN4 is homologous to yeast 5'→3' exoribonucleases Xrn1p and Rat1p, which function in mRNA and rRNA degradation, respectively; however, EIN5/XRN4 can only rescue the *xrn1p* mutant and not the *rat1p* [54]. Similar to Xrn1p, EIN5 localizes sub-cellularly to the cytoplasm [55]. *EBF1/2* transcripts, which are ethylene-inducible, accumulate in the *ein5/xrn4* background. Consequently, EIN3 protein does not accumulate in the *ein5*/*xrn4* background in the presence of ethylene, leading to the ethylene-insensitive phenotype of the mutant [54]. Similarly, many other ethylene-inducible transcripts are expressed at much lower levels in the *ein5* background relative to wild type [54, 55]. EIN5/XRN4 does not appear to directly degrade *EBF1*/*2* transcripts, because the half-life of *EBF1*/*2* transcripts in the *ein5* mutant background is the same as in the wild type [55]. Therefore, the accumulation of *EBF1*/*2* mRNAs may be due to increased transcription with *EIN5*/*XRN4* promoting a repressor of *EBF1*/*2* transcription.

**The *EER* Genes Represent a Class of Potential Ethylene Signaling Components**

One of the most recent classes of putative ethylene signaling components identified is the group of *ENHANCED ETHYLENE RESPONSE* genes (*EER*'s), identified through genetic screens for seedlings that exhibit enhanced triple response phenotypes, relative to wild type [56-60]. The *eer* mutants are not constitutive ethylene response mutants, so they act similarly to wild type in the absence of ethylene, but respond more rapidly to increasing levels of exogenous ethylene. *EER1* encodes a PP2A phosphatase, which was found to associate with the CTR1 kinase domain *in vitro* [57] and could potentially function in activating CTR1, similar to PP2A's role in activating Raf-1 [57]. *EER3* encodes a previously uncharacterized

prohibitin [59]. In mammalian systems, prohibitin functions to form transcriptional complexes [59]. *EER4* encodes a transcription factor containing a C-terminal putative TATA BOX BINDING FACTOR (TFIID)-interacting domain [58]. In yeast, TFIID binds to the TATA box and initiates formation of the RNA Polymerase II complex [59]. Christians and Larsen report that EER3 can directly interact with EER4 in the yeast-two-hybrid assay. If EER3 and EER4 have conserved functions relative to mammalian and yeast systems respectively, this interaction could be required for the recruitment of transcriptional machinery for the expression of genes that will repress ethylene responses. *EER5* encodes a protein with a proteasome COP9 initiation factor (PCI/PINT)-associated module (PAM) domain similar to those found in components of the COP9 signalosome (CSN). EER5 protein analysis suggests that EER5 interacts with the C-terminus of EIN2 and with the CSN, suggesting that EER5 functions between EIN2 and the modification or degradation of target proteins [60].

*Ethylene Signaling in Other Plants and Organisms*

Wang et al. tested for ethylene-binding in many plant species from the non-vascular moss *Physcomitrella patens* to the popular aquarium plants from the genus *Vallisneria* to the Chinese evergreen *Juniperus chinensis*, and found that all plants tested did bind ethylene [24]. While the sequence information for many of these plant genomes lags, available data support the idea that the ethylene signaling pathway, as observed in *Arabidopsis*, is highly conserved among plants. For instance, an *Arabidopsis etr1-1* transgene (which confers ethylene insensitivity in *Arabidopsis*) renders tomato seedlings insensitive to ethylene and delays the onset of tomato

ripening. The same *etr1-1* mutation when transferred to petunias can delay the abscission of flower petals [61]. Additionally, ethylene receptor homologs have been identified in many plants including tomato, passion fruit, marsh dock, peaches, and geraniums [62]. Downstream components such as CTR1, EIN2 and EIN3 homologs have also been identified in other plants, further supporting conservation of the ethylene signaling pathway [24, 35, 63]. Because ethylene was identified as a phytohormone, it was of interest to determine what other organisms, outside of the plant kingdom, have ethylene signaling activity. The most extensive sequence analysis of non-plant species has been by searching for homologs to the ethylene binding domain (EBD) of the ETR1 receptor. (Querying against the EBD and not full length ETR1 avoids the retrieval of histidine kinases that are not specific to ethylene binding.) This approach led to the identification of several EBD's in cyanobacteria as well as other eubacteria such as Proteobacteria [24]. Corresponding to the ethylene binding data (except in the case of Chara), no EBD's have been found in the available metazoa, fungi or green algae genomes [24]. It has been shown that the *Synechocystis slr1212* EBD protein can bind ethylene [19], but it remains unclear whether that binding has biological significance and whether there is an intact ethylene signaling system in prokaryotes. Such a system would require a CTR1-independent signaling pathway as CTR1 is not found in prokaryotes.

*Cross-talk With Other Hormones*

In recent years many exciting discoveries have been in made in the other phytohormones, which had previously been lagging behind our understanding of the ethylene signaling pathway due mostly to the lack of such a distinct and well

documented phenotype (i.e. the triple response phenotype) to utilize for genetic screens. For example, degradation of hormone-response repressors by the 26 S Proteosome seems to be key for auxin, jasmonic acid (JA) as well as gibberrelic acid (GA) signaling. The F-box protein TIR1 has been shown to be an auxin receptor [64, 65]. COI1 is an F-box protein that can bind JA and upon JA binding targets the jasmonic acid repressor family, the JAZ proteins, for degradation via the 26 S Proteosome [66]. Likewise the soluble GA receptor GID1 binds to the DELLA proteins, which repress gibberellin responses, and targets them for degradation [67]**.** Abscisic acid (ABA) has a unique signaling system, and recently two putative ABA receptors have been characterized. The H-subunit of $Mg^{2+}$-protoporphyrin not only plays a role in chlorophyll biosynthesis and plastid-to-nucleus retrograde, but can also bind ABA and promotes ABA signaling [68]. The G-coupled protein, GCR2 is a plasma membrane bound protein that can bind physiologically relevant levels of ABA and may serve in an ABA-receptor capacity [69, 70]. The more we learn about the signaling pathway of the different phytohormones, the clearer it becomes that these pathways integrate into much more complex relationships than we can currently understand, acting to antagonize each other under specific conditions while acting synergistically under other conditions. As an example, ethylene and JA have been found to synergistically act in pathogen attack but antagonize each other in apical hook formation and in wound responses [71]. The cross-talk between JA and ethylene can occur prior to ethylene biosynthesis as JA-ACC conjugates have been found in plants and suggest that conjugation may provide a mechanism that regulates availability of both substrates [71]. JA and ethylene cross-talk can also occur at

downstream point within both pathways. Exogenous application of either JA or ethylene induce the expression of the transcription factor *ETHYLENE RESPONSE FACTOR1* (ERF1), and application of both hormones has an additive effect on *ERF1* expression which in turn leads to a subset of ethylene and jasmonic acid responses [72].

ABA may also have multiple points of cross talk with ethylene signaling. ABA can induce the expression of multiple ACC synthase genes [73]. However, ethylene-insensitive mutants, such as the *etr1-2* receptor mutant, are hypersensitive to ABA, suggesting that ABA can also affect signaling downstream of ethylene biosynthesis. The *etr1-2* mutant seed accumulates more biological active ABA than WT seeds and less inactive ABA conjugates [74]. These are only two examples of how JA and ABA can influence the ethylene-signaling pathway at multiple points. A complete understanding of how these tightly regulated hormone pathways integrate into a larger network of all the hormones, leading to specific plant responses, remains an inexplicable question.

## *Conclusions*

Many of the components involved in ethylene signaling have been identified, and through epistasis analysis have been placed into the ethylene signal transduction pathway. This pathway has expanded from a linear model to a more complex, intricate model with multiple points of regulation both by feedback mechanisms as well as input from other hormones and signaling pathways. Genetic and biochemical data suggest that there are additional components of the pathway yet to be identified.

Additionally, the mechanisms that transmit the signal from one component to the next, in particular from the receptors to CTR1, CTR1 to EIN2, and EIN2 to the downstream components, remain unknown. The foundation of the pathway has been established, and now by identifying additional components of the pathway through new screens, utilizing new protein-protein interaction tools for *in planta* visualization, and developing new genetic approaches to study the already known proteins of the pathway, we will begin to fill in the gaps within our current understanding but likely also expose larger and more complex questions about the pathway.

# Chapter 2: AWE1is a Novel Protein That Functions in Hypocotyl Elongation and Cell Expansion

*Introduction*

Multiple lines of evidence suggest that additional components of the ethylene signal transduction pathway have yet to be elucidated. A receptor quadruple loss-of-function mutant (*etr1 etr2 ers2 ein4*) has a more severe phenotype than the constitutive response mutant, *ctr1*, suggesting an unidentified CTR1-independent pathway downstream of the receptors [16]. *ctr1* mutants still respond to exogenous ethylene [16], suggesting that disruption of CTR1 activity does not entirely disrupt ethylene signaling. Finally, a pull-down experiment with Arabidopsis ETR1 yielded a large complex of proteins, including CTR1, the other ethylene receptors, as well additional proteins not yet identified [36, 75](GE Schaller, personal communication.)

Traditionally forward genetic screens have been used to identify components of the ethylene signaling pathway. However, those screens became saturated over time with more than 40 ethylene-insensitive *ein2* alleles and 9 constitutive ethylene responding *ctr1* alleles being isolated [33, 42]. Therefore in order to elucidate unidentified components using a genetics approach, we have employed new screens. Using forward genetics our lab continues to identify potential components of the pathway by screening for suppressors of the ETR1 receptor ethylene-insensitive mutant *etr1-2* (Appendix A). Additionally our lab took a reverse genetics approach,

employing the yeast-two-hybrid assay, to screen for proteins that interact with the ETR1 ethylene receptor. The soluble portion of the ethylene receptor ETR1 (residues 293-729) was screened against an *Arabidopsis* etiolated seedling cDNA library (~$10^6$ clones screened). Twenty-nine clones were identified as potential ETR1-interactors, because they interacted with the ETR1 bait but not with the non-specific control bait, human lamin. Out of the twenty-nine clones isolated, ASSOCIATES-WITH-ETR1 (AWE1), was of special interest because in addition to a strong interaction with ETR1, the AWE1-prey interacted with a small portion of CTR1(amino-terminal residues 308 through 569), the next known downstream component in the ethylene signal transduction pathway (Wen-ming Ding, Masters thesis). Investigation of the *AWE1* gene and protein product suggests that AWE1 functions in hypocotyl elongation and cell expansion through both ethylene-dependent and ethylene-independent processes.

*Results*

**Cloning the *AWE1* cDNA**

For further *AWE1* analysis and downstream sub-cloning purposes I wanted to obtain the annotated full-length *AWE1* coding sequence. The *AWE1* clone isolated from the yeast-two-hybrid screen was only a partial sequence, and there were no full-length EST's available when I began my studies. Previous attempts by others to clone the annotated *AWE1* full-length coding sequence were unsuccessful. Two sets of primers designed to amplify the annotated full-length *AWE1* coding sequence had

resulted in products that carried mutations in the predicted 'ATG' start codon. In an attempt to avoid this problem, I designed a primer twenty nucleotides upstream of the predicted start codon and using RT-PCR was able to clone the *AWE1* coding sequence, as annotated by the TAIR website (www.arabidopsis.org), from wild-type rosette leaves for downstream analyses, such as sub-cloning purposes.

### *AWE1* Encodes a Protein with Homologs in Cyanobacteria and Plants

*AWE1* (*At3g29185.1*) is a single-copy gene in *Arabidopsis* that encodes a protein of 396 amino acids. The primary amino acid sequence lacks any recognized, conserved motifs (Figure 2-1a). The first forty-one residues of the encoded product comprise a weakly predicted chloroplast transit peptide sequence (Figure 2-1a) (ChloroP1.1)[76]. The AWE1 protein is predicted to be a soluble protein that localizes sub-cellularly in the chloroplast, vacuole, and cytoplasm according to the Bioarray Resource sub-cellular prediction program (www.bar.utoronto.ca) [77].

*AWE1* has two predicted gene models; the first being *At3g29185.1*. The second, *At3g29185.2*, is predicted to encode a 411 amino acid protein with a transmembrane domain (Figure 2-1b). Currently there is no *Arabidopsis* EST data to support the second gene model. The clone isolated from the initial yeast-two-hybrid screen encoded the last 343 amino acids of *At3g29185.1*, and the cDNA that I cloned from *Arabidopsis* rosette leaves also encodes the *At3g29185.1* model.

Previously it was reported that AWE1 and the **e**thylene **b**inding **d**omain (EBD) of ETR1 have homologs in cyanobacteria and other plants but not in protists, fungi, or animals, suggesting that the protein-protein interaction might be highly conserved (Shockey, dissertation 2004). I ran the blastP and tblastn programs

# FIGURE 2-1

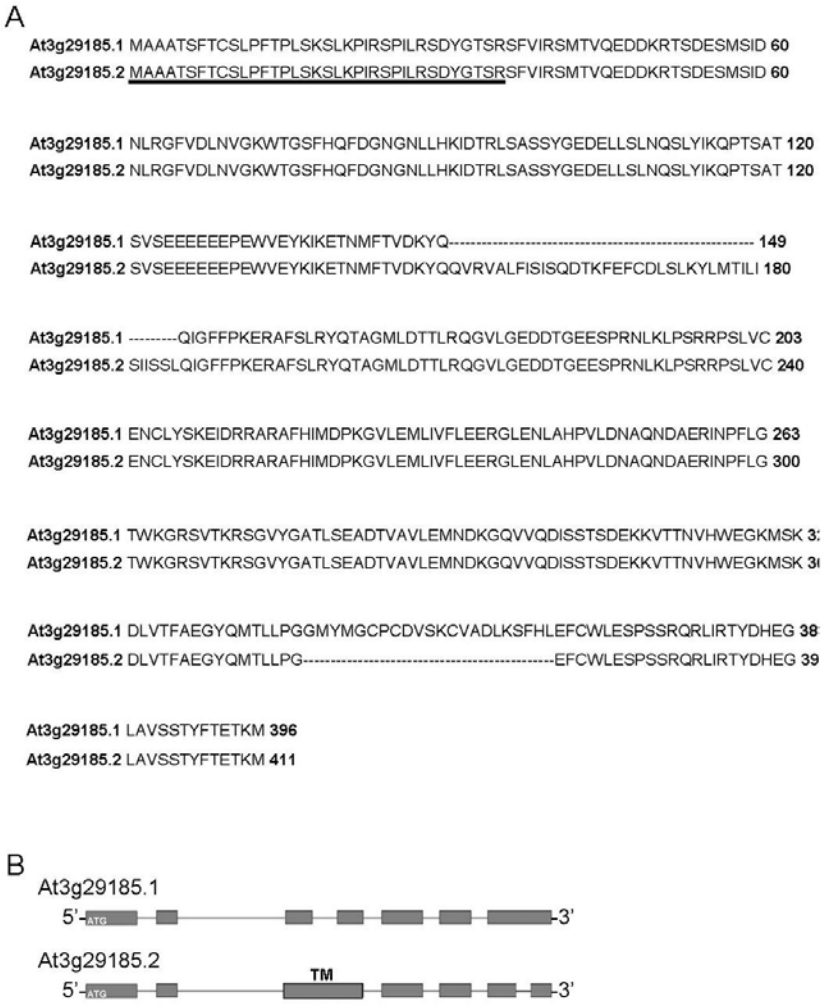



**Figure 2-1:** AWE1 encodes a novel protein with no conserved motifs. **A&B.** There are two predicted gene models for *AWE1*. *At3g29185.1* encodes a protein of 396 amino acids in length while the predicted At3g29185.2 protein is 411 amino acids in length and predicted to include an intron with a transmembrane domain (TM). The underlined region in **A** (residues 1 to 42) is a predicted chloroplast transit peptide sequence (ChloroP1.1).

querying the At3g29185.1 amino acid sequence against the non-redundant protein sequence database and the nucleotide collection database respectively, to identify potential plant homologs in more recently sequenced genomes. I detected hits in *Lycopersicon esculentum* (tomato) and *Oryza sativa* (rice) as previously reported. Additionally, there were hits in the dicot *Populus tricocharpa* (California poplar), the monocot *Sorghum bicolor* (sorghum), two mosses *Physcomitrella patens* and the club moss *Selaginella moellendorffii* , and cyanobacteria *Anabaena variabilis* (filamentous cyanobacteria), *Synechocystis sp. PCC 6803* (marine cyanobacteria), and *Gloeobacter violaceus* (rod-shaped cyanobacteria) (Table 2-I). Interestingly, all of the hits detected have good e-value scores (Table 2-I, column 4), but lack roughly the first 40 to 45 residues present in AWE1, roughly 20% of the AWE1 sequence (Table 2-I, column 3). For example, the predicted *Populus trichocarpa* peptide sequence spans from AWE1 residues 55 to 396 (Table 2-I column 3). I did not identify any AWE1 homologous sequences (cut-off e-value of <1) in protists, yeast or animals. Recent work by Wang et al. (2006) showed a similar pattern for the ETR1 EBD [24]. ETR1 EBD homologs were found in cyanobacteria and plants, but were not identified in protists, yeast, or animals (cut-off e-value of <1)[24]. However information on potential ETR1 EBD homologs in the above more recently sequenced genomes had not been verified. I repeated the blast searches using the protein sequence for the ETR1 EBD (residues 1-128 as defined by Wang et al. (2006)[24] and specifically searched for hits in the above listed organisms. I found ETR1 EBD homologous sequences in all of the organisms listed above (Table 2-I). Finally I ran the tblastn program using the ETR1 EBD sequence as a query. As previously

TABLE 2-I

| **Organism** | **Blast program** | **Residues of AWE1** that the sequence aligns with (1-396 possible) | **E-value of AWE1 blast result** | **Residues of EBD** that the sequence aligns with (1-128 possible) | **E-value of ETR1 EBD blast result** |
|---|---|---|---|---|---|
| *Populus trichocarpa* (poplar tree) | blastP and tblastn | 55-396 | 2e-150 | 1-128 | 4e-83 |
| *Lycopersicon esculentum* (tomato) | tblastn | 42-396 | 1e-145 | 2-128 | 9e-62 |
| *Sorghum bicolor* (grain sorghum) | blastP | 61-396 | 9e-133 | 1-128 | 9e-76 |
| *Oryza sativa* (rice) | blastP and tblastn | 54-396 | 2e-131 | 1-128 | 8e-64 |
| *Physcomitrella patens* (moss) | blastP | 61-396 | 3e-85 | 1-128 | 9e-67 |
| *Selaginella moellendorffii* (club moss) | blastP | 150-187; 190-238; 251-300; 313-396 | 2e-60 | 7-128 | 8e-70 |
| *Anabaena variabilis* (filamentous cyanobacteria) | tblastn | 12-103; 199-396 | 8e-13 | 23-117 | 3e-19 |
| *Synechocystis sp PCC 6803* (marine cyanobacteria) | tblastn | 32-396 | 8e-07 | 24-117 | 2e-14 |
| *Gloeobacter violaceus* (rod shaped, cyanobacteria) | tblastn | 68-396 | 1e-04 | 16-86 | 4e-05 |

**TABLE2-I:** AWE1 and the ethylene-binding domain of ETR1 have homologs in plants and cyanobacteria. The At3g29185.1 amino acid sequence was queried using the blastP or tblastn programs (**column 2**) to identify candidate AWE1 homologs that had not previously been reported (Shockey, dissertation 2004). Although the e-value scores for each hit were good (**column 4**), none of the organisms (**column 1**) had full length homologs of AWE1 (**column 3**). For example, *Populus trichocarpa* (**column 1**) had a very good e-value score of 2e-150 (**column 4**) but the *Populus trichocarpa* predicted peptide sequence only stretched from AWE1 residues 55-396 (**column 3**). Most of the sequences resulting from blast shared homology to the last ~80% of the AWE1 amino acid sequence. In the case of *Selaginella moelendorffii,* I combined the sequences from all four hits (spanning AWE1 residues 150-187, 190-238, 251-300, and 313-396, third column) and ran a blast protein-protein alignment between AWE1 and the combined *Selaginella moelendorffii* sequences to retrieve the e-value. The e-values of homologous sequences to the ethylene binding domain of ETR1 (ETR1 residues 1-128) are shown in **columns 5** and **6**.

reported, I found no ETR1 EBD homologs in animals, fungi, or protists (e-value <1). This parallels the blast results for AWE1. Although the primary amino-acid sequence yields no clues to function, a secondary structure prediction program PHYRE (Protein Homology-analogY Recognition Engine) [78] predicts a lipocalin-like fold in the carboxy-terminal 200 amino acids of AWE1 as the predicted secondary structure of AWE1 aligns (with an e-value of 1.4e-21) to the solved crystal structure of a putative lipocalin-like protein from *Nostoc punctiforme pcc 73102*. Lipocalins are generally cytosolic or secreted, extracellular-localized proteins that are found in organisms from prokaryotes to mammals and generally bind small, hydrophobic molecules [79].

**Confirming the Protein-Protein Interactions *In Planta***

Next I wanted to confirm the ETR1-AWE1 and CTR1-AWE1 physical interaction, observed in yeast, in plants. For this I cloned *AWE1*, *ETR1*, and *CTR1* into split YFP vectors to test for protein-protein interactions *in planta* using bimolecular fluorescence complementation analysis (BiFC). The split YFP system is thoroughly described in Walter et al. (2004) [80]. The YFP (YELLOW FLUORESENT PROTEIN) molecule is split into an amino-terminal half (nYFP) which is fused to one protein of interest and a carboxy-terminal half (cYFP) which is fused to a second protein of interest. If the two proteins interact *in planta*, then the nYFP and cYFP will assemble into a complete YFP molecule which can be detected by fluorescence microscopy. ETR1 and CTR1 were each cloned into the vector carrying the nYFP (pSPY**N**E) and AWE1 into the vector carrying the cYFP tag (pSPY**C**E) (Figure 2-2a). Each of the YFP constructs and the *p19* (RNA silencing suppressor) vectors were individually transformed into the *Agrobacterium strain*

*C85C1* and grown up in culture separately, because each vector carried a kanamycin-resistance marker for selection of transformants. Then all three cultures (one carrying the nYFP clone, one the cYFP clone, and the third carrying the p19 plasmid) were resuspended together for infiltration into two-week old tobacco plants. (Tobacco was used for this study instead of *Arabidopsis* due to the ease of infiltration into tobacco leaves for this transient expression study. Additionally tobacco cells are larger than *Arabidopsis* pavement cells, and therefore easier to view under the microscope.) Four days after infiltration I detected yellow fluorescence above the background level (background being either un-infiltrated leaves or leaves infiltrated with ETR1-nYFP alone) in tobacco leaves infiltrated with ETR1-nYFP and AWE1-cYFP, indicative of interaction. The frequency of the interaction was low as I observed signal above background in ~33% of the leaves, 4 out of 12. The YFP pattern in the pavement cells (Figures 2-2d and e) resembled over-expression of ETR1 in tobacco pavement cells alone (Figures 2-2b and c). The fluorescence was more easily detected in mesophyll cells in which the YFP pattern appeared as a diffuse network of yellow (Figures 2-2j and k). Such a diffuse pattern might be expected of an endomembrane-localized protein, ETR1, and a cytosolic protein (potentially AWE1) [26]. For comparison figure 2-2h and 2-2i show the interaction between two endomembrane proteins, ETR1 and RTE1, which form a more defined net-like pattern expected of endomembrane localized proteins [26]. Finally, a weak fluorescent signal above background levels (Figures 2-2f and g) was detected for AWE1 and CTR1 (Figures 2-2l and m) in epidermal pavement cells, in a similar pattern as observed in the ETR1 and AWE1 analysis (Figures 2-2d and 2-2e.) The frequency of observation was low.

# FIGURE 2-2

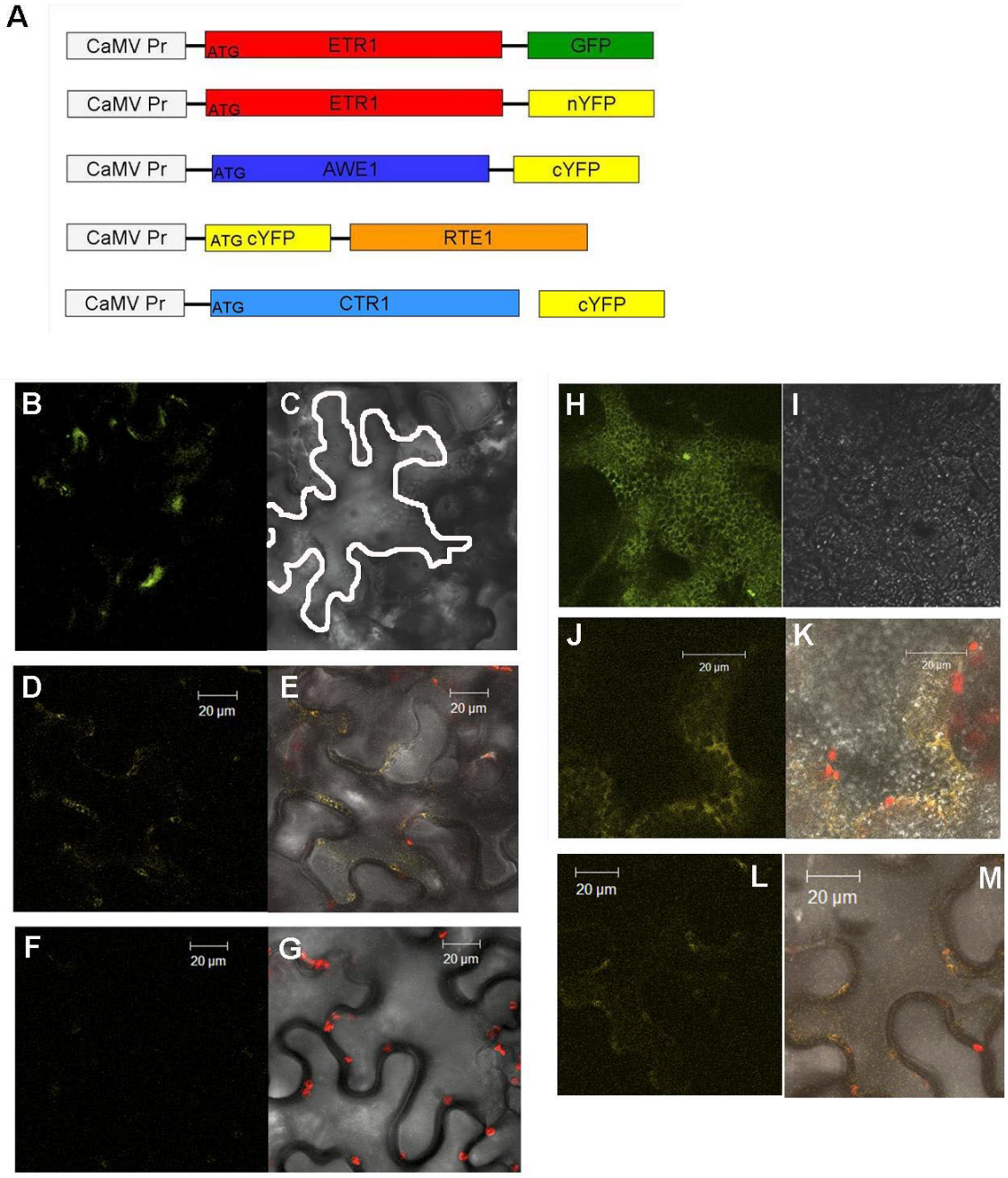

A
CaMV Pr
ATG
ETR1
GFP
CaMV Pr
ATG
ETR1
nYFP
CaMV Pr
ATG
AWE1
cYFP
CaMV Pr
ATG cYFP
RTE1
CaMV Pr
ATG
CTR1
cYFP
B
C
D
20 µm
E
20 µm
F
20 µm
G
20 µm
H
I
J
20 µm
K
20 µm
20 µm
L
20 µm
M

**Figure 2-2:** Confirming the AWE1-ETR1 and AWE1-CTR1 interactions in planta using BiFC. **A.** Fusions used for testing the interactions. ETR1-GFP was used for the localization of over-expressed ETR1 in tobacco. The nYFP and cYFP stand for the amino-terminal and carboxy-terminal halves of YFP, which come together to form one molecule of YFP if the two proteins interact. **B-M.** Confocal images of the abaxial surface of tobacco leaves. **B&C.** Localization of ETR1-GFP in tobacco epidermal pavement cells. The left panel representing the GFP signal and the right panel showing bright field image (photos courtesy of Chunhai Dong.) The white line in C is the outline of one pavement cell. **D&E.** White arrows point to yellow fluorescence detected in tobacco pavement cells infiltrated with ETR1 and AWE1, suggesting ETR1-AWE1 protein-protein interaction (488 nm laser, intensity of 20%.) **F&G.** This yellow-fluorescent pattern is not seen in tobacco plants infiltrated with ETR1-nFYP alone (488nm laers, intensity of 23%). **H&I**. Yellow fluorescence detected in tobacco mesophyll cells infiltrated with two endomembrane bound proteins, ETR1 and RTE1 (photos courtesy of Chunhai Dong). The pattern is tight and reticulate. **J&K**. Yellow fluorescence detected in tobacco mesophyll cells infiltrated with one endomembrane-bound protein, ETR1, and AWE1 (488nm laser, intensity of 16%). The yellow pattern in **J** is more diffuse than in **H**, which supports a membrane-protein interacting with a cytosolic-localized protein. **L&M.** Weak yellow-fluorescent signals were detected above background levels (white arrows) in the pavement cells of tobacco infiltrated with AWE1 and CTR1 (488 nm laser, intensity 24%). The pattern was similar to the YFP pattern seen in pavement cells testing ETR1 and AWE1 interaction (D&E).

About 30% of the infiltrated leaves had detectable fluorescence. In this replicate, two of the five leaves analyzed had detectable fluorescence. While I did detect a small amount of chlorophyll auto-fluorescence through the YFP filter in the tobacco infiltrated with ETR1-nYP alone (Figures 2-2f and g), I have not observed the distinct YFP patterns observed in Figures 2-2d and e in the ETR1-nYFP alone, negative control (five leaves analyzed).

### *awe1-1* Mutants are Impaired in Hypocotyl Elongation and Cell Expansion

The BiFC results suggest that AWE1 can interact with ETR1 and CTR1 *in planta*. Because *etr1* loss-of-function mutants are hypersensitive to ethylene and *ctr1* loss-of-functions mutants display constitutive responses to ethylene, I tested *awe1* loss-of-function mutants for enhanced ethylene responses to test for AWE1's involvement in ethylene signaling. I characterized an *awe1* loss-of-function mutant and *AWE1* over-expression transgenic lines. *awe1-1* is a SALK T-DNA insertion line [81] carrying a T-DNA in the third intron of the *AWE1* coding sequence (Figure 2-3a). *awe1-2* is an *Arabidopsis* tilling line [82] that encodes an S269L amino acid substitution in a conserved serine residue. *awe1-3* is a second T-DNA insertion line (SALK), with the T-DNA in the 3'UTR. The *awe1-1* mutant line was used for initial analysis, because the T-DNA insertion site made this line the most likely to have reduced *AWE1* expression.

RT-PCR analysis of RNA extracted from individual plants confirms that the *awe1-1* mutant is a loss-of-function allele with largely reduced levels of full length *AWE1* transcripts (Figure 2-3b).

# FIGURE 2-3

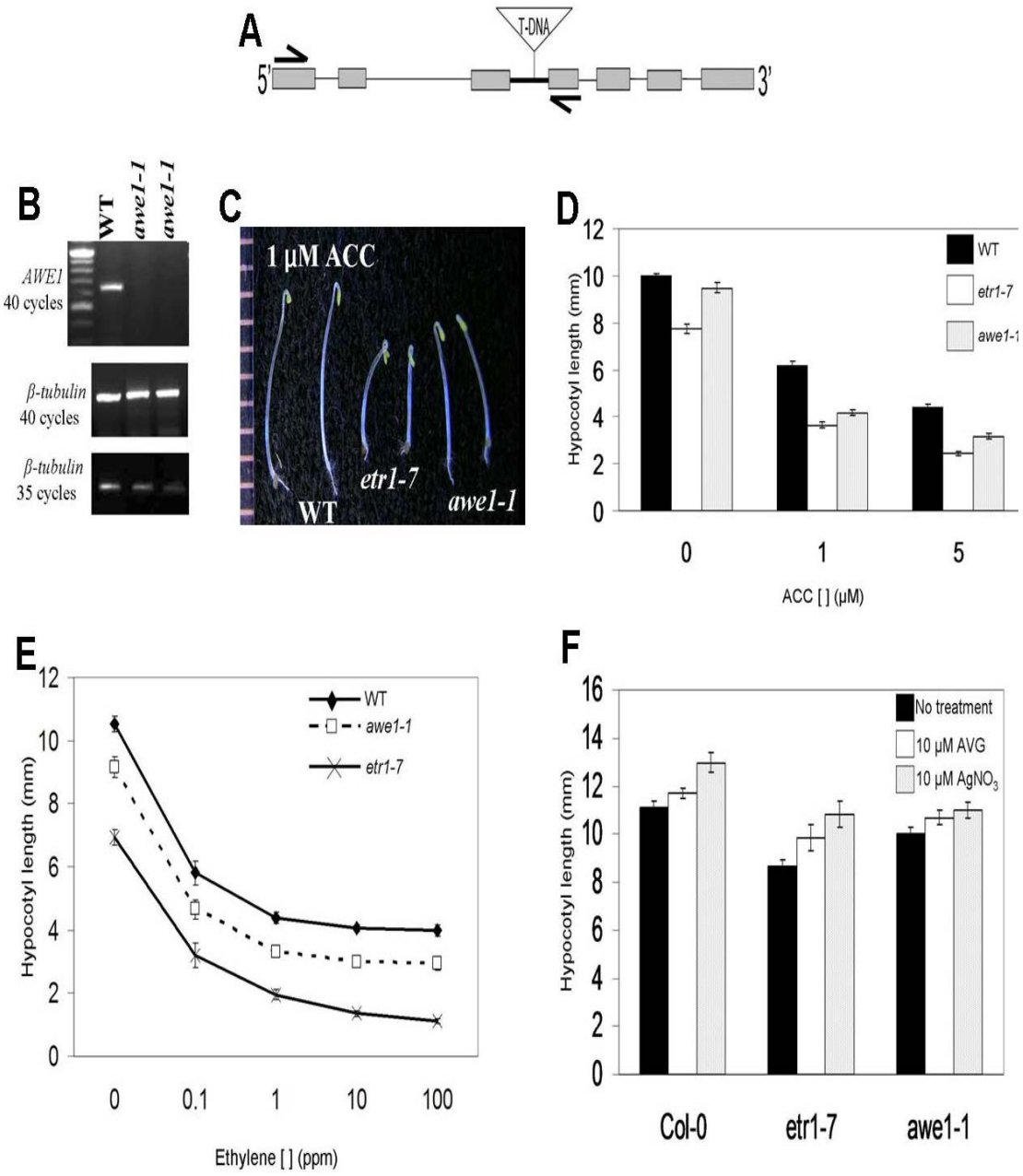

A
T-DNA
5'
3'
B
WT
awe1-1
awe1-1
AWE1
40 cycles
β-tubulin
40 cycles
β-tubulin
35 cycles
C
1 μM ACC
WT
etr1-7
awe1-1
D
Hypocotyl length (mm)
WT
etr1-7
awe1-1
ACC [ ] (μM)
E
Hypocotyl length (mm)
WT
awe1-1
etr1-7
Ethylene [ ] (ppm)
F
Hypocotyl length (mm)
No treatment
10 μM AVG
10 μM AgNO3
Col-0
etr1-7
awe1-1

**Figure 2-3:** *awe1-1* mutants are hypersensitive to ethylene. **A.** The *awe1-1* mutation is a SALK-derived T-DNA insertion in the 3rd intron of the gene. **B** RT-PCR (from RNA of 1, 3-week old rosette per sample) showing that transcript levels are significantly reduced in the mutant background. (The positions of *AWE1* primers used for the RT-PCR are indicated in figure A by black, half arrows). **C&D.** Dark-grown *awe1-1* mutant seedlings have shorte hypocotyls than wild-type seedlings when grown on the ethylene-precursor 1- Amino-1-cyclopropane carboxylic acid (ACC), but the *awe1-1* hypocotyls are not as short as the hypocotyls of the ethylene-hypersensitive ethylene recepto *etr1-7* mutant. **E.** Hypocotyl lengths of dark-grown *awe1-1* mutant seedlings were also shorter than wild-type when grown in the presence of exogenous ethylene in the ethylene dose response assay. **F.** *awe1-1* and *etr1-7* dark-gro seedlings slightly respond to the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) as well as to silver, which blocks ethylene binding by the receptors. Error bars on graphs represent standard error of the mean (n>20 seedlings for each data point).

*awe1-1* mutant seedlings exhibited shortened hypocotyls when grown in the dark on medium containing the ethylene precursor *1-amino-1*-cyclopropane carboxylic acid (ACC) (Figures 2-3c and d) or in the presence of exogenous ethylene (Figure 2-3e), suggesting the *awe1-1* mutant might be hypersensitive to ethylene. *awe1-2* and *awe1-3* mutants responded similarly to wild- type in the dark-grown seedling assays (Appendix b). This was not completely surprising as a single amino acid substitution in *awe1-2* may not be sufficient to disrupt AWE1 function, and the insertion of a T-DNA in the 3' UTR in *awe1-3* might not affect *AWE1* expression levels. Consequently, the *awe1-2* and *awe1-3* mutants were not characterized further.

When the *awe1-1* mutant seedlings were grown on medium containing the ethylene biosynthesis inhibitor, aminoethoxyvinylglycine (AVG) [83] or on medium containing silver ($AgNO_3$), which inhibits ethylene binding by the receptor [19, 84], the hypocotyl shortening was partially alleviated, similarly to the ethylene receptor loss-of-function mutant *etr1-7* (Figure 2-3f), implying that the *awe1-1* hypocotyl phenotype is at least partially due to impaired ethylene signaling.

Since ethylene is known to promote the senescence of plant tissue [85], I tested whether the *awe1-1* mutant had an enhanced or premature senescence response to ethylene. I tested for the premature senescence of cotyledons and rosette leaves in *awe1-1* mutant plants relative to wild-type when grown in the presence of ~100 ppm exogenous ethylene for three days. *awe1-1* mutant cotyledons exhibited slight yellowing relative to wild-type cotyledons, but not to the extent of a known ethylene-hypersensitive mutant, *etr1*-7 (Appendix b). I did not observe obvious premature senescence in the adult rosettes of the *awe1-1* mutant relative to wild-type, unlike the

*etr1-7* mutant which displayed premature senescence (Appendix b). This may indicate that AWE1 functions in only a subset of ethylene responses, which does not include senescence.

Although the *awe1-1* rosettes do not display premature senescence in the presence of ethylene, the mutant plants have smaller, more compact rosettes than wild-type plants (Figures 2-4a and b). Ethylene is known to inhibit cell expansion, and constitutive ethylene response mutants have small compact rosettes as a consequence [32]. Bright-field images of the abaxial surface of *Arabidopsis awe1-1* leaves revealed that the pavement cells of the *awe1-1* mutant are slightly smaller than wild-type cells (Figure2-4c), and this may attribute, at least in part, for the smaller rosettes observed in the *awe1-1* mutants.

Next I wanted to verify that the above phenotypes are indeed a consequence of the *awe1* mutation and not the result of an independent T-DNA insertion in another locus of the same T-DNA line. This was of concern, because I did not have a second allele for *awe1*. Since there are currently no candidate *awe1* knock-out lines available, I took a second approach to verifying the *awe1-1* phenotypes. I made an *AWE1* over-expression construct, which was able to rescue the mutant *awe1-1* phenotypes in the four independent lines that I analyzed in the seedling triple (Figure 2-5a and 2-5b.) These results suggest that disruption of the *awe1* locus in the *awe1-1* background is causing the observed phenotypes.

# FIGURE 2-4

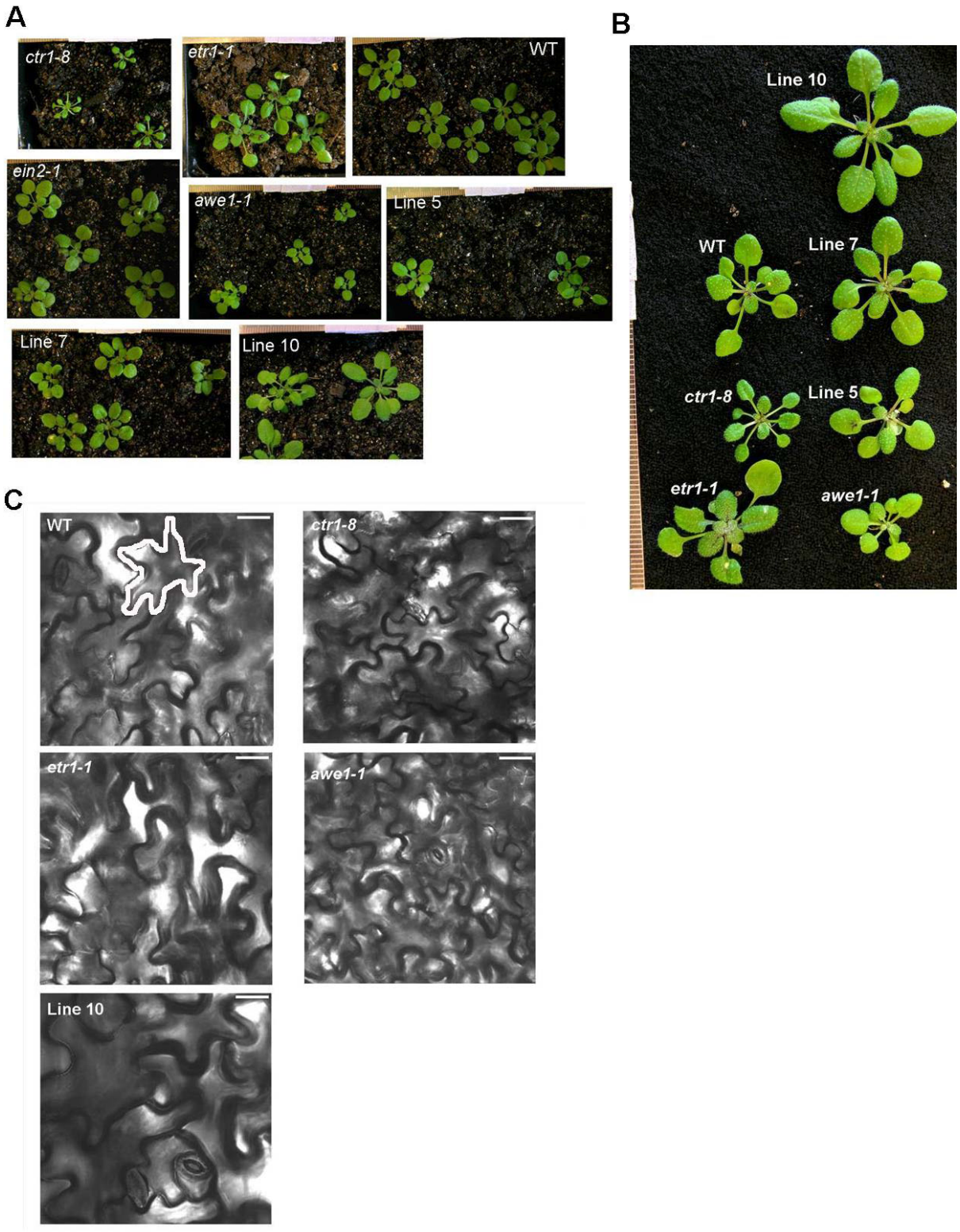

A
ctr1-8
etr1-1
WT
ein2-1
awe1-1
Line 5
Line 7
Line 10
B
Line 10
WT
Line 7
ctr1-8
Line 5
etr1-1
awe1-1
C
WT
ctr1-8
etr1-1
awe1-1
Line 10

**Figure 2-4:** *awe1-1* mutants have smaller rosettes than wild-type plants, which may be a consequence of cell expansion. **A&B.** *awe1-1* 3-week old rosettes have smaller, more compact rosettes than wild-type plants or the ethylene-insensitive mutants *etr1-1* and *ein2-1* but not as severe as the constitutive ethylene-response mutant *ctr1-8*. Over-expression of *AWE1* in the *awe1-1* mutant background can rescue this phenotype as observed in the 3 independent *awe1-1* lines over-expressing *AWE1* (#5, #7, and #10) seen here. **C.** Bright field images of the abaxial surface of the oldest leaf from each of the rosettes in **A**, shows that the cells of the *awe1-1* mutant rosettes are slightly smaller than wild-type and *etr1-1*. The *AWE1* over-expression line shown has pavement cells that look similar to wild type. (The pavement cells of three leaves were examined for each genotype. The white scale bar = 20μm.) (The white line indicates the outline of one *Arabidopsis* pavement cell.)

**Over-expression of *AWE1* Alleviates *etr1-7* Hypocotyl Shortening**

*awe1-1* loss-of-function mutant seedlings had slightly shorter hypocotyls than wild-type seedlings when grown in the presence of ethylene, so I wanted to test for the converse, ethylene-insensitivity in *AWE1* over-expression lines. I analyzed the dark-grown seedlings of five wild-type lines over-expressing *AWE1* when grown on medium containing ACC or in the presence of exogenous ethylene (Figure 2-5c and 2-5d)(Appendix b). RT-PCR results for three of the lines (lines 1, 2, and 4) left me unsure of whether *AWE1* was being over-expressed (Appendix b), but RT-PCR for lines six and line seven confirmed over-expression of *AWE1* (Figure 2-5e). All five independent wild-type transgenic lines displayed hypocotyl lengths similar to wild-type untransformed seedlings when grown in the dark in the presence of ethylene (Figures 2-5c, d and Appendix b). However, over-expression of *AWE1* in the ethylene-hypersensitive *etr1-7* mutant restored seedling hypocotyl lengths to nearly wild-type (Figures 2-6a thru d). To further investigate the effectiveness of *35S::AWE1* in restoring the *etr1* hypocotyl lengths to wild-type-like, I over-expressed *AWE1* in a triple receptor loss-of-function mutant lacking *ETR1* (*etr1 etr2 ein4)* and a double receptor loss-of-function mutant, *etr2 ein4,* with wild-type ETR1. Hypocotyl lengths of the transgenic triple receptor mutant (*etr1 etr2 ein4)* lines carrying the *AWE1* over-expression construct were similar to the hypocotyl lengths of the *etr2 ein4* double mutant when grown in the dark on ACC, suggesting that *AWE1* over-expression can compensate for the loss of *etr1* with regards to hypocotyl length (Figure 2-7a). The hypocotyl lengths of *etr2 ein4* double mutant seedlings over-expressing *AWE1* were comparable to the hypocotyl lengths of the untransformed

# FIGURE 2-5

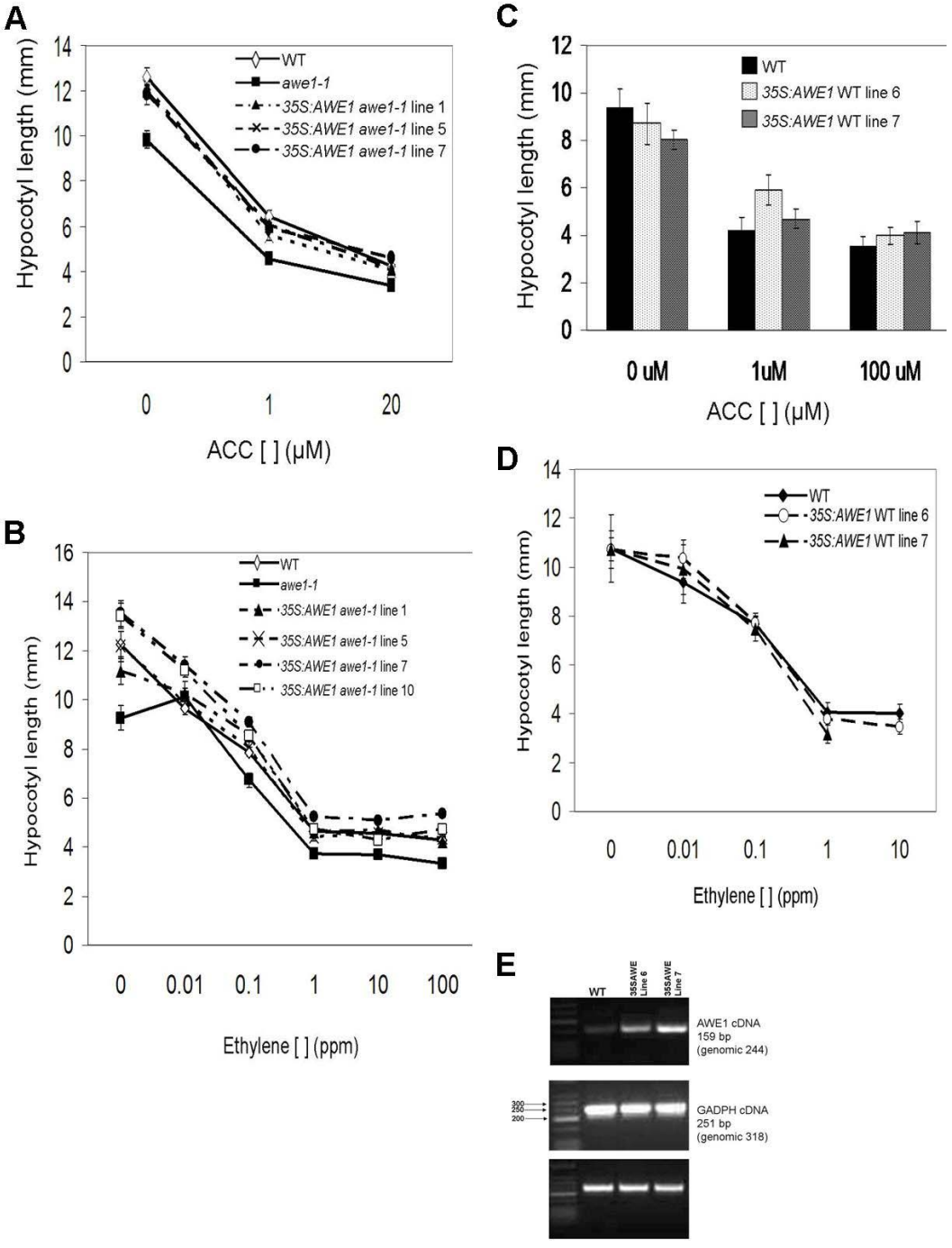
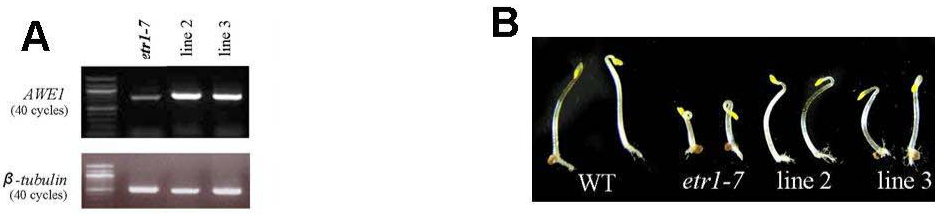
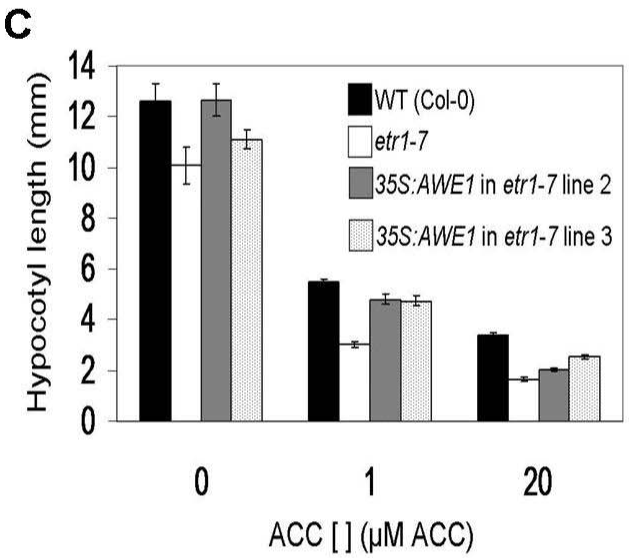

A
Hypocotyl length (mm)
WT
awe1-1
35S:AWE1 awe1-1 line 1
35S:AWE1 awe1-1 line 5
35S:AWE1 awe1-1 line 7
ACC [ ] (μM)
B
Hypocotyl length (mm)
WT
awe1-1
35S:AWE1 awe1-1 line 1
35S:AWE1 awe1-1 line 5
35S:AWE1 awe1-1 line 7
35S:AWE1 awe1-1 line 10
Ethylene [ ] (ppm)
C
Hypocotyl length (mm)
WT
35S:AWE1 WT line 6
35S:AWE1 WT line 7
0 uM
1uM
100 uM
ACC [ ] (μM)
D
Hypocotyl length (mm)
WT
35S:AWE1 WT line 6
35S:AWE1 WT line 7
Ethylene [ ] (ppm)
E
WT
35SAWE Line 6
35SAWE Line 7
AWE1 cDNA
159 bp
(genomic 244)
GADPH cDNA
251 bp
(genomic 318)

**Figure 2-5:** Over-expression of *AWE1* rescues the hypocotyl shortening of the *awe1-1* mutant but does not confer ethylene insensitivity in the WT background. **A&B.** Three independent *awe1-1* transgenic lines carrying the *AWE1 over-expression* construct respond to either ACC (A) or exogenous ethylene (B) similar to wild-type. Error bars represent standard error of the mean for $n \geq 19$ $T_2$ seedlings in panel A and $n=10$ $T_3$ seedlings in panel B. **C&D.** Hypocotyl lengths of two independent wild-type transgenic lines over-expressing *AWE1*, grown in the dark, either on ACC (C, error bars represent standard deviation of $n \geq 13$ $T_2$ seedlings) or in the presence of exogenous ethylene (D, error bars represent standard deviation of $n=8$ $T_2$ seedlings). **E.** RT-PCR showing that *AWE1* is over-expressed in wild-type transgenic lines 6 and line 7. Top panel is RT-PCR using *AWE1*-specific primers while the bottom two panels are products using *GADPH*-specific primers showing that all samples had similar levels of RNA.

# FIGURE 2-6


A
etr1-7
line 2
line 3
AWE1
(40 cycles)
β-tubulin
(40 cycles)
B
WT
etr1-7
line 2
line 3


C
Hypocotyl length (mm)
14
12
10
8
6
4
2
0
WT (Col-0)
etr1-7
35S:AWE1 in etr1-7 line 2
35S:AWE1 in etr1-7 line 3
0
1
20
ACC [ ] (μM ACC)

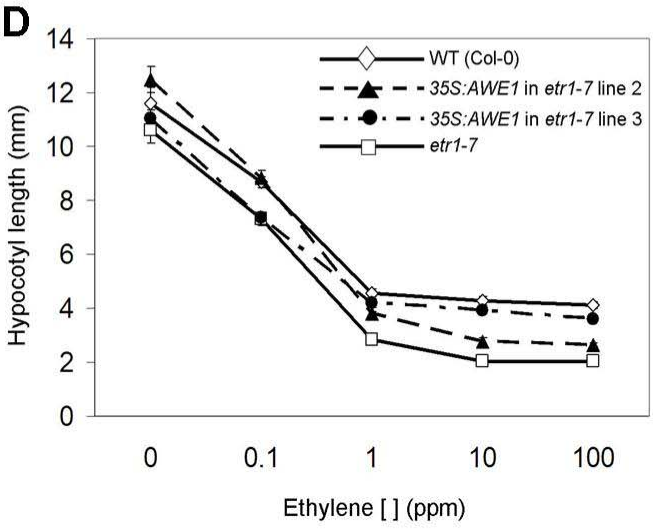

D
Hypocotyl length (mm)
14
12
10
8
6
4
2
0
WT (Col-0)
35S:AWE1 in etr1-7 line 2
35S:AWE1 in etr1-7 line 3
etr1-7
0
0.1
1
10
100
Ethylene [ ] (ppm)

**Figure 2-6**: *AWE1* over-expression can compensate for the loss of ETR1. **A.** RT-PCR of *etr1-7* transgenic lines over-expressing *AWE1*. The top panel is RT-PCR product with *AWE1*-specific primers, and the lower panel is a βeta-tubulin control showing similar RNA starting levels in all samples. **B.** Photograph of dark-grown *etr1-7* seedlings over-expressing *AWE1* when grown on 1μM ACC. Lines 2 and 3 are longer than the *etr1-7* untransformed seedlings. **C&D.** Hypocotyl lengths of two independent *etr1-7* transgenic lines over-expressing *AWE1* when grown in the dark, either on ACC (C, error bars represent standard error of the mean for $n \geq 19$ seedlings) or in the presence of exogenous ethylene (D, error bars represent standard error of the mean for $n \geq 24$ seedlings).

# FIGURE 2-7

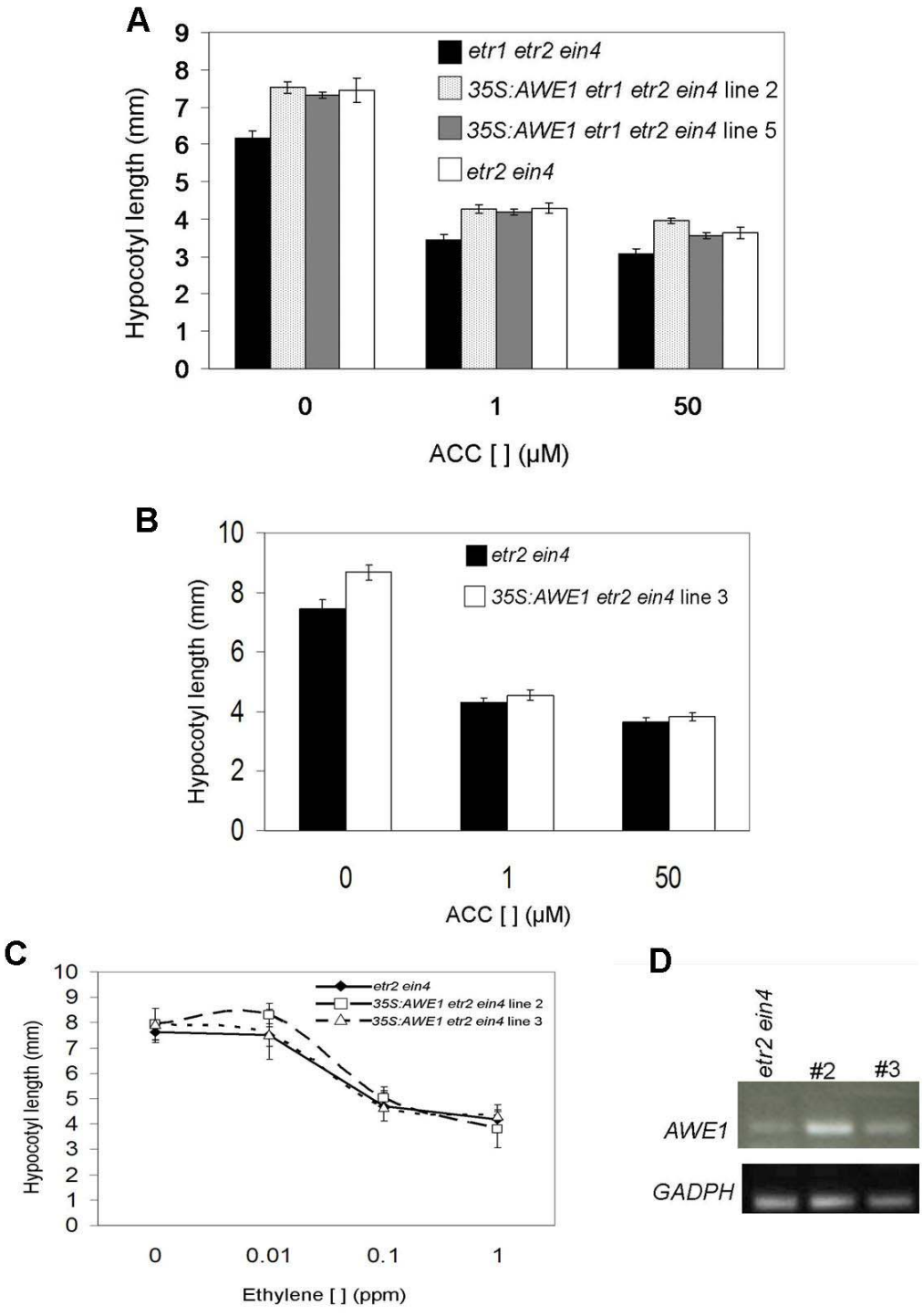

A
Hypocotyl length (mm)
etr1 etr2 ein4
35S:AWE1 etr1 etr2 ein4 line 2
35S:AWE1 etr1 etr2 ein4 line 5
etr2 ein4
ACC [ ] (μM)
B
Hypocotyl length (mm)
etr2 ein4
35S:AWE1 etr2 ein4 line 3
ACC [ ] (μM)
C
Hypocotyl length (mm)
etr2 ein4
35S:AWE1 etr2 ein4 line 2
35S:AWE1 etr2 ein4 line 3
Ethylene [ ] (ppm)
D
etr2 ein4
#2
#3
AWE1
GADPH

**Figure 2-7:** *AWE1* over-expression can restore the hypocotyl lengths of the *etr1 etr2 ein4* triple mutant to *etr2 ein4*-like but has no affect on the hypocotyls of the *etr2 ein4* double mutant. **A.** Graph showing hypocotyl lengths of three independent *etr1 etr2 ein4* transgenic $T_3$ lines over-expressing *AWE1*. The three lines behave similarly to the *etr2 ein4* double mutants over increasing concentrations of ACC. (Error bars represent standard error of the mean for $n \geq 24$ $T_3$ seedlings.) **B-C.** Graphs showing hypocotyl lengths of transgenic *etr2 ein4* lines over-expressing *AWE1* when plated on (B, error bars represent standard error of the mean for $n \geq 24$ $T_3$ seedlings) or ethylene (C, error bars represent standard deviation of n=8 $T_3$ seedlings.) **D.** RT-PCR verifying that *AWE1* is over-expressed in the transgenic lines. Top panel is RT-PCR using *AWE1* primers (35 cycles) and bottom panel is RT-PCR using *GADPH* primers (30 cycles) as an RNA control.

control *etr2 ein4* double mutant seedlings when grown on medium containing ACC (Figure 2-7b). Preliminary results suggest similar results when these seedlings are grown in exogenous ethylene (Figure 2-7c). I used RT-PCR to verify the over-expression of *AWE1* in both *etr2 ein4* lines (Figure 2-7d).

### *awe1* Double Mutant Analysis

I next analyzed *awe1* double mutants to determine where AWE1 may function in the ethylene signaling pathway. I crossed the *awe1-1* mutant to the ethylene insensitive *etr1-1* mutant. *awe1-1 etr1-1* double mutant dark-grown seedlings were insensitive to exogenous ethylene, behaving similarly to the *etr1-1* single mutant (Figure 2-8a). Interestingly, the *awe1-1 etr1-1* double mutant rosettes were small and chlorotic in color, similar to the *awe1-1* single mutant (Figure 2-8b). At the same time, I tested for *awe1-1*'s ability to suppress the ethylene-insensitivity conferred by the *ein2-1* mutation. Double *awe1-1 ein2-1* mutant seedlings were ethylene-insensitive, similar to the *ein2-1* single mutant (Figure 2-8c). The *awe1-1 ein2-1* double mutant rosettes looked similar to the *awe1-1* single mutant (Figure 2-8d). Because *awe1-1* could not suppress the strong insensitivity of *etr1-1* in the hypocotyl seedling analysis, it suggests that the *awe1-1* effects on hypocotyl elongation are ethylene-independent, or that the loss of *awe1* cannot compensate for the strong ethylene-insensitive mutants *etr1-1* and *ein2-1*, or potentially AWE1 could act upstream of ETR1. The rosette phenotypes of both the *awe1-1 etr1-1* and *awe1-1 ein2-1* double mutants support an ethylene-independent function of AWE1 in regulating cell expansion of rosette leaves. Analysis of *awe1-1 etr1-7* and *awe1-1 ctr1-3* double mutants will be useful in determining whether *AWE1*'s function is in

# FIGURE 2-8

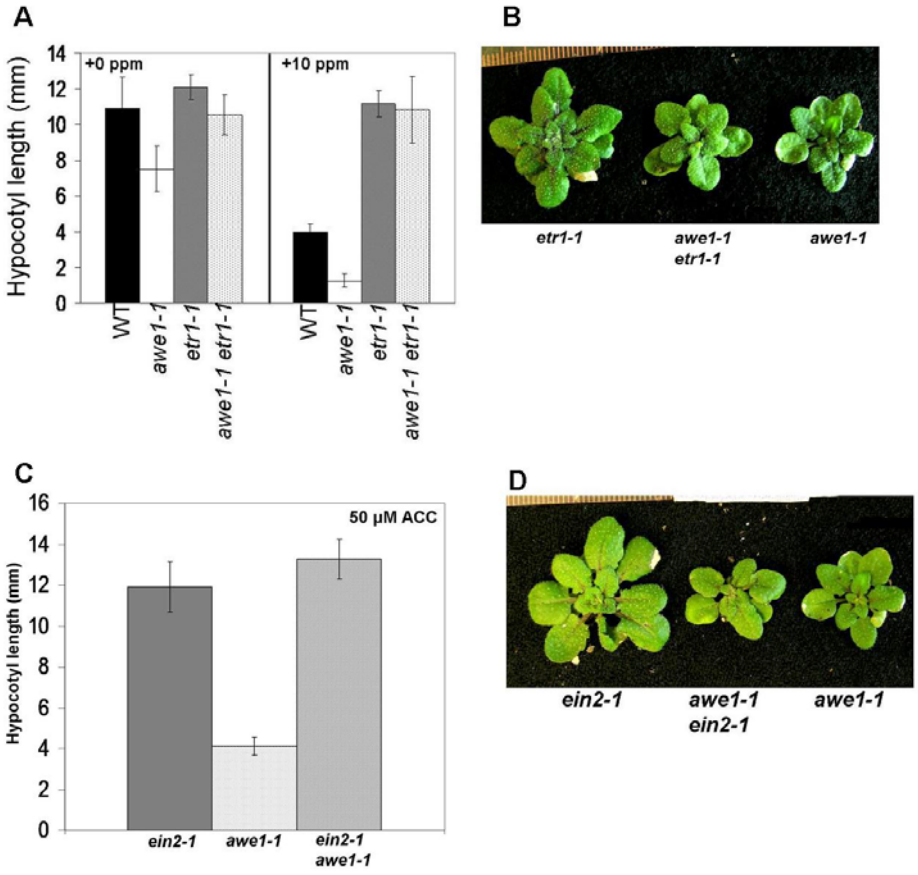



**Figure 2-8:** The hypocotyl shortening of *awe1-1* dark-grown seedlings is blocked by the ethylene-insensitive *etr1-1* and *ein2-1* mutants. **A&B.** The *awe1-1 etr1-1* double mutant dark-grown seedlings had long hypocotyls when grown on ACC, similar to the *etr1-1* single mutant. The *awe1-1 etr1-1* seedlings were slightly shorter than *etr1-1* mutants alone when grown in the absence of ACC. The *awe1-1 etr1-1* rosettes looked more similar to *awe1-1* (B). **C&D.** The *awe1-1 ein2-1* double mutant dark-grown seedlings behaved similarly to the ethylene-insensitive *ein2-1* mutant seedlings when grown on ACC. Error bars in A and B represent standard deviation of n=10 seedlings.

ethylene signaling. If the double mutants have additive effects, relative to the single mutants, this will imply that *AWE1* functions in an ETR1- and CTR1-independent pathway.

**Sub-cellular Localization of the AWE1 Protein**

Based on localization prediction programs AWE1 may localize to the cytosol, chloroplast, or vacuole (or all three). There seems to be a correlation between AWE1 and ETR1 both physically, based on yeast-two-hybrid and BiFC analysis, and genetically, based on over-expression analysis; therefore, I hypothesized that AWE1 would localize peripherally to the ER where the ETR1 receptor and the CTR1 protein are localized [21, 36]. To investigate the sub-cellular localization of AWE1 in detail, I used a genomic AWE1 fragment consisting of the genomic portion of AWE1 (from 600 bases upstream of the 'ATG' start codon to the last codon prior to the *AWE1* 'TGA' stop codon, fused to a carboxy-terminal RFP tag, followed by ~250 bases of the *AWE1* 3'UTR (Figure 2-9a). I tested for and observed rescue of the *awe1-1* phenotypes with the $Pr_{AWE1}$::AWE1-RFP construct, suggesting that the construct is functional (Figures 2-9b and c). In guard cells, I observed AWE1-RFP localization (false-green) in the chloroplast as well as in what appears to be the cytoplasm (Figures 2-9d and e). In pavement cells, I also observed RFP-fluorescence (again false-green) in the chloroplast (red auto-fluorescence) (Figures 2-9f thru i) as well as a weak signal which may be cytosolic (Figures 2-9f and g). Because AWE1 protein was found to sub-cellularly localize to the chloroplast and the *awe1-1* mutant rosettes were small and chlorotic, I next examined the chloroplasts of *awe1-1* mutant leaves. There were no observable differences in the number of

# FIGURE 2-9

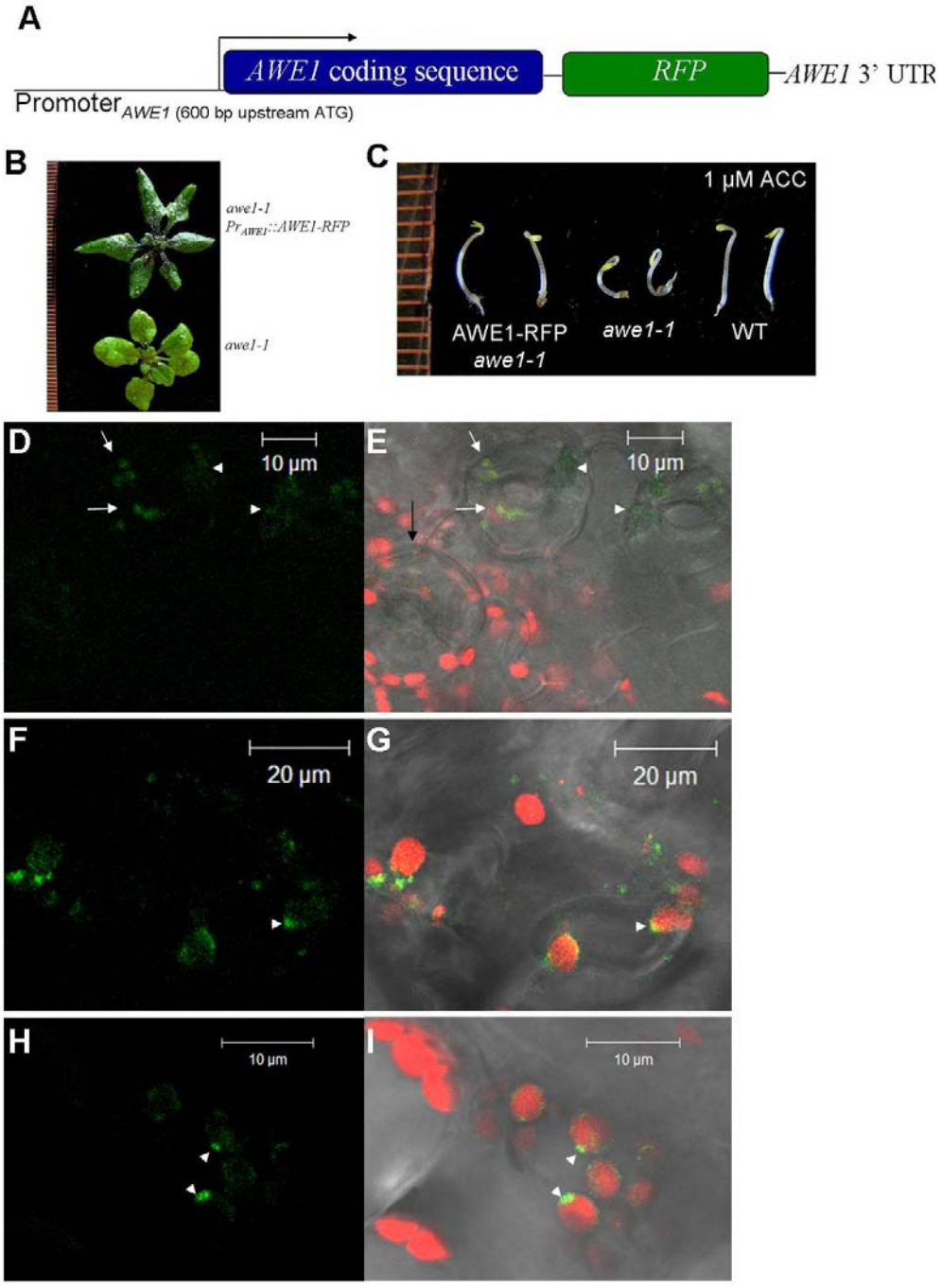

A
AWE1 coding sequence
RFP
AWE1 3' UTR
Promoter AWE1 (600 bp upstream ATG)
B
awe1-1
Pr AWE1::AWE1-RFP
awe1-1
C
1 µM ACC
AWE1-RFP
awe1-1
awe1-1
WT
D
10 µm
E
10 µm
F
20 µm
G
20 µm
H
10 µm
I
10 µm

**Figure 2-9:** Localization of AWE1. **A.** Expression of the *AWE1* coding sequence was driven by a native *AWE1* promoter (600 bases upstream of the *AWE1* predicted 'atg' start site) and fused to a carboxy-terminal RFP tag followed by *AWE1* 3'UTR. **B&C.** The AWE1-RFP transgene rescued the *awe1-1* mutant rosette and seedling phenotypes. **D-H.** Confocal images of the abaxial surface of AWE1-RFP transformed *awe1-1 Arabidopsi*s rosette leaves. **D.** RFP (false green) channel and **E.** overlay of RFP channel, chlorophyll auto-fluorescence (red) and bright field channels of a leaf surface focusing on three guard cells. Green signal was detected in the chloroplast (white arrows) and additionally, more weakly detected in what may be the cytoplasm (white arrowheads) of two guard cells, while no RFP-fluorescence (green) is detected in the third guard cell (black arrow). **F&G.** Green signal was detected surrounding and in the chloroplast (white arrowheads) of pavement cells as well as weak signal detected in what appears to be the cytoplasm. punctate dots were visible in some pavement cells suggesting that AWE1-RFP may also localize to the golgi, although co-localization has yet to be shown. **H&I.** In some pavement cells observed, the green signal does not appear in the entire chloroplast but appears to surround the chlorophyll (white arrows), a pattern similar to the membrane envelope of the chloroplasts [86].

chloroplasts in the *awe1-1* mutant compared to wild type when I looked at the newest/youngest leaf of each plant (two plants per genotype); however, the chloroplasts in the *awe1-1* mutant appeared to be slightly larger than wild-type or *awe1-1* transgenic lines over-expressing *AWE1* (Figure 2-10). I repeated this study using the oldest leaf from the same group of plants, observing one leaf per plant and three plants per genotype, but did not detect the same difference in chloroplasts size between the *awe1-1* mutant and other genotypes (Appendix b). Any AWE1 function in chloroplasts development might correlate to the age, maturity of the leaf.

*Discussion*

### *AWE1* is a Novel Gene Found in Cyanobacteria and Plants

*AWE1*, a single-copy gene in *Arabidopsis* with homologs in other plants as well as cyanobacteria, was identified in a yeast-two-hybrid screen for ETR1-interacting proteins. Primary sequence analysis of the encoded AWE1 protein yields no conserved motifs. The secondary structure prediction program, PHYRE, predicts a lipocalin-like structure in the carboxy-terminal half of AWE1 based on the similarity of the predicted secondary structure of AWE1 to the crystal structure of a putative lipocalin from *Nostoc punctiforme PCC 71302*. I identified sequences from the monocot *Sorghum bicolor*, the dicot *Populus trichocarpa*, the mosses *Physcomitrella patens* and *Selagineall moellendorffii,* and the cyanobacteria *Anabaena variabilis, Gloebacter violaceus,* and *Synechocystis sp. PCC 6803* that are similar to the AWE1 protein sequence (from AWE1 residues ~50 to 396), and the secondary structure of the *Oryza sativa, Physcomitrella patens*, *Selaginella*

# FIGURE 2-10

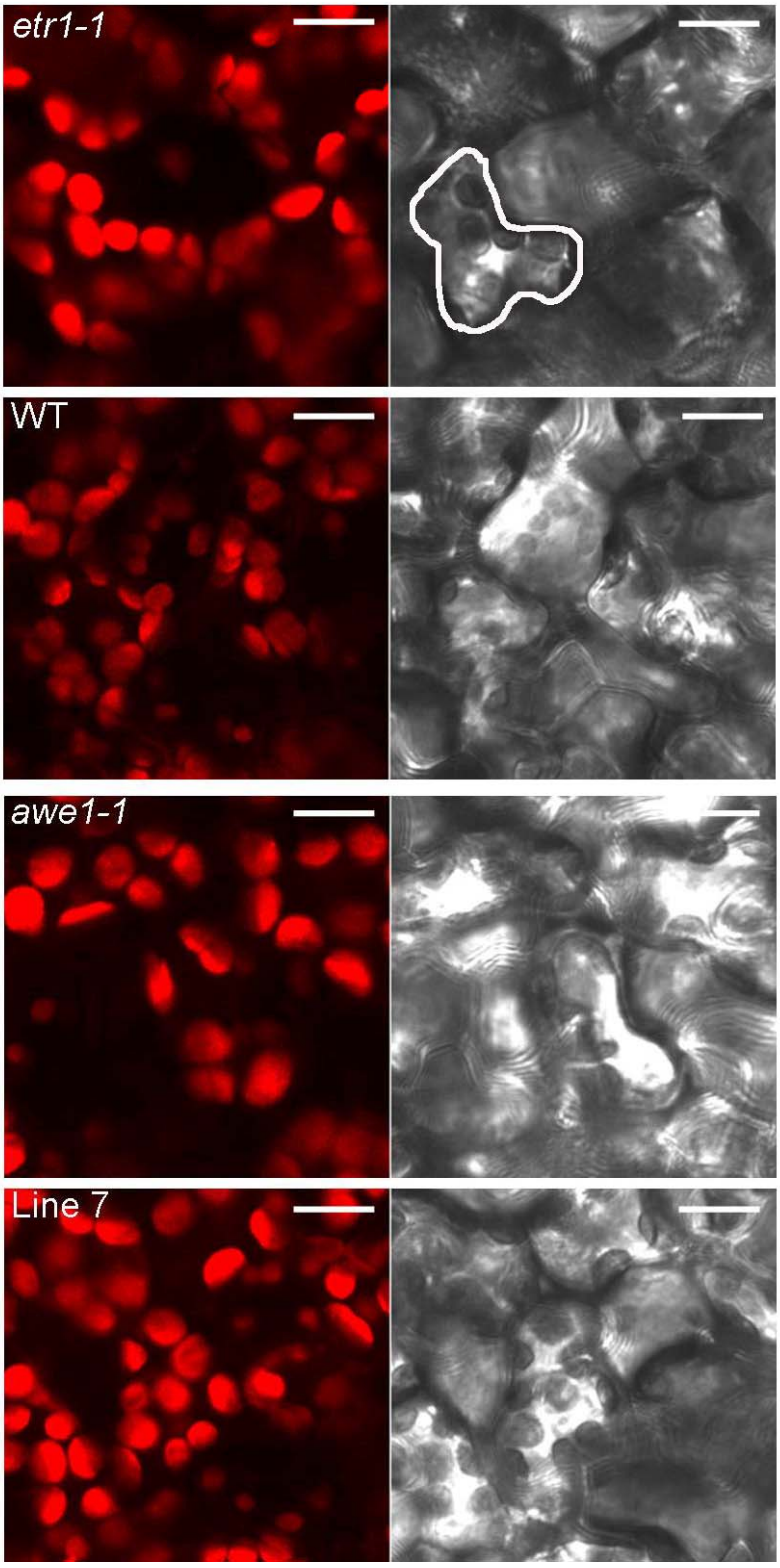


**Figure 2-10:** The *awe1-1* mutant leaves have larger chloroplasts than wild-type. Mesophyll cells of the same leaves used for Figure 3-5c were analyzed with a 488nm laser to stimulate auto-fluorescence of the chlorophyll. *awe1-1* chloroplasts were larger than wild-type chloroplasts, and this phenotype was rescued by the *AWE1* over-expression construct as seen in *OxAWE1* line 7. The left images are chlorophyll auto-fluorescence, while the right images show bright field images of the surface. The scale bar = 10μm. The white line in the *etr1-1* bright field image is the outline of one mesophyll cell.

*moellendorffii*, *Anabaena variabilis* and *Gloeobacter violaceus* primary sequences all predict a putative carboxy-terminal lipocalin-like motif [78]. (The *Sorghum bicolor*, *Populus trichocarpa*, and *Synechocystis* sequences were not queried.) Lipocalins are small proteins that have low sequence similarity (~21%) but a highly conserved secondary structure consisting of eight anti-parallel β-sheets that fold onto each other, forming a barrel that stores small molecules with low solubility or molecules that are chemically sensitive such as retinoid, vitamins, steroids, fatty acids, or odorants for transport [79]. Lipocalins are found in many organisms from prokaryotes to mammals [79]. At present AWE1 homologs and homologs of the ethylene-binding domain (EBD) of ETR1 have been identified in cyanobacteria and plants, but not in green algae, fungi or animals [24]. This parallel between ETR1 and AWE1 is unlike other known components of the ethylene signal transduction pathway known to physically or genetically interact with ETR1, such as CTR1 [34] and RTE1 [31], which both have homologs in eukaryotes, including mammals, but not in prokaryotes [31, 32]. These data are consistent with the possibility that AWE1 and ETR1 physical association may have co-evolved as a highly conserved interaction.

**Confirming the AWE1 Protein-Protein Interactions *In Planta***

To confirm the initial ETR1 and AWE1 protein-protein interactions observed in yeast, as well as the CTR1 and AWE1 interactions, I utilized BiFC tools in *Nicotiana benthamiana* to detect interactions between the full-length ETR1, CTR1, and AWE1 proteins. (The yeast-two-hybrid assay used to test for the initial protein-protein interactions was limited in that only the soluble portion of the ETR1 receptor and a small portion of the CTR1 amino-terminal region (residues 308-569) could be

used.) I observed fluorescent YFP signals above background levels, albeit with weak intensity, implying that the capacity exists for AWE1-ETR1 and AWE1-CTR1 interactions to occur when expressed by their native promoters in *Arabidopsis* cells. The weak intensity of the signal could correlate with the low frequency of the infiltration or with unstable protein expression in this transient assay. Each of the three vectors used for BiFC analysis (the nYFP-containing vector, the cYFP-containing vector, and the p19 RNA silencing suppressor) have to enter the same cell in order for the YFP halves to come together and be expressed. Because each of the three vectors has kanamycin selection markers, all three vectors were transformed separately into *Agrobacterium* cells. Consequently all three independent *Agrobacterium* strains must infect the same tobacco cells for fluorescence to occur. The BiFC fluorescence patterns appeared similar to the fluorescence localization pattern of ETR1-GFP when over-expressed in tobacco pavement cells. The intensity of the YFP signal when testing for ETR1 and AWE1 interaction was stronger in the mesophyll cells. Additionally the YFP signal in the mesophyll cells appeared in a pattern that was more diffuse than the reticulate pattern of YFP signal resulting from the interaction between two endomembrane-localized proteins, ETR1 and RTE1. This would be expected if the YFP signal is coming from an endomembrane-localized protein, like ETR1, and a protein localized to the cytosol, potentially AWE1 [26]. This analysis complements the previous yeast-two-hybrid findings; however, the BiFC system also has limitations. Arabidopsis proteins are being over-expressed in a non-native system (tobacco), and there is likely a natural affinity of the nYFP and cYFP halves for each other. Showing protein-protein interaction in Arabidopsis

without over-expressing the proteins would be ideal. We do not have an antibody to the AWE1 protein, which would be useful for detecting AWE1 in the ETR1-complex. Ongoing research in our lab (in collaboration with labs at USDA) aims to identify the proteins in the ETR1-complex and proteins that associate with CTR1 through immunoprecipitation of each protein with its interacting partners, followed by mass spectrometry analysis. If AWE1 associates with both ETR1 and CTR1 in Arabidopsis, then AWE1 may be identified in this mass spectrometry approach. In the future it would also be useful to test for AWE1's physical interaction with other histidine kinases to verify the specificity of AWE1 for ETR1. These results do not rule out that AWE1 normally associates with a histidine kinase and is interacting with ETR1, a histidine kinase, when its true interaction partner is not present.

**AWE1 Functions in Hypocotyl Elongation and Cell Expansion**

Based on the sequence analysis and the protein-protein interactions with ETR1 and CTR1, I hypothesized that *awe1* loss-of-function mutants would be hypersensitive to ethylene, similar to *etr1* null and *ctr1* loss-of-function mutants. *awe1-1* dark grown-seedlings had shorter hypocotyls than wild-type seedlings when grown on medium containing ACC or in the presence of exogenous ethylene. This is similar to, although not as severe as, the *etr1-7* loss-of-function mutant, which is hypersensitive to ethylene. The hypocotyl shortening of both *etr1-7* and *awe1-1* was slightly rescued when seedlings were grown on medium containing the ethylene biosynthesis inhibitor AVG or on medium containing $AgNO_3$ which blocks ethylene binding by the receptors. The alleviation suggests that the hypocotyl shortening for both mutants is in part due to ethylene-hypersensitivity. *AWE1* expression in dark

grown Arabidopsis seedlings is reduced by >1.5 fold when grown in the presence of exogenous ethylene [45] supporting a role of AWE1 in ethylene-induced hypocotyl growth. However, because neither the *etr1-7* nor the *awe1-1* recovered to complete wild-type hypocotyl length when ethylene biosynthesis was blocked, the results suggest that the shortened hypocotyl phenotype of both mutants may also be due to impairments of an ethylene-independent pathway or that ethylene biosynthesis is not completely blocked. To test whether the *awe1* hypocotyl response observed is within the ETR1 signaling pathway, it would be useful to analyze the *awe1-1 etr1-7* double loss-of-function mutant to see if there is an additive effect or if the effect is similar to the more severe, *etr1-7*, single mutant.

In addition to shorter hypocotyls, *awe1-1* rosettes were smaller and more compact than wild-type plants, which is an effect observed in constitutive ethylene response mutants most likely due to ethylene's inhibition of cell expansion [32, 87]. Analysis of the size of pavement cells suggests that the *awe1-1* smaller stature is due to cell expansion defects, similar to the *ctr1* constitutive response mutant.

Unlike the *ctr1-8* constitutive response mutant, *awe1-1* rosette leaves were chlorotic (lighter in color) than wild-type plants. In Mandarin oranges, ethylene has been documented as decreasing the number of chloroplasts by promoting disintegration of the chloroplast's inner membranes [88], yet *ctr1* mutants do not have chlorotic phenotypes. AWE1 might function in repression of ethylene-induced chloroplast degradation in a manner that is independent of known ethylene signaling components. Recent findings such as the sub-cellular localization of the ethylene response factor-like protein, ERF15, in the chloroplast [89] and preliminary results

from a collaboration between our lab and Dr. Zhiyong Wang's lab (Carnegie Institute for Science, Stanford, California) that the chloroplast-localized protein FIFTY-FOUR-CHLOROPLASTS (FFC) [90] might be rapidly altered by ethylene (25-minute ethylene treatment) suggests that there might be ethylene-related protein modifications (through signaling) in the chloroplast during ethylene responses.

**AWE1 Protein Sub-cellular Localization in the Chloroplast**

Correlating with the chlorosis of the *awe1-1* mutant, I found that the AWE1 protein displayed sub-cellular localization mostly in the chloroplast with a small fraction of the protein possibly localizing to the ER. ETR1 is known to localize to the ER and Golgi membranes (with its histidine kinase and receiver domains in the cytosol) but has not been found to localize to the chloroplast [21, 36]. While most of the RFP fluorescence I observed was in the chloroplasts, a small portion did appear outside of the chloroplasts, potentially ER. Interestingly multiple chloroplast-specific proteomic studies, utilizing tandem mass spectrometry, have not identified AWE1 among proteins localized within the chloroplast envelope [91] or the entire chloroplast [89]. This may suggest that AWE1 is not always localized in the chloroplast or that the AWE1 protein is found in multiple organelles within the cell. There are previously recorded examples of dual-targeting of proteins. FstZ, which functions in chloroplast division has two functional splice forms, including one form that lacks the chloroplast-target peptide sequence. Consequently FstZ is sub-cellularly localized to either the chloroplast or the cytoplasm depending on whether the chloroplast-transit peptide sequence is spliced out or not [92]. Because I did observe a small fraction of AWE1 protein outside of the chloroplast, dual-targeting of

the AWE1 protein cannot be ruled out. Collectively the following information about AWE1 - the homologous sequences identified in other organisms lack the chloroplast transit peptide region, a partial *AWE1* clone lacking the chloroplast transit peptide sequence was initially isolated, and the annotated *AWE1* 'ATG' start codon proved difficult to clone - might indicate that there is a form of the AWE1 protein that is not localized within the chloroplast. AWE1 has two methionine residues downstream of the chloroplast-transit peptide sequence, at positions 43 and 57, which could serve as potential alternative start codons for an AWE1 protein. Dual-targeting would help explain the physical interaction observed between AWE1 and the ER/Golgi-bound ETR1 receptor if this interaction is indeed occurring in *Arabidopsis*.

There are other possibilities as to the dual localization of the AWE1 protein. AWE1 may not be an abundantly expressed protein in general or the AWE1-RFP protein could be mis-localized due to the RFP tag. However, the AWE1-RFP construct rescued the hypocotyl shortening and the rosette phenotypes of the *awe1-1* mutant, implying that the AWE1-RFP fusion is likely functioning correctly. We used the genomic *AWE1* sequence consisting of ~600 bases upstream of the *AWE1* 'ATG' start codon and including all exons and introns to the stop codon. In the future it would be interesting to use the *AWE1* cDNA sequence with the chloroplast-transit peptide sequence fused to RFP and also the *AWE1* cDNA starting with one of the two "ATG" codons that are downstream of the chloroplast-transit peptide to determine if the latter fusion (lacking the chloroplast-transit peptide sequence) can rescue the *awe1-1* mutant and if so, detect where the protein is expressed sub-cellularly in *Arabidopsis* cells.

Because most of the AWE1 protein localized to the chloroplast, and the *awe1* mutant had a chlorotic phenotype, I examined the chloroplasts of the *awe1-1* mutant relative to wild type. While I could not detect abnormalities in the chloroplast shape, I did see that the chloroplasts in the newest leaves of *awe1-1* mutants were larger than chloroplasts in the newest leaves of wild-type leaves, or *awe1-1* transgenic plants over-expressing *AWE1*. However I did not observe a difference in chloroplast size when looking at the oldest, most mature leaves of the plants (Appendix b). A decrease in chloroplasts would lead to a decrease in chlorophyll concentration and likely the onset of chlorosis. The *awe1-1* mutant leaves are chlorotic, which is unlike *ctr1* and *etr1* loss-of-function mutants. This difference combined with the inability of *etr1-1* or *ein2-1* to block the chlorotic phenotype of the *awe1-1* mutant suggests that the chlorotic phenotype is an ethylene-independent phenotype.

### *AWE1* Over-expression Effects are Observed in *etr1* Mutants but not in Wild Type

The sub-cellular localization of AWE1 protein, mostly to the chloroplast, led me to question the biological significance of the ETR1 and AWE1 protein-protein interactions observed in the BiFC analysis. However, when I analyzed transgenic lines over-expressing *AWE1*, I observed a correlation between phenotype of transgenic lines and lack of the ETR1 receptor, which could be a consequence of direct interactions between AWE1 and ETR1. Transgenic wild-type plants over-expressing *AWE1* looked identical to wild-type untransformed controls. However, over-expression of *AWE1* in the *etr1-7* mutant restored the *etr1-7* mutant seedling hypocotyl lengths to near wild-type lengths. Additionally over-expression of *AWE1*

in the *etr1 etr2 ein4* triple mutant restored the hypocotyl lengths of the triple mutant to the hypocotyl lengths of the *etr2 ein4* double mutant, while over-expression in the *etr2 ein4* double mutant appeared to have no affect. These results indicate that *AWE1* over-expression can compensate for the lack of ETR1 in hypocotyl growth. This is very interesting considering that growing *etr1-7* seedlings on an ethylene-biosynthesis inhibitor does not restore *etr1-7* hypocotyl lengths to wild-type like, but the over-expression of *AWE1* does. It may suggest that AWE1 acts with ETR1 in an ethylene-independent pathway. AWE1 was previously found to weakly interact with the ERS1 receptor but not with the ETR2 receptor in the initial yeast-two-hybrid analysis, providing preliminary support for the specificity of the AWE1 and ETR1 interaction (Shockey, 2004 dissertation). Another possibility is that the ETR1 receptor is required to inhibit AWE1 function in hypocotyls, and in the *etr1* null backgrounds, AWE1 does not stop functioning as it would in wild-type conditions.

**Double Mutant Analysis Suggests AWE1 Function in Rosettes is Ethylene-Independent**

*awe1-1 etr1-1* and *awe1-1 ein2-1* double mutant seedlings were insensitive when grown in the dark in the presence of ethylene. This result suggests that *AWE1* acts upstream of *ETR1* in hypocotyl elongation, or that the *awe1-1* allele cannot suppress the strong ethylene-insensitivity conferred by the *etr1-1* mutation in hypocotyl elongation, or that *AWE1's* action is ethylene-independent and can be masked by ethylene-insensitive mutants. Interestingly the *awe1-1 etr1-1* and *awe1-1 ein2-1* double mutant adult plants looked similar to the *awe1-1* single mutant. Collectively these results suggest that *AWE1's* function in hypocotyl elongation may be different than its function in rosettes. AWE1's function in the hypocotyl may be ethylene-dependent, because the *awe1-1* mutation did not have an

antagonistic effect on *etr1-1* or *ein2-1* hypocotyl length. Sub-cellular localization of the AWE1 protein in hypocotyls might provide additional insight into a potential ethylene-dependent role of AWE1 in ethylene signaling in hypocotyls.

**Potential Functions of AWE1**

A clearer understanding of AWE1's role in hypocotyl elongation and cell expansion might be obtained by having an *awe1* allele that has complete loss of *AWE1* expression (a knock-out allele). Because I have yet to identify a second *awe1* allele that yields the above mentioned phenotypes (or potentially even more severe phenotypes) I am currently analyzing artificial microRNA lines, in which I designed the artificial microRNA with specificity to the *AWE1* transcript. The artificial microRNA should, in theory, reduce *AWE1* expression by targeting the *AWE1* transcript for degradation [90]. If RT-PCR analysis reveals that these artificial microRNA lines have reduced *AWE1* expression, I will then analyze these lines for defects in hypocotyl elongation and cell expansion.

Additionally, the possibility remains that in the plant, under normal growth conditions, AWE1 may physically interact with a histidine kinase other than ETR1. In the future, testing for physical interaction between AWE1 and other histidine kinases, specifically any histidine kinases that are localized to the chloroplast, will be useful to provide support or negate the hypothesis that AWE1 is non-specifically interacting with ETR1. It still remains unclear whether the *awe1-1* loss-of-function and *AWE1* over-expression phenotypes recorded here are due specifically to impairments in ethylene signaling or not. Testing for additive effects of *awe1-1 etr1-7* and *awe1-1 ctr1-3* double mutants should help to clarify this point.

Possibly constructing an *AWE1* clone that lacks the sequence encoding for the chloroplast-transit peptide and testing for rescue of the *awe1-1* seedling and rosette phenotypes will inform us of whether AWE1 protein is dually targeted and whether the targeting is dependent on the age of the plant. If the above-mentioned, truncated AWE1 clone can rescue an *awe1-1* mutant, then it would be interesting to determine if the same truncated AWE1 can interact with the receptors, where it is localized sub-cellularly, and if it can, when over-expressed, compensate for the hypocotyl shortening of the *etr1-7* mutant.

If the chloroplast target peptide sequence is needed for proper AWE1 function, and the AWE1-ETR1 interaction seems to be specific and real, then what would be the function of the AWE1 and ETR1 interaction? It is speculated that some chloroplast-targeted proteins require phosphorylation of the chloroplast transit peptide sequence [94]. Perhaps the ETR1 histidine kinase activity could have a role in phosphorylating AWE1. Adult *etr1-7* transgenic plants carrying an ETR1 transgene, mutated on the histidine residue, have chlorotic leaves and smaller rosettes than wild-type plants [95]. Cho and Yoo (2007) conclude that the mutated ETR1 histidine kinase domain had a dominant negative effect on the other receptors, but it could potentially be a dominant negative affect on AWE1. Additionally the AWE1-ETR1 physical interaction is lost when the putative ETR1-histidine kinase phosphate acceptor residue is mutated from a D to an E, thought to mimic constitutive phosphorylation of the site (Shockey, 2004 dissertation). These findings could imply

that there is a role of the ETR1 histidine kinase activity on AWE1 function, possibly for proper sub-cellular targeting of the AWE1 protein.

In the future, confirming the interactions between ETR1 and AWE1 in Arabidopsis with the native promoter driving expression of each, and the sub-cellular localization of over-expressed AWE1 in the *etr1-7* background would be useful to provide support for or negate the idea of a specific AWE1 and ETR1 interaction.

If the FFC protein modification identified by Wang et al. through 2-D DIGE analysis is determined to be real (based on replicate studies) (preliminary, unpublished information), then testing for AWE1 interaction with FFC, and potential localization patterns of FFC and AWE1 in the presence or absence of ethylene may begin to build a backbone for an ethylene-regulated chloroplast signaling pathway.

Based on the findings reported here, AWE1 seems to have at least a genetic interaction with ETR1 in hypocotyl growth, which may be through physical protein-protein interaction. Because the *awe1-1* mutation does not antaognize the ethylene-insensitivity observed in *etr1-1* or *ein2-1* hypocotyls, *awe1-1*'s function in hypocotyl elongation may be ethylene-dependent (see model figure 2-11.) The phenotype of *etr1* lines over-expressing *AWE1* suggests that ETR1 is required for regulating AWE1 function in the presence of ethylene (see model figure 2-12.) Currently there is no evidence to suggest that AWE1's function in rosettes is ethylene-related. Because *etr1-1* and *ein2-1* normally display delayed senescence in the presence of ethylene, testing for senescence of the *awe1-1 etr1-1* and *awe1-1 ein2-1* may provide a link between AWE1 and ethylene-related senescence or provide additional support for a model in which AWE1's function in adult plants is not directly ethylene related.

# FIGURE 2-11

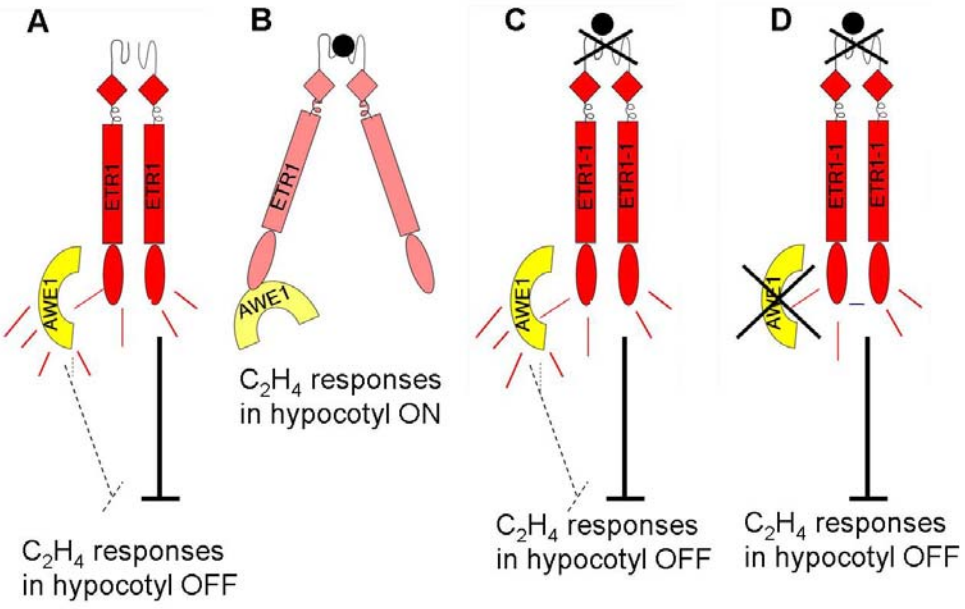



**Figure 2-11**: Model of AWE1 function in hypocotyl elongation of dark grown *Arabidopsis* seedlings. **A.** Genetic data suggest that AWE1 is a negative regulator of ethylene responses in the hypocotyl and may act in conjunction with ETR1 in repressing ethylene responses. **B.** AWE1 function in the hypocotyl may require ETR1 for proper regulation in the presence of ethylene. **C.** If AWE1 requires ETR1 for turning off function, then in the *etr1-1* background, AWE1 would always be functioning. **D.** *awe1-1 etr1-1* double mutant analysis suggest that AWE1 cannot compensate for the ethylene-insensitivity of *etr1-1* in hypocotyls, perhaps because *etr1-1* is likely keeping all other receptors, along with its own signaling "on" in order to repress ethylene responses.

# FIGURE 2-12

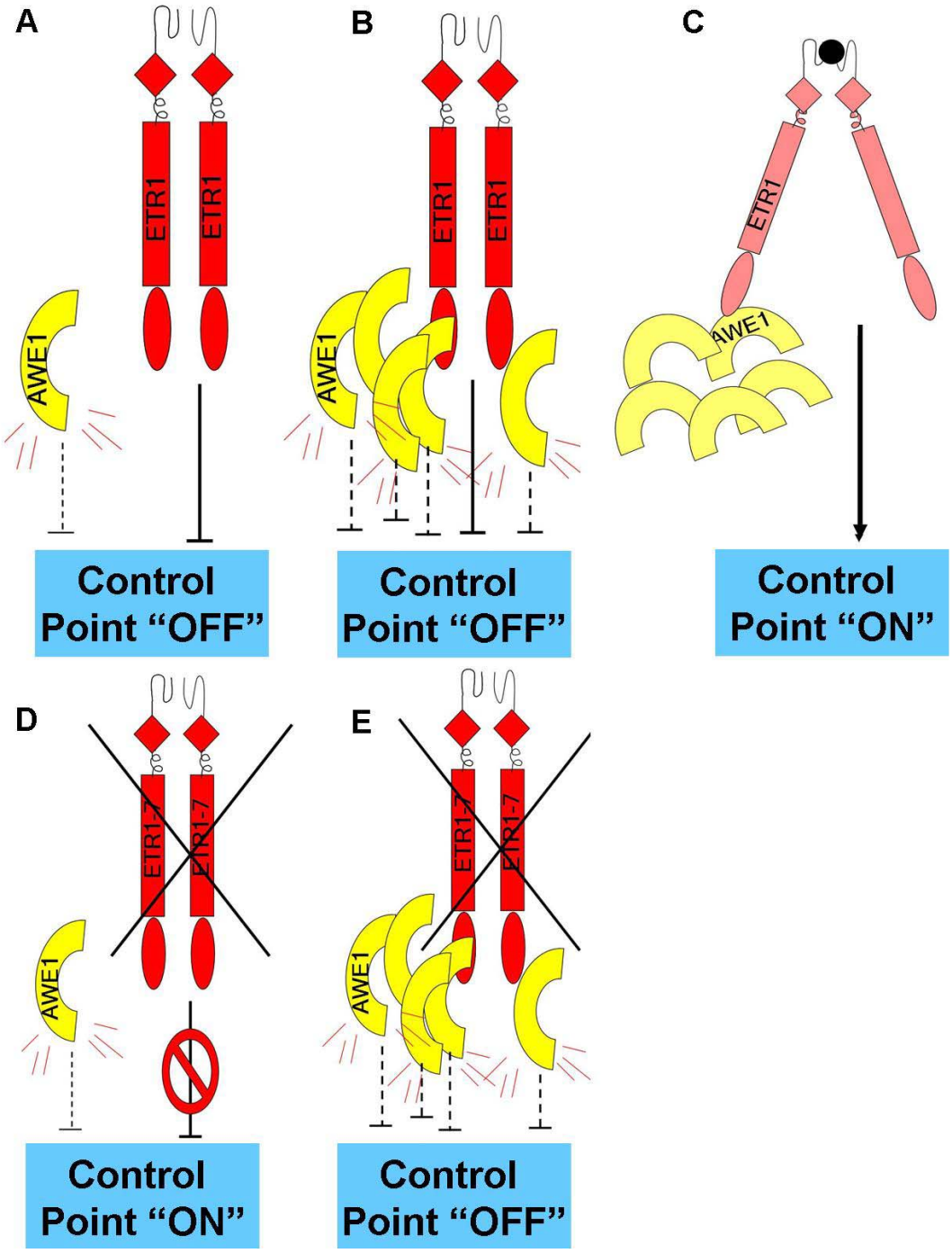

A
ETR1
ETR1
AWE1
Control
Point "OFF"
B
ETR1
ETR1
AWE1
Control
Point "OFF"
C
ETR1
AWE1
Control
Point "ON"
D
ETR1-7
ETR1-7
AWE1
Control
Point "ON"
E
ETR1-7
ETR1-7
AWE1
Control
Point "OFF"

**Figure 2-12**: Model of AWE1's function based on the ability of *over-expressed* AWE1 to compensate for a lack of ETR1 in hypocotyl lengths. **A&B**. Under normal conditions, or when AWE1 is over-expressed in the wild-type background, AWE1 is functioning to repress a subset of ethylene responses. **C.** Once ethylene binds, the ETR1 receptor stops signaling and turns off the AWE1 signal (even in the over-expressed lines), possibly through protein-protein interaction. Because over-expression of *AWE1* does not confer ethylene-insensitivity this model includes a limited or set quantity of the downstream substrate or control point. In other words, AWE1's activity is limited by the next downstream control point. **D&E.** ETR1 and AWE1 act in concert on the downstream control point. In D, no ETR1 protein is present to stop the AWE1 signal/function, therefore, even in the presence of ethylene, AWE1 is still working to repress ethylene responses, but due to lack of ETR1, the repression of the downstream control point is incomplete. In E, the additional copies of AWE1 protein, due to over-expression, are all functioning even in the presence of ethylene allowing for compensation of *etr1* through repressing the ETR1-specific responses. *AWE1* over-expression brings the *etr1-7* mutant to wild-type like and not ethylene-insensitive possibly due to the limited quantity of the downstream control point.

*Experimental Procedures*

**Sequence Analysis**

blastP and tblastn searches were done at the NCBI Blast (blast.ncbi.nlm.nih.gov/Blast.cgi) and the Joint Genome Institute (www.jgi.doe.gov/genome-projects/) websites, using the At3g29185.1 amino acid sequence, as predicted by The Arabidopsis Informational Resource (TAIR) (www.arabidopsis.org), as the query sequence. The same amino acid sequence was used in the PHYRE program (www.sbg.bio.ic.ac.uk/~phyre/) which predicts secondary structures [78]. Finally the At3g29185.1 amino acid sequence was queried in the ChloroP1.1 program to search for predicted chloroplast transit peptide sequences [76].

**Plant Growth Conditions and Measurements**

The *Arabidopsis thaliana* Columbia ecotype (Col-0) was the wild-type strain used for all experiments in this study. The *awe1-1* SALK T-DNA line (SALK_030444) was backcrossed once to Col-0 wild-type before analysis. For dark-grown seedling response assays, *Arabidopsis* seeds were sterilized in a 50% bleach solution (with 10 μM Tween 20 (Fisher) added per 1 ml of sterilization solution) at room temperature for five minutes, with gentle mixing, then spun down briefly in a microfuge, and finally rinsed with water four times before being plated on Murashige and Shookg (MS) (Sigma Aldrich) medium containing 0.9% agar. After plating, the seeds were stratified at 4˚C for 3 days. Then the seeded plates were placed in light for five to six hours, wrapped in foil, and grown in the dark at 20˚C for 3.5 days. For the triple

response assay, seeds were germinated on MS media supplemented with ACC (Sigma Aldrich), AVG (Sigma Aldrich), or $AgNO_3$ (Sigma Aldrich) at the reported concentrations or on MS plates grown in air-tight mason jars with exogenous ethylene added through a rubber septum. Seedlings were removed from the MS medium for photographs. The hypocotyl lengths were then measured from the digital photographs using the ImageJ software. Adult *Arabidopsis* and *Nicotiana benthamiana* were grown in soil at 16 hour light/8 hour dark cycles (fluorescent light) at 20˚C. *N. benthamiana* plants were used two to three weeks after germination.

For genotyping purposes DNA was extracted from $F_3$ pooled seedlings or individual plants. Primers flanking the T-DNA insertion site were used to detect wild-type *AWE1* with forward primer 5'-CTGTGTTTCTGCTTCAATGGG-3' and reverse primer: 5'-TCTAACCCTCTTTCCTCGAGG-3'. To detect the T-DNA insertion (*awe1-1*) allele, the forward primer above and the SALK T-DNA primer Lba 5'-TGGTTCACGTAGTGGGCCATCG-3', which anneals to the T-DNA sequence were used. For genotyping *etr1-1* dCAPS primers [93] were used. The forward primer was 5'-GCGATTGCGTATTTTTCGAT-3' and the reverse primer was 5'GTCCATAAGTTAATAAGATGAGTTGA-3'. The amplified *etr1-1* PCR fragment was digested with the *Nsi*I restriction enzyme while the wild-type *ETR1* fragment was not. Primers used for genotyping the *ein2-1* allele were previously described in Resnick et al. (2006).

**RNA Extraction and cDNA Cloning**

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). The primers used to amplify the *AWE1 cDNA* from *Arabidopsis* RNA extracts were designed so that the sense primer annealed twenty bases upstream of the annotated 'ATG' start site and carrying a *Cla*I restriction site (underlined) in the 5' end: 5'-ATCGATTCACTCACTCGCTC-3'. The anti-sense primer annealed to the 3' end of the predicted *AWE1* coding sequence and carried an *Xba*I restriction site (underlined): 5'-AGATCTCTCACATTTTTGTCTCAG-3'. The *cDNA* was cloned into the pGEM-T vector (Promega) for downstream use. For analysis of transcript levels in *awe1* T-DNA mutants the same primers flanking the T-DNA insertion site and used for genotyping were also used for RT-PCR to check for transcript levels in the *awe1-1* mutant. Additionally, primers annealing to the 3'-end of the *AWE1* sequence (amplifying a cDNA fragment from *AWE1* nucleotides 905 to 1064) were used for RT-PCR of over-expression lines. The sense primer was 5'-GGAAAGGTCGTTCGGTAACA-3' and the antisense was 5'-TCCCAGTGCACATTTGTTGT-3'. Control primers used for RT-PCR were either primers annealing to *GADPH* (*At1g13440*): 5'-CAAGGAGGAATCTGAAGGCAAAATGA-3' and 5'-CAACCACACACAAACTCTCGCCG-3', primers annealing to β-tubulin (*At5g23860*): 5'-CGTGGATCACAGCAATACAGAGCC-3' and 5'-CCTCCTGCACTTCCACTTCGTCTTC-3', or primers annealing to *ACTIN3* (At3g53750) 5'-GTATGTGGCTATTCAGGCTG-3' and 5'-CTGGCGGTGCTTCTTCTCTG-3'.

**Plant Transformation Constructs**

The *AWE1* coding sequence was amplified (minus the *AWE1* stop codon) 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): sense 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCTATGGCTGCTGCTACTTCC-3' and antisense 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCATTTTTGTCTCAGTAAAATAAGT-3'. The *AWE1* coding sequence was transferred from the pDONR221 (entry) vector (Invitrogen) into various destination vectors such as the pB2GW7,0 (over-expression vector)(Plant Systems Biology) and the pSPYCE-35S (split YFP vector used for BiFC analysis)[80]. Primers used to amplify CTR1 for cloning into the nYFP vector pSPYNE-35s were (sense primer) 5'-ACAAGTTTGTACAAAAAAGCAGGCTCTCCAACCACCATGGAAATGCCCGGTAGAAG-3' and (antisense) 5'-TCCGCCACCACCAACCACTTTGTACAAGAAAGCTGGGTACAAATCCGAGCGGTTGGGCG-3'. CTR1 was cloned into the entry pDONR221 vector (Invitrogen), followed by subsequent cloning into the pSPYNE-35S split YFP vector [80]. ETR1 was previously cloned into the pDONR221 vector and available for recombination into the pSPYNE-35S vector. Cloning of the AWE-RFP sub-cellular localization constructs consisted of Gateway 3-piece cloning techniques (Invitrogen). AWE1-RFP#7 primer and the above *AWE1* antisense primer (carrying the $attB_2$ site) were used to amplify the genomic region starting about 700 base pairs upstream of the

'ATG' start codon through the last codon prior to the *AWE1* stop codon. The AWE1-RFP#7 primer sequence is: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCTTCTAGATCTAGTTGTTAACGA-3'. The fragment was amplified and cloned into the entry vector pDONRP4-P1R (Invitrogen). Primers AWE1-RFP#3 and AWE1-RFP#4 were used to clone the 3'UTR about 250 bases downstream of the *AWE1* stop codon. AWE1-RFP#3: 5'-GGGGACAGCTTTCTTGTACAAAGTGGCCATGGCTGCTGCTACTTCCTTCC-3' and AWE1-RFP#4 : 5'-GGGGACAACTTTGTATAATAAAGTTGGCGATTGATATGCAGTGAAG-3'. This fragment was amplified and cloned into the entry vector pDONRP2R-P3. Finally both entry clones along with RFP, which was previously cloned into the pDONR221 entry vector, were collectively and directionally cloned (using the specific recombination sites within each entry vector) into the destination vector pDESTR4-R3 for transformation into *Arabidopsis* The *DH5α e.coli* strain was used for subcloning. Agrobacterium strains used for plant transformations were either *GV3101* (for transformation into *Arabidopsis*) or *C85C1* (for transformation into *Nicotiana benthamiana*.) The floral dip method was used for transformation of the AWE1-RFP construct into *Arabidopsis awe1-1* plants [97]. Transformants were selected with the herbicide Finale (active ingredient glufosinate) (Bayer). For infiltration of *Nicotiana bentamiana,* the abaxial leaf surface of ~two week old plants were infiltrated with the *C85C1 Agrobacterium strain* as described in Walter et al. (2004). Each BiFC construct was independently transformed into *C85C1*, because all vectors used for the experiment carried the kanamycin resistance marker NPTIII for

selection in bacteria. Liquid cultures (5 mL) were started forty-eight hours prior to infiltration for each agrobacterium being used as well as for the p19 helper plasmid. After one day of incubation at 30˚C, 1 mL of each culture was transferred to a larger aliquot, 50 mL LB (with the antibiotics kanamycin, rifampicin, tetracycline as well as acetyosyringone, for stimulatation of the *Agrobacterium vir* genes ) and grown for another 24 hours. Approximately 18 hours later, the cells were spun down and resuspended altogether in a 10mL infiltration media consisting of 10mM MES, 10mM $MgCl_2$, and 10µM Aceytosyringone. This solution was incubated at room temperature, with gentle shaking, for six hours before infiltration. Using a syringe, the abaxial leaf surfaces were infiltrated with the media carrying the *Agrobacteria*.

**BiFC and Sub-cellular Localization Analysis**

For BiFC analysis, the abaxial surfaces of infiltrated tobacco leaves were observed seventy-two hours post infiltration using a Zeiss LSM 510 Confocal Microscope with a 488nm Argon laser. For AWE1 sub-cellular localization studies, abaxial leaf surfaces of ~two-week old stably transformed *Arabidopsis* lines were analyzed using the same Zeiss microscope but with the 543 nm laser.

# Chapter 3: The Role of the CTR1 Amino-terminal Domain in Ethylene Signaling

*Introduction*

CTR1, a serine/threonine protein kinase, is the next known downstream component from the ethylene receptors, and actively represses ethylene responses through kinase activity [32, 33]. The CTR1 protein is comprised of a 550 residue amino-terminal region, thought to have a regulatory function, fused to a carboxy-terminal kinase domain that shares 41% identity to the mammalian Raf kinases (Figure 3-1) [32]. CTR1 is a putative MAPKKK, although a CTR1-mediated MAP kinase cascade has yet to be elucidated. The CTR1 amino-terminal region physically interacts with the histidine kinase domains of the two subfamily I receptors, ETR1 and ERS1 [34]. This interaction seems to be essential for repressing ethylene responses. A specific point mutation, the only known missense mutation in the amino-terminal region (*ctr1-8*, G354E), disrupts CTR1's physical association with the receptors both in yeast and *in vivo*, and the *ctr1-8* mutant plant displays a constitutive ethylene response phenotype [33](Shockey and Chang, unpublished). There are additional *ctr1* loss-of-function alleles that confer constitutive ethylene responses in the plant. The *ctr1-1* mutation, D694E, results in the loss of $\geq$ 99.9% of CTR1 kinase activity *in vitro* [33, 36] (Shockey and Chang, unpublished). The *ctr1-3* mutation (R435stop) causes early truncation of the protein so that the carboxy-terminal catalytic kinase domain is not translated [33]. Collectively, analysis of the

*ctr1* alleles implies that CTR1 has a key, non-redundant role in repressing ethylene responses.

Apart from the essential interaction of the CTR1 amino-terminal region with the subfamily I receptors and the requirement of CTR1 kinase activity for repressing ethylene responses, little else is understood about CTR1 function and regulation.

Extensive analysis of the ETR1 and ERS1 histidine kinase domains reveals that the receptor histidine kinase activity is mostly dispensable for transmitting the ethylene signal to CTR1 [98, 99]. Thus the receptors must activate and deactivate CTR1 signaling through a different, unknown mechanism(s). The lack of interaction between the subfamily I receptors and the CTR1-8 mutant protein suggests that CTR1's physical association with ETR1 and ERS1 receptors is essential for CTR1 activation. However, we do not know if this association is the only requirement for CTR1 activation.

The CTR1 substrate also remains unknown; however, we speculate that the substrate may associate with the ER, where CTR1 peripherally associates as a component of the ETR1 receptor complex [36]. Because CTR1 is a putative MAP kinase kinase kinase, many groups hypothesize that the CTR1 substrate is a MAP kinase kinase. Ouaked et al. (2003) presented *in vitro* kinase data supporting the role of a MAP kinase cascade in ethylene signal transduction, showing that the *Medicago* MAP kinase kinase SIMKK and the MAP kinase SIMK both have increased kinase activity in the presence of ACC [40]. Similarly, the *Arabidopsis* homolog of the MAP kinase SIMK, MPK6, has increased kinase activity in a *ctr1* mutant background when compared to wild type [41]. Ouaked's and Yoo's separate findings both

support a role for the Arabidopsis MAP kinase, MPK6, in promoting ethylene responses [40, 41]. While Ouaked et al. (2003) draw the MPK6 and MAPKK's downstream of CTR1 (being repressed by CTR1 in the absence of ethylene), Yoo et al. (2008) propose two separate MAP kinase cascades regulating downstream responses: MPK6 in a cascade that promotes ethylene responses and CTR1 in a separate pathway that represses downstream responses. However, confirming a MAP kinase cascade, whether activated or repressed by CTR1, or acting in parallel to CTR1, is challenging due to the high redundancy of MAP kinase kinases and MAP kinases (10 and 23 respectively) in *Arabidopsis* [100].

Finally, an additional question about CTR1 signaling is whether or not CTR1 has any auto-regulation of its own kinase. The Raf kinases have an amino-terminal region that auto-inhibits the Raf kinase activity through direct intramolecular interactions between the two domains [101]. We also know that Raf associates with the Ras GTPase for activation [102]. Once this interaction dissociates, the Raf amino-terminal region is freed to interact and inhibit its own kinase activity [101]. Interestingly the crystal structure of the ETR1 receiver domain is similar to that of Ras [103] thus making a parallel between the Ras-Raf interaction and ETR1-CTR1 interaction attractive. Additionally, i*n vitro* data reveal that, similar to Raf, the CTR1 amino-terminal region physically associates with the CTR1 kinase domain [59](Shockey and Chang, unpublished.) These findings and the loss of receptor-CTR1 interaction in the *ctr1-8* mutant background, led us to propose a model in which the CTR1 amino- terminal region has two regulatory functions. One function is to maintain the CTR1 interaction with the ETR1 and ERS1 receptors, which, based

on *ctr1-8* data seems to be essential for CTR1 kinase activity in the absence of the ethylene ligand. The second proposed function for the CTR1 amino-terminal region is autoinhibition of CTR1 kinase activity. We propose that, once the receptors bind ethylene, the CTR1 <-> receptor interaction is lost. This may allow the now-freed CTR1 amino-terminal region to autoinhibit CTR1 kinase activity through physical interaction (Figure 3-1). I was interested in exploring this model *in vivo*. This led to testing whether CTR1 must associate with the receptors to act on downstream substrates, and to test this, I fused CTR1 to the transmembrane region of the ETR1 receptor, which should constitutively target CTR1 to the receptor complex. To test for auto-inhibitory function of the CTR1 amino-terminal region on CTR1 kinase activity I compared transgenic *Arabidopsis* lines carrying either a CTR1 truncated construct (lacking the CTR1 amino-terminal region) to lines carrying a full-length CTR1 construct.

*Results*

**Over-expression of the CTR1 Kinase Domain is not Enough to Confer Ethylene Insensitivity**

We reasoned that by removing the CTR1 amino-terminal region and expressing only the CTR1 kinase domain (CTR1 KD), would allow the CTR1 kinase to be constitutively active, repressing ethylene responses and consequently render transgenic seedlings insensitive to ethylene. To test for the potential auto-inhibitory function of the CTR1 amino-terminal region, I expressed a truncated CTR1 protein, consisting of the kinase domain, residues 550 to 821, and lacking the amino-terminal, putative auto-inhibitory region, in wild-type *Arabidopsis* plants. I analyzed nine

# FIGURE 3-1

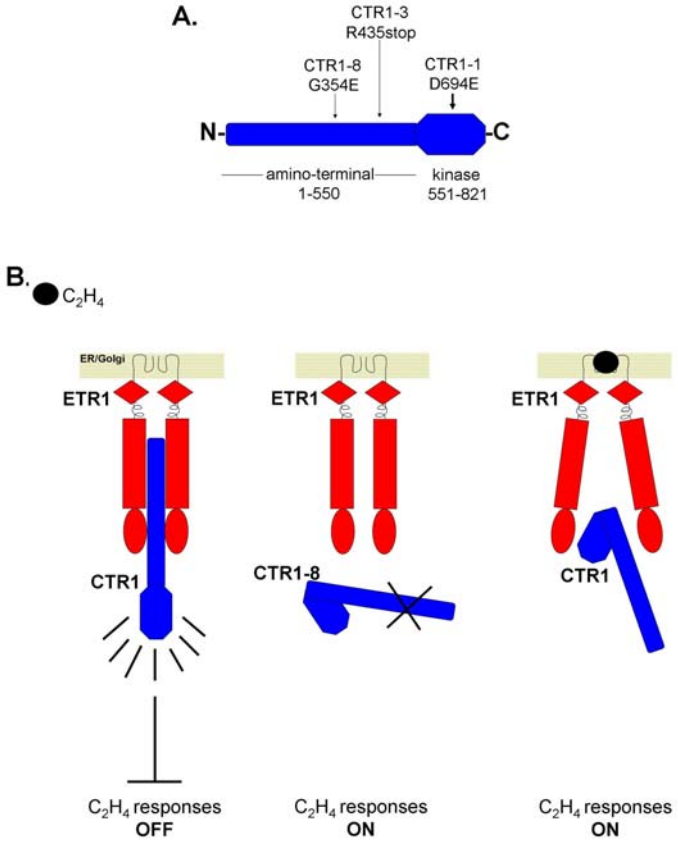



**Figure 3-1:** CTR1 is a negative regulator of ethylene responses. **A.** CTR1 is an 821 amino acid protein comprised of a putative amino-terminal auto-regulatory region from residues 1-550, and a carboxy terminal kinase domain, residues 551-821. Three well characterized mutations are marked: *ctr1-8* is the only known missense mutation in the amino-terminal region, *ctr1-3* results in early truncation of the protein, *ctr1-1* mutation disrupts kinase activity. **B.** The ETR1 receptor and CTR1 work to repress ethylene responses. ETR1 interacts with the CTR1 amino-terminal region, and we speculate that this interaction is lost upon ethylene binding to the receptor, allowing for a conformational change within CTR1, consequently causing inhibition of CTR1 kinase activity.

independent transgenic lines for seedling responses to exogenous ethylene (as well as to ACC). Only two lines (lines 3 and 6) showed a consistent slight increase in hypocotyl length relative to wild-type seedlings (data not shown). Because the native CTR1 protein in the wild-type background could potentially inhibit or interfere with the kinase activity of the over-expressed CTR1 KD, I transformed the fusion into the *ctr1-3* loss-of-function mutant. The *35S::CTR1KD* transgene slightly alleviated the constitutive triple response of the four homozygous independent transgenic *ctr1-3* mutant lines analyzed although the seedlings never recovered to wild type lengths (Figure 3-2b). Increasing the concentration of ACC in the medium had no visible effects on the average hypocotyl length of the *ctr1-3* transgenic lines. This is different than the untransformed wild type and *ctr1-3* controls and could be indicative of a constitutively active CTR1 kinase in the *ctr1-3* transgenic lines. However, the phenotype was mild and transgenic *ctr1* lines did not recover to wild-type-like.

One potential explanation for a lack of phenotype is that the truncated CTR1 KD protein may not localize near its substrate. This speculation was built upon previous yeast-two-hybrid results which revealed that the CTR1 KD by itself cannot interact directly with the ethylene receptors, ETR1 and ERS1 [34]. If the CTR1 substrate is at the ER, as we speculate, then the over-expressed CTR1 KD may spatially be unable to act upon that substrate due to its own mislocalization.

**Targeting the CTR1 KD to the Receptor Complex Confers Slight Increases in Hypocotyl Length**

To address this potential inability of the CTR1 KD to interact with its substrate due to lack of CTR1 KD interaction with the ethylene receptors, I fused the

# FIGURE 3-2

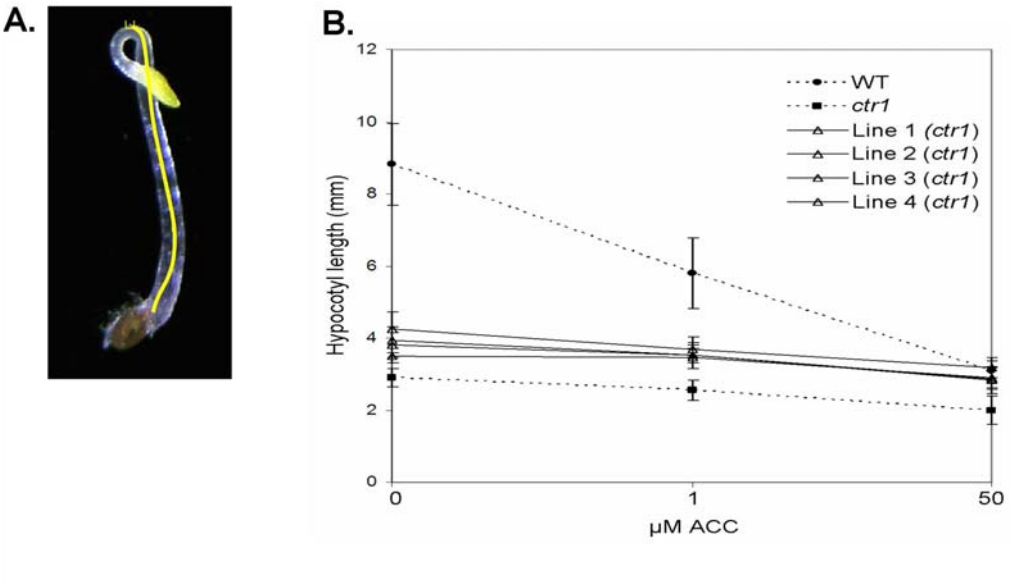



**Figure 3-2:** Expression of the CTR1 KD cannot cause ethylene insensitivity or restore ctr1 loss-of-function mutant hypocotyl lengths to wild type. **A.** The hypocotyls of etiolated *Arabidopsis* seedlings shorten in the presence of exogenous ethylene (part of the triple response phenotype.) The yellow line represents the hypocotyl length which was measured for each seedling. (The apical hook is not included in the hypocotyl measurement.) **B.** Four independent, homozygous transgenic *ctr1-3* lines (empty triangles) harboring the CTR1 KD transgene had longer hypocotyls than the untransformed *ctr1-3* seedlings (black squares). However the CTR1 KD transgene could not rescue the *ctr1-3* transgenic lines to wild type hypocotyl lengths (black circles).

CTR1 KD to the transmembrane spanning region of the ETR1 receptor, which should in theory target the CTR1 KD to the ETR1 receptor complex at the ER/Golgi. Two truncated forms of the ETR1 ethylene receptor, both containing the ETR1 transmembrane spanning region, were used for the fusions (Figure 3-3). The first ETR1 truncation, residues 1-106 ($ETR1^{1-106}$), contained only the ETR1 N-terminal transmembrane domains, with no putative "linker region" between the final transmembrane domain of ETR1 and the start codon of CTR1. Because, in theory, this ETR1-CTR1 fusion might restrict any conformational movements of the cytosolic, carboxy-terminal CTR1 fusion, I made a second ETR1 truncation, residues 1-349 ($ETR1^{1-349}$), consisting of the ETR1 transmembrane domains through the cytsolic, coiled coil region, allowing for a putative "linker" region (ETR1 residues 107 to 349). (This $ETR1^{1-349}$ truncation was previously expressed in *Arabidopsis*, and immunoblot analysis revealed that $ETR1^{1-349}$ localizes to the membrane extracts of Arabidopsis tissue, similar to full length ETR1 [104]. Both ETR1 truncations were fused to either the truncated CTR1 KD, or the full length CTR1 protein (CTR1FL) (Figure 3-3a). I used the CTR1FL fusions as a control for comparison to the CTR1 KD fusions. In analysis of transgenic lines, any phenotype that I attribute to an over-active CTR1 KD should not be visible in transgenic lines carrying the CTR1FL fusions, if the CTR1 amino-terminal region is an auto-inhibitor of the kinase. Expression of all constructs was driven by the CaMV 35S promoter. (Cloning procedures are summarized in Figure 3-7.) Initially, I transformed the $ETR1^{1-106}$-CTR1FL and $ETR1^{1-349}$-CTR1FL transgenes into the *ctr1-8* background, looking for rescue of the *ctr1* constitutive triple response phenotype by the full length CTR1

fusion. (I initially tested for functionality of the transgene in the less severe *ctr1-8* background instead of the stronger *ctr1-3* mutant, because *ctr1-8* plants are easier to transform and produce more seeds than *ctr1-3* plants.) Both transgenes containing CTR1FL partially restored the triple responses of the constitutively responding *ctr1-8* seedlings (Figure 3-3c and d). The ETR1$^{\mathbf{1\text{-}349}}$-CTR1FL transgene restored the hypocotyl lengths slightly more than the ETR1$^{\mathbf{1\text{-}106}}$-CTR1FL transgene. Transgenic lines carrying either transgene (ETR1$^{1\text{-}106}$-CTR1FL or ETR1$^{1\text{-}349}$-CTR1FL) responded when ACC was added to the medium suggesting that the full length CTR1 was functioning similarly to wild-type CTR1 (Figure 3-3c and d). Therefore these transgenes (ETR1$^{1\text{-}106}$-CTR1FL and ETR1$^{1\text{-}349}$-CTR1FL) could serve as controls for comparisons to constructs carrying only the CTR1 kinase domain: ETR1$^{1\text{-}106}$-CTR1KD and ETR1$^{1\text{-}349}$-CTR1KD.

I transformed all four constructs: ETR1$^{1\text{-}106}$-CTR1KD, ETR1$^{1\text{-}106}$-CTR1FL, ETR1$^{1\text{-}349}$-CTR1KD, and ETR1$^{1\text{-}349}$-CTR1FL into wild-type plants to determine if the CTR1KD-fusion transgenes could confer ethylene insensitivity in *Arabidopsis* seedlings. Since the ETR1$^{1\text{-}349}$-CTR1FL transgene functioned more closely to wild type than the ETR1$^{1\text{-}106}$-CTR1FL transgene, I focused on those transgenic lines harboring the ETR1$^{1\text{-}349}$-CTR1FL (as the control) in comparison to the lines carrying the ETR1$^{1\text{-}349}$-CTR1KD fusion. I looked for ethylene-insensitivity in transgenic seedlings grown on 5 μM ACC (a concentration of ACC at which I knew that the ETR1$^{1\text{-}349}$-CTR1FL behaved similar to wild type CTR1 when expressed in the *ctr1-8* background, Figure 3-3b and d). The five independent wild-type transgenic lines harboring the ETR1$^{1\text{-}349}$-CTR1FL transgene displayed no ethylene-insensitivity while

# FIGURE 3-3

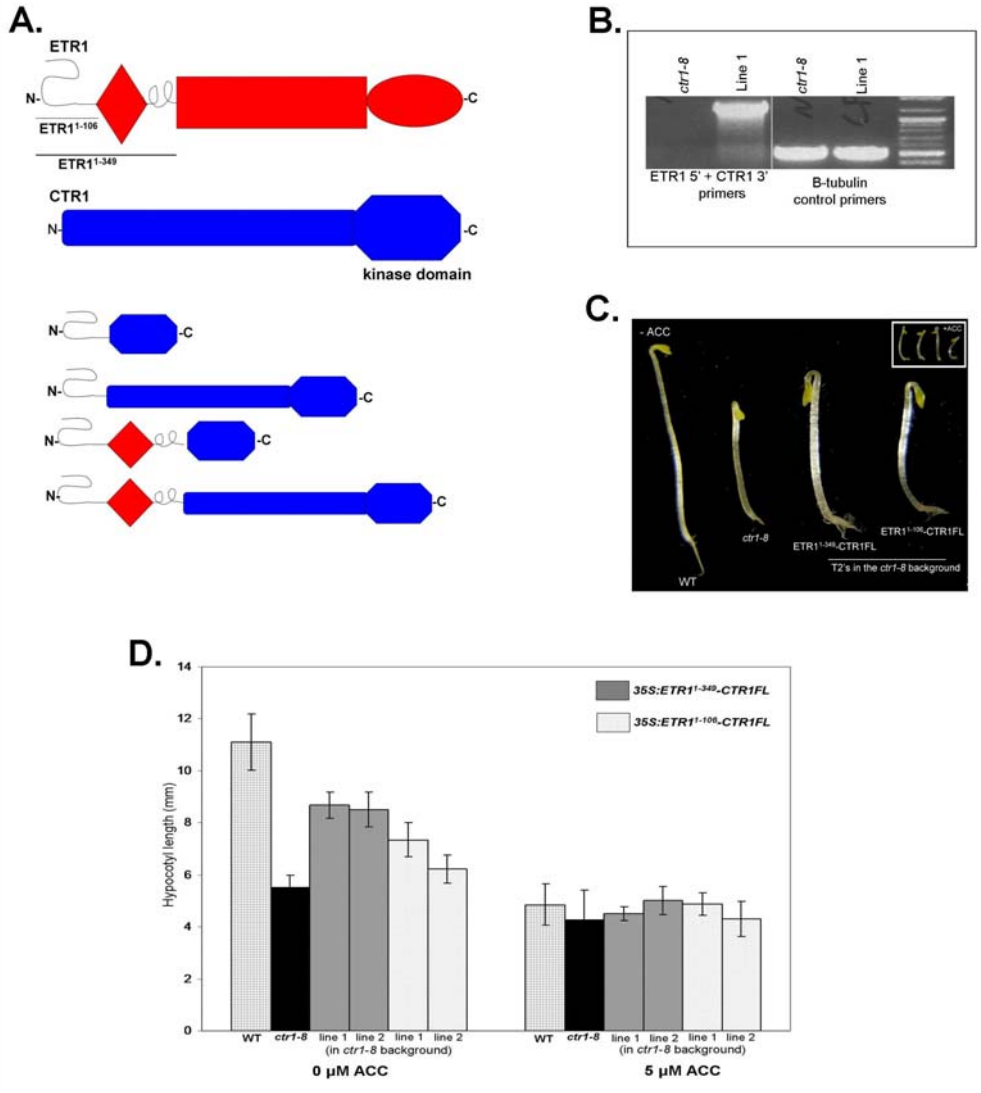

A.
ETR1
N-
-C
ETR1 1-106
ETR1 1-349
CTR1
N-
-C
kinase domain
N-
-C
N-
-C
N-
-C
N-
-C
B.
ctr1-8
Line 1
ctr1-8
Line 1
ETR1 5' + CTR1 3' primers
B-tubulin control primers
C.
- ACC
+ ACC
WT
ctr1-8
ETR1 1-349-CTR1FL
ETR1 1-106-CTR1FL
T2's in the ctr1-8 background
D.
35S:ETR1 1-349-CTR1FL
35S:ETR1 1-106-CTR1FL
Hypocotyl length (mm)
0
2
4
6
8
10
12
14
WT
ctr1-8
line 1
line 2
line 1
line 2
(in ctr1-8 background)
0 µM ACC
WT
ctr1-8
line 1
line 2
line 1
line 2
(in ctr1-8 background)
5 µM ACC

**Figure 3-3:** Chimeric fusions used to target CTR1 to the receptor complex. **A.** Four ETR1 truncations fused to CTR1 were constructed to localize CTR1 to the receptor complex in plants: ETR1$^{1\text{-}106}$-CTR1FL, ETR1$^{1\text{-}106}$-CTR1KD, ETR1$^{1\text{-}349}$-CTR1FL, ETR1$^{1\text{-}349}$-CTR1KD. **B.** RT-PCR showing expression of ETR1$^{1\text{-}349}$-CTR1FL in the *ctr1-8* background. The 5' primer was specific for ETR1 and the 3' primer was an internal primer of CTR1, so the product should only be present in transgenic lines and is not present in the *ctr1-8* control. β-tubulin specific primers were used as a control for the RT-PCR. **C and 3D.** The ETR1$^{1\text{-}106}$-CTR1FL and ETR1$^{1\text{-}349}$-CTR1FL transgenes can partially restore the constitutive dark grown triple response phenotype of the *ctr1-8* mutant. **3C inse**t: All seedlings respond to exogenous ACC, suggesting the transgene carrying the CTR1FL are functional. **3D:** Error bars represent the standard deviation of $13 \leq n \leq 15$ seedlings measured of each genotype per treatment.

the seven independent wild-type lines carrying the 35S::ETR1$^{1-349}$-CTR1KD were insensitive to exogenous ethylene (Figure 3-4a and b, and data not shown). Comparisons between those transgenic lines carrying the ETR1$^{1-349}$-CTR1FL and those carrying the ETR1$^{1-349}$-CTR1KD imply that removal of the CTR1 amino-terminal region yields a constitutively active CTR1 kinase, providing *in planta* support of our model that the CTR1 kinase is regulated by its amino-terminal region.

Additionally, comparisons between transgenic lines carrying the ETR1$^{1-349}$-CTR1KD and those harboring the over-expressed CTR1KD alone, show that the ETR1$^{1-349}$-CTR1KD transgene conferred a higher degree of ethylene-insensitivity (Figure 3-4c), providing indirect support for a model in which the CTR1 substrate localizes to the receptor-CTR1 complex at the ER/Golgi.

**Over-expression of the CTR1 KD and CTR1 FL in *ctr1-3* Suggests Additional Components are Required for CTR1 Regulation**

In order to eliminate the possibility that the wild type CTR1 interferes with the phenotypes that I am attributing to the transgenes, I transformed the ETR1$^{1-349}$-CTR1FL and ETR1$^{1-349}$-CTR1KD constructs into the *ctr1-3* severe loss-of-function mutant. Unlike in the wild-type transgenic lines, the *ctr1-3* transgenic lines carrying either the ETR1$^{1-349}$-CTR1FL construct or the ETR1$^{1-349}$-CTR1KD construct behaved similarly (Figure 3-5a). Expression of either transgene slightly alleviated the constitutive responses in the *ctr1-3* background, but neither could restore the *ctr1-3* mutant to wild type. This is unlike the ability of the ETR1$^{1-349}$-CTR1FL to restore the *ctr1-8* mutant to wild-type-like hypocotyl lengths and suggests that the ETR1$^{1-349}$-CTR1FL cannot compensate for the more severe ethylene responses in the *ctr1-3*

# FIGURE 3-4

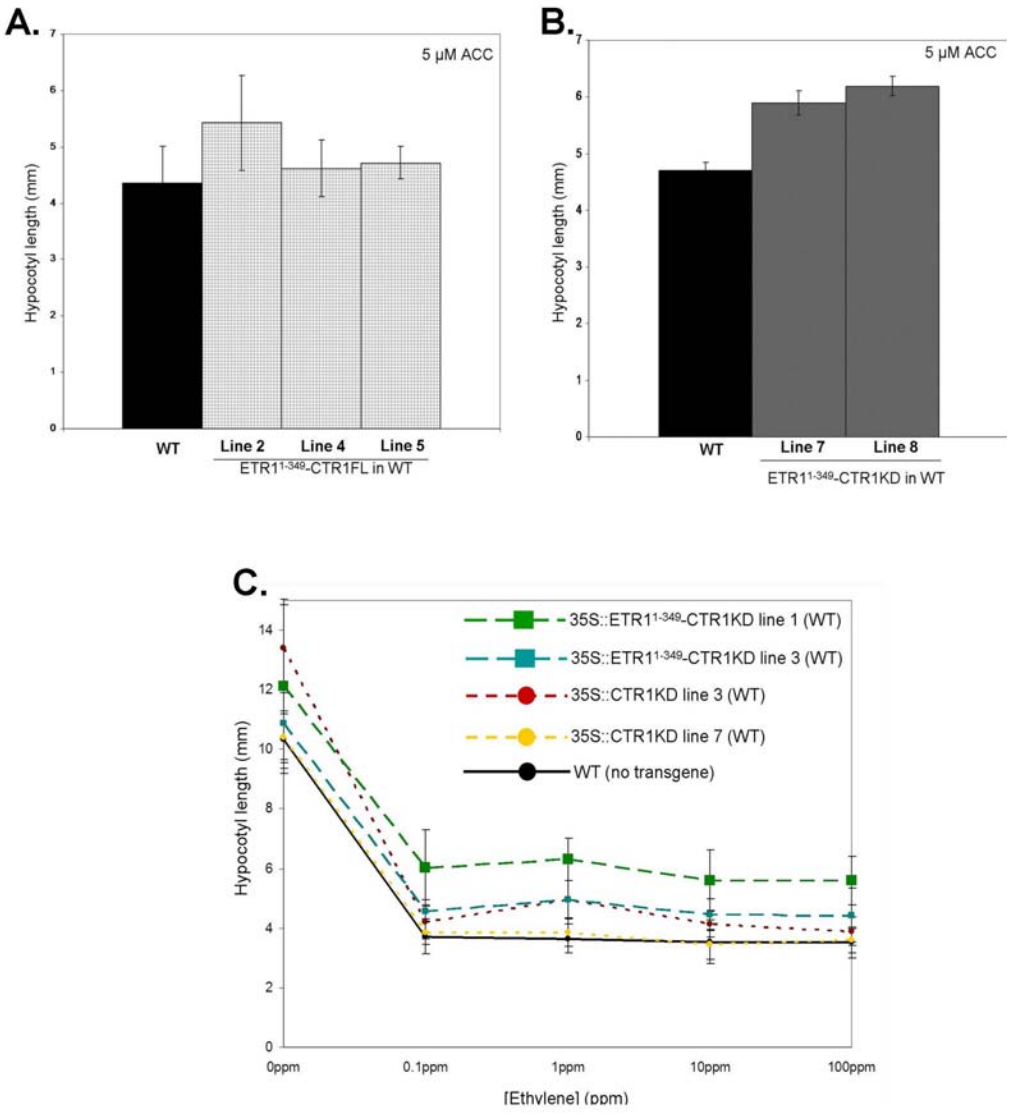

A.
5 μM ACC
Hypocotyl length (mm)
WT
Line 2
Line 4
Line 5
ETR1^1-349-CTR1FL in WT
B.
5 μM ACC
Hypocotyl length (mm)
WT
Line 7
Line 8
ETR1^1-349-CTR1KD in WT
C.
35S::ETR1^1-349-CTR1KD line 1 (WT)
35S::ETR1^1-349-CTR1KD line 3 (WT)
35S::CTR1KD line 3 (WT)
35S::CTR1KD line 7 (WT)
WT (no transgene)
Hypocotyl length (mm)
0ppm
0.1ppm
1ppm
10ppm
100ppm
[Ethylene] (ppm)

**Figure 3-4:** Over-expression of the targeted CTR1 KD confers partial ethylene insensitivity. **A.** The ETR1$^{1-349}$-CTR1FL transgene had no affect on hypocotyl growth of wild type seedlings. (Five independent transgenic lines are represented by the checkered, gray bars. The non-transformed wild type control is represented by the black bar.) **B.** Transgenic lines carrying the ETR1$^{1-349}$-CTR1KD transgene had longer hypocotyls than the wild type untransformed plants (black bar Figure 4: Over-expression of the targeted CTR1 KD confers ethylene-insensitivity. **C.** Wild-type transgenic lines over-expressing the ETR1$^{1-349}$-CTR1KD had longer hypocotyls than those lines over-expressing the CTR1 KD alone when grown in the presence of exogenous ethylene. (Error bars for figures 3-4a-c represent standard deviation of $n \geq 14$ seedlings measured for each data point.)

# FIGURE 3-5

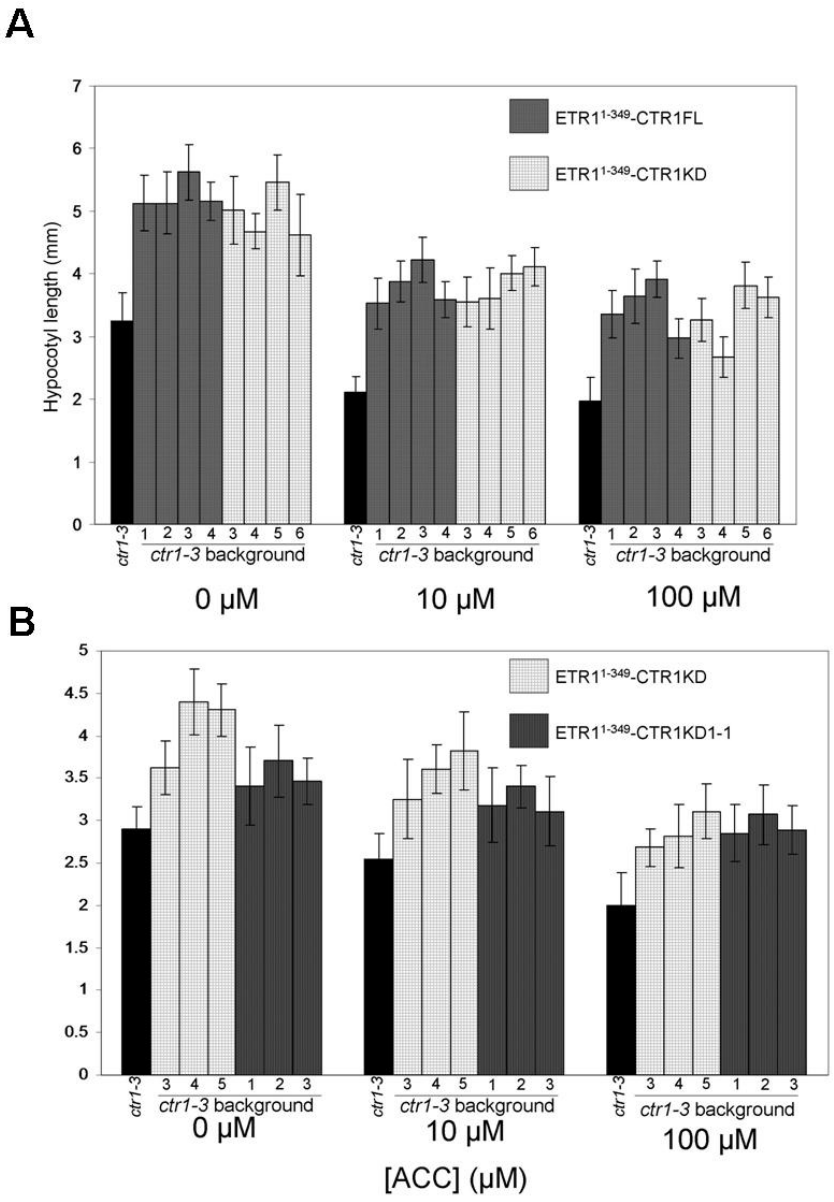

A
Hypocotyl length (mm)
ETR1^1-349-CTR1FL
ETR1^1-349-CTR1KD
ctr1-3
ctr1-3 background
0 µM
10 µM
100 µM
B
ETR1^1-349-CTR1KD
ETR1^1-349-CTR1KD1-1
[ACC] (µM)

**Figure 3-5:** The ETR1$^{1-349}$-CTR1FL and ETR1$^{1-349}$-CTR1KD transgenes partially alleviate the constitutive response in the *ctr1-3* mutant, but this alleviation is not completely due to CTR1 auto-kinase activity. **A.** Comparison of four independent homozygous *ctr1-3* transgenic lines carrying the ETR1$^{1-349}$-CTR1FL transgene (dark gray bars) compared to four independent homoyzgous *ctr1-3* lines carrying the ETR1$^{1-349}$-CTR1KD transgene (light gray bars) and the *ctr1-3* untransformed control (black bar.) The two transgenes behave similarly in the *ctr1-3* background, alleviating the hypocotyl shortening in the mutant but responding to increasing concentrations of ethylene. **B.** Comparisons of the ETR1$^{1-349}$-CTR1KD transgenic lines (light gray bars) to lines carrying the ETR1$^{1-349}$-CTR1KD with a D694E mutation that should disrupt transgene kinase activity (dark gray bars). The point mutation disrupts the transgenes ability to confer hypocotyl elongation when grown in ACC- conditions [31]. However, the transgene still allows slight hypocotyl elongation relative to the *ctr1-3* null, (black bar.) Error bars represent standard deviation of n>14 seedlings.

mutant. However, the difference in hypocotyl lengths between the *ctr1-3* transgenic lines and the *ctr1-3* untransformed control plants suggests that the CTR1 kinase in both the ETR1$^{1-349}$-CTR1FL and ETR1$^{1-349}$-CTR1KD is active, even in the presence of ACC.

To determine if the slight alleviation of hypocotyl length is due to CTR1 kinase activity, I transformed an ETR1$^{1-349}$-CTR1KD transgene carrying the *ctr1-1* D694E mutation, which should disrupt ≥99.9% of the CTR1 kinase activity [33](Shockey and Chang, unpublished) into the same *ctr1-3* background. These transgenic lines, although not as long as those lines carrying the ETR1$^{1-349}$-CTR1KD, did have longer hypocotyls than the *ctr1-3* untransformed seedlings (Figure 3-5b). This suggests that while constitutive CTR1 kinase activity may be partially responsible for the hypocotyl elongation observed, there are other unidentified mechanisms also responsible for the partial alleviation of the constitutive phenotype in the *ctr1-3* background.

*Discussion*

Most models of CTR1 regulation are based on our current understanding of how the mammalian Raf kinases are regulated. Previous work in our lab showed that the CTR1 amino-terminal region can directly interact with the CTR1 kinase domain *in vitro* [59](Shockey and Chang unpublished), and this is similar to Raf [101]. Because this intramolecular interaction inhibits the Raf kinase activity, I tested for potential auto-inhibitory effects of the CTR1 intramolecular interaction *in vivo* by over-expressing the CTR1 kinase domain alone. I found that transgenic *Arabidopsis* seedlings over-expressing the CTR1 KD alone responded similarly to the

untransformed control seedlings when grown on medium containing ACC. This result suggested that the initial model, in which expressing the CTR1 KD without the CTR1 amino-terminal region, should confer ethylene-insensitivity, was incorrect or incomplete (Figure 3-6a). There are several potential explanations for the lack of an effect: the CTR1 amino-terminal region is not an auto-inhibitor of its own kinase, the transgene may not have been expressed in any of the lines tested, or the CTR1 KD alone cannot act upon its substrate *in vivo*. This latter possibility could result from a lack-of-interaction between the CTR1 KD and the ethylene receptor complex. If the CTR1 substrate is a part of the ethylene receptor complex, which is where CTR1 resides sub-cellularly [36], then the truncated CTR1 KD, which cannot interact with the receptors [34], may be mis-localized relative to the wild-type CTR1 protein and unable to interact with its substrate. In this scenario, I would not expect to see ethylene-insensitivity in transgenic plants over-expressing the CTR1 KD, even if the kinase was constitutively active.

To test this idea of CTR1 KD mislocalization relative to the ETR1 complex and potentially the CTR1 substrate due to lack of the CTR1 amino-terminal region (Figure 3-6b), I fused CTR1, either the KD or full-length CTR1 (CTR1 FL), to the carboxy-terminal end of each of two ETR1 receptor truncations which included the ETR1 transmembrane domains only ($ETR1^{1-106}$) or the ETR1 transmembrane domains through the cytosolic GAF and coiled coil domains ($ETR1^{1-349}$). (If the ETR1-CTR1 KD fusions conferred ethylene insensitivity in transgenic *Arabidopsis* plants, and the ETR1-CTR1FL fusions did not, this would provide evidence for a role

# FIGURE 3-6

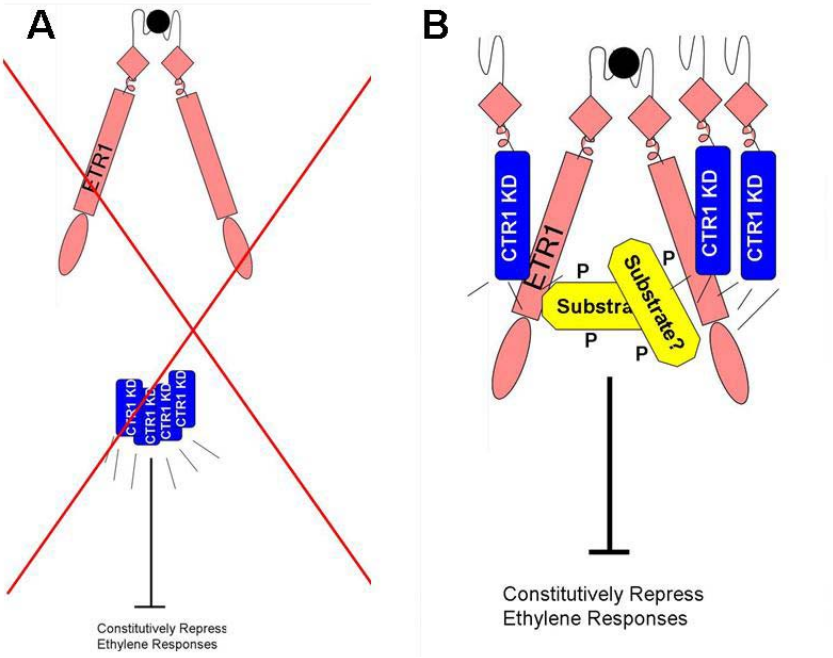



**Figure 3-6:** Removing the CTR1 amino-terminal region is not sufficient to cause a constitutively active CTR1 kinase domain. **A.** The initial hypothesis was that expressing the CTR1 kinase domain without the CTR1 amino-terminal region would cause ethylene-insensitivity if the amino-terminal region served to auto-inhibit kinase activity. The work described here suggests that the CTR1 amino-terminal region has additional or different roles, potentially in CTR1 kinase activation, through interaction between the CTR1 amino-terminal region and the ethylene receptor complex. This former model does not seem to be complete (indicated by the red "X"). **B.** To test the necessity of the CTR1 kinase to interact with the ethylene receptor complex in order to act upon its substrate, I targeted the kinase using the transmembrane portion of ETR1 and looked for ethylene insensitivity.

of the CTR1 amino-terminal region as an autoinhibitor of kinase activity. See model in Figure 3-7).

Both the ETR1$^{1-106}$-CTR1FL and the ETR1$^{1-349}$-CTR1FL transgenes partially rescued the triple response of *ctr1-8* mutant seedlings, suggesting that the constructs were partially functional. *ctr1-8* lines carrying the ETR1$^{\mathbf{1-349}}$-CTR1FL transgene displayed longer hypocotyls than *ctr1-8* lines carrying the ETR1$^{\mathbf{1-106}}$-CTR1FL, suggesting that the ETR1$^{\mathbf{1-349}}$-CTR1FL transgene may more closely resemble wild type CTR1 (potentially due to the putative "linker" region between the ETR1 transmembrane domains and the start codon of CTR1 FL in the ETR1$^{1-349}$-fusion.) Therefore I chose to focus on those fusions containing the ETR1 1-349 truncation: ETR1$^{\mathbf{1-349}}$-CTR1FL and ETR1$^{\mathbf{1-349}}$-CTR1KD.

In the wild-type background, the transgenic lines carrying the ETR1$^{\mathbf{1-349}}$-CTR1KD transgene had slightly longer hypocotyls than wild-type while transgenic lines carrying the ETR1$^{\mathbf{1-349}}$-CTR1 FL looked similar to wild-type. Assuming that the CTR1 FL transgene was expressed, these results provide support of our hypothesis that the CTR1 amino-terminal region is required for both inhibition of CTR1 kinase activity and spatial localization of the CTR1 kinase relative to its substrate.

However, the data generated from these same transgenes in the *ctr1-3* loss-of-function background suggest that additional proteins are likely required for CTR1 regulation. The ETR1$^{1-349}$-CTR1 KD slightly alleviated constitutive ethylene responses in the *ctr1-3* mutant (both in the absence and presence of ACC). Transgenic lines carrying the ETR1$^{1-349}$-CTR1FL transgene behaved similarly, suggesting that the alleviation cannot be attributed to the truncated CTR1 kinase in

# FIGURE 3-7

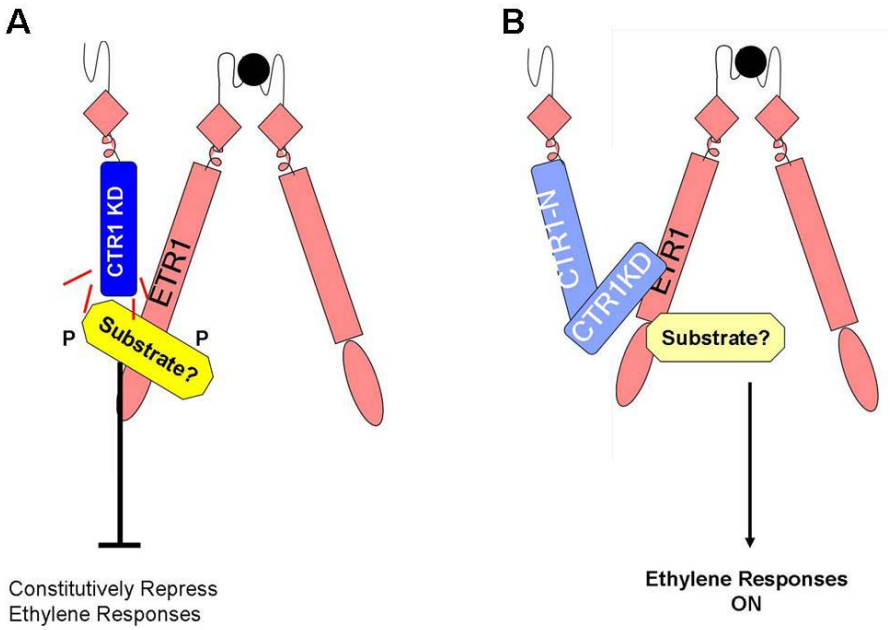



**Figure 3-7**: Testing a two-fold model, that the CTR1 kinase substrate is localized to the ethylene receptor complex and that the CTR1 amino-terminal region auto-inhibits kinase activity once ethylene binds. **A.** Transgene targeting the CTR1 kinase domain to the receptor complex. If the CTR1 substrate is present at the receptor complex and the CTR1 amino-terminal region is an auto-inhibitor of kinase activity, this trangene should render plants ethylene-insensitive. **B.** A control transgene to test the significance of the CTR1 amino-terminal region when CTR1 is targeted to the receptor complex.

the transgenic lines harboring the ETR1$^{1-349}$-CTR1 KD construct (or that any constitutive CTR1 kinase activity due to a lack of CTR1 amino-terminal region is not causing the phenotype.) Interestingly, the ETR1$^{1-349}$-CTR1 FL fusion could not restore CTR1 function in the *ctr1-3* mutant, unlike the partial-rescue of the less-severe *ctr1-8* mutant. (The CTR1-8 kinase can function *in vitro* [32].) None of the *ctr1-3* transgenic lines that I observed recovered to wild-type hypocotyl lengths; however, all of the *ctr1-3* transgenic lines had longer hypocotyls than the *ctr1-3* untransformed mutant. The longer hypocotyls were not altered in the absence or presence of ACC, and this may indicate that the transgenes are constitutively signaling, to a degree, in the *ctr1-3* background. If so, then both transgenes are constitutively active (see model in Figure 3-8), which would provide support for a model in which the CTR1 substrate is at the receptor complex and that CTR1 must dissociate with the complex for deactivation.

To test whether the phenotypes were due to CTR1 kinase activity, I analyzed transgenic *ctr1-3* lines that carried the ETR1-CTR1FL fusion carrying the *ctr1-1* mutation which should disrupt CTR1 kinase activity, ETR1$^{\mathbf{1-349}}$-CTR1KD (D694E)[33]. The transgenic *ctr1-3* lines carrying this transgene also had subtle restoration of the hypocotyls of the *ctr1-3* mutant but not to the extent of the *ctr1-3* transgenic lines carrying ETR1$^{1-349}$-CTR1KD and ETR1$^{1-349}$-CTR1FL.

These results suggest that the phenotypes I had initially attributed to a constitutively active CTR1 kinase domain were not entirely due to CTR1 kinase activity. However trans-phosphorylation of the ETR1$^{\mathbf{1-349}}$-CTR1KD(D694E) could also potentially lead to CTR1 signaling, and cannot be ruled out. Huang et al. (2003)

# FIGURE 3-8

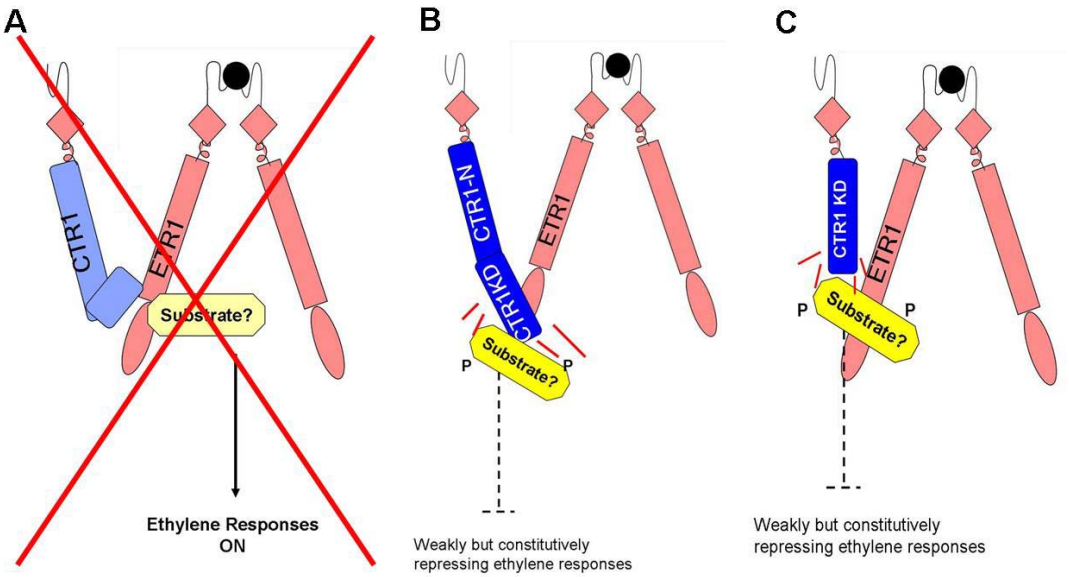



**Figure 3-8:** The presence or absence of the CTR1 amino-terminal region in the transgene does not alter the ethylene responses in the *ctr1-3* background. **A.** Model showing my original hypothesis that the CTR1FL transgene would not be active when ethylene bound to the receptor. This model does not seem to be entirely correct or complete (indicated by the red "X.") **B.** The actual results suggest that the CTR1 FL is active to the same extent of the CTR1 KD transgene, shown in **C**. This result may indicate that once the CTR1 kinase domain is recruited to the receptor complex, the CTR1 amino-terminal region's function is complete and the CTR1 amino-terminal region is not required for auto-inhibition of kinase. Alternatively the fusion of the amino-terminal region to the ethylene receptor may disrupt the ability of the CTR1 amino-terminal region to inhibit kinase activity.

showed that there is a very low level (0.1%) of trans-activation of a CTR1-1 kinase by a CTR1 wild-type kinase *in vitro* [33], and even if CTR1 activity is not present in the *ctr1-3* background, other MAPKKK's may be active. *ctr1-3* plants are very stressed throughout their life cycles. Potentially signaling proteins (such as other MAPKKK's) could be activated in the *ctr1-3* background that would not be activated in the wild type background under normal environmental conditions. Collectively the ability to trans-activate *in vitro* and the potential abnormal increased activation of proteins in the *ctr1-3* background might lead to trans-activation of the ETR1$^{1\text{-}349}$-CTR1 (D694E) chimera by other MAPKKK's *in vivo*. I did a blast search and found that the CTR1 KD shares over 60% identity with at least 5 other *Arabidopsis* kinases, including the MAPKKK's EDR1 and MAP3K delta-1. Trans-phosphorylation could explain the slight hypocotyl elongation seen in those *ctr1-3* transgenic lines carrying the mutated transgene.

Finally, why could the ETR1$^{1\text{-}349}$-CTR1FL transgene rescue the *ctr1-8* mutant but not the more severe *ctr1-3* mutant? One possibility is that in the *ctr1-8* background, the CTR1-8 protein is localized to the cytosol, unable to interact with the receptors while the truncated CTR1-3 protein (residues 1-434), which lacks the kinase domain (assuming that the protein product is produced in plants) has about 80% of the CTR1 amino-terminal region which may still interact with the receptors. The CTR1-3 protein might titrate out any interactions of the ETR1$^{1\text{-}349}$-CTR1FL with the receptors or other regulatory components, potentially leading to mis-regulation of CTR1 in the ETR1$^{1\text{-}349}$-CTR1 FL fusion.

This work does not provide the concrete *in vivo* support of a model in which the CTR1 amino-terminal region auto-inhibits CTR1 kinase activity. The lack of an antibody against CTR1 makes it more challenging to interpret the data. An antibody would have allowed me to determine protein levels in the plants and potentially detect correlations between phenotype and protein levels. The slight alleviation of constitutive ethylene responses suggest that the chimeras are being expressed at least at very low levels. The data also suggest that additional components, besides autoregulation, are required for CTR1 activation and regulation as over-expressing the CTR1 kinase domain is not enough to confer ethylene-insensitivity. *In vitro* analysis of Raf-1 activation suggests that the PP1 and PP2A phosphatases are essential for dephosphorylating specific Raf-1 serine residues in order for Raf-1 activation to occur [105]. In *Arabidopsis* the *eer1/rcn1* mutant, which encodes a PP2A, was identified in a screen for *enhanced ethylene response* mutants [56, 57]. This suggests a role for PP2A in negatively regulating ethylene responses. The *Arabidopsis* PP2A was found to associate with the CTR1 KD but not the CTR1 amino-terminal region *in vitro* [57]. Although the biological significance of this interaction remains unknown, the interaction could serve to dephosphorylate specific CTR1 KD residues required to activate the CTR1 kinase [57].

It remains unknown as to whether or not the receptors continuously interact with the CTR1 amino-terminal region both in ethylene-bound and non-ethylene bound conditions. We know that Raf assosciates with the Ras GTPase for activation. Additionally and surprisingly the crystal structure of the ETR1 receiver domain is similar to that of Ras [103] thus making a parallel between the Ras-Raf interaction

and ETR1-CTR1 interaction more attractive. A potential hypothesis is that once the receptors bind ethylene they lose the interaction with CTR1 (potentially through a conformational change.) This would allow the CTR1 amino-terminal region to interact and inhibit the CTR1 kinase activity, very similar to Raf regulation. I used multiple systems to test for loss of ETR1 and CTR1 interaction in yeast and in plants (see Appendix c). Unfortunately, the systems ended up being non-suitable, and the answer to this question remains inconclusive. If ETR1 does lose interaction with CTR1 upon ethylene binding, then there should be additional components that keep CTR1 localized to the ER. (CTR1 has no known or predicted modification sites, such as myristoylation, palmytoylation, etc.) Multiple 14-3-3 proteins have been identified in cDNA library screens as CTR1-interactors in the yeast-two-hybrid assay [106]. Interestingly, 14-3-3 proteins are essential for activating Raf-1 kinase activity and potentially essential for sequestering inactive Raf. 14-3-3 and Raf-1 form a complex which is recruited to Ras for activation. Upon Ras/Raf-1 interaction, the 14-3-3 proteins are displaced. This displacement may be facilitated by membrane-bound phosphatidylserine (PS). Once 14-3-3 is displaced from the complex, the Raf-1 protein is no longer protected from the serine phosphatases such as PP2A [101]. Perhaps these 14-3-3 proteins with putative binding sites in both the CTR1 amino- and carboxy- terminal regions are essential for CTR1 retention at the ER and CTR1 activation (or re-activation of CTR1 once ethylene responses have been carried out (Figure 3-9)).

As new techniques are developed, such as the recently available split luciferase reporter system for *Arabidopsis* [107], we will be able to more closely test

# FIGURE 3-9

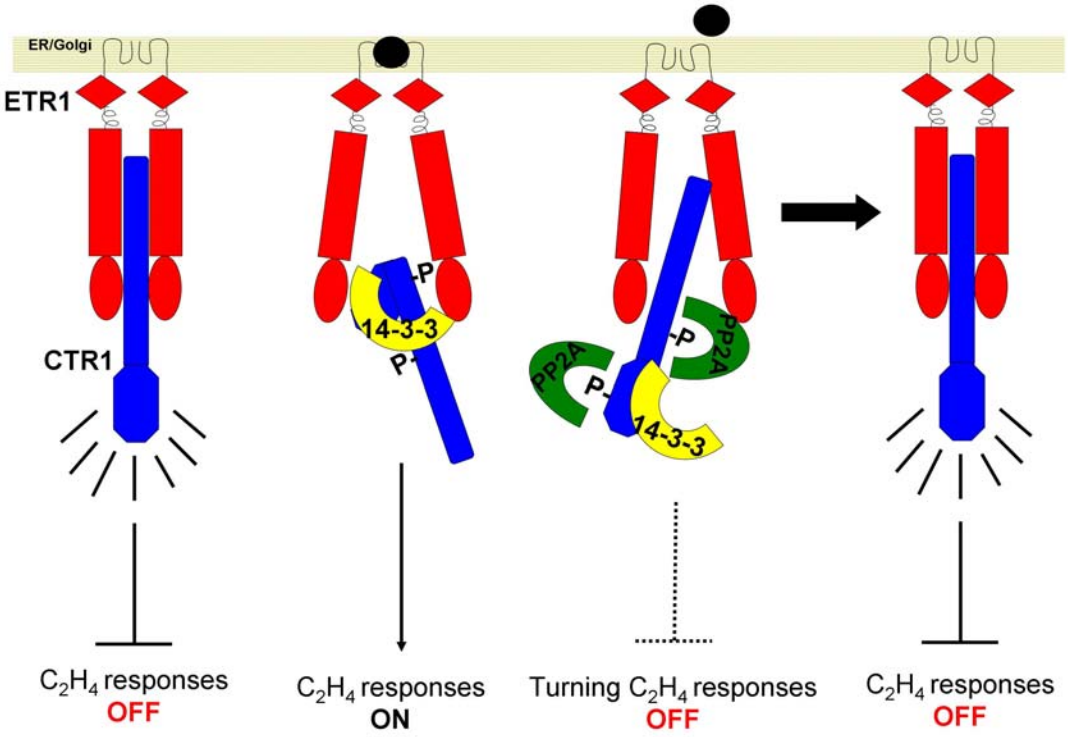

ER/Golgi
ETR1
CTR1
-P
14-3-3
PP2A
$C_2H_4$ responses
OFF
$C_2H_4$ responses
ON
Turning $C_2H_4$ responses
OFF
$C_2H_4$ responses
OFF

**Figure 3-9:** Model of CTR1 regulation. The CTR1 amino-terminal region may function in inhibiting CTR1 kinase activity once ethylene binds to the receptors; however, my work suggests that additional components are needed for the activation and repression of CTR1 kinase activity. PP2A proteins have been identified in the activation or re-activation of the CTR1 mammalian homolog Raf-1. The PP2A proteins act on two specific serine residues, one in the amino-terminal region and one in the kinase region of Raf-1 for Raf-1 activation. An *Arabidopsis* PP2A, RCN1, was found to interact with the CTR1 kinase domain *in vitro* [79]. 14-3-3 proteins are required for sequestering inactive Raf-1 and upon the proper signal the 14-3-3 proteins dissociate from Raf-1, potentially exposing the serine residues for PP2A activity. Several 14-3-3 proteins have been identified in yeast-two-hybrid screens as potential interactors with CTR1 [83], and the CTR1 sequence suggests a 14-3-3 binding site on the amino-terminal region and a 14-3- binding site on the carboxy-terminal kinase domain. These PP2A and 14-3-3 proteins may function in activation and deactivation of CTR1 kinase activity.

for dynamic interactions between the receptors, CTR1, and other receptor-complex components, advancing the model of CTR1 regulation and determining how similar (or not) CTR1 regulation is compared to the regulation of Raf-1 and other MAPKKK's.

*Experimental Procedures*

**Plant Strains, Growth Conditions, and Measurements**

The *Arabidopsis thaliana* Columbia ecotype (Col-0) was used. Adult plants were grown in soil at 16 hour light/8 hour dark days (fluorescent light) at 20˚C. Seedlings were grown on Murashige and Skoog (MS) medium containing 0.9% agar. Seeds were stratified for 3 days at 4˚C and then grown for 3.5 days in 24 hr dark.
For the triple response assay, seeds were germinated on MS supplemented with ACC (Sigma Aldrich) at the reported concentrations. Hypocotyl lengths were measured by removing seedlings from MS or MS + ACC plates with forceps and laying the seedlings out on a black cloth alongside a ruler. All seedlings comprising each data point were laid out together and photographed. Once all of the photos were taken, the ImageJ software was used for measuring the hypocotyl region (from the bottom of the hypocotyl region, just above where the root begins to the tip of the apical hook (Figure 3-2a).

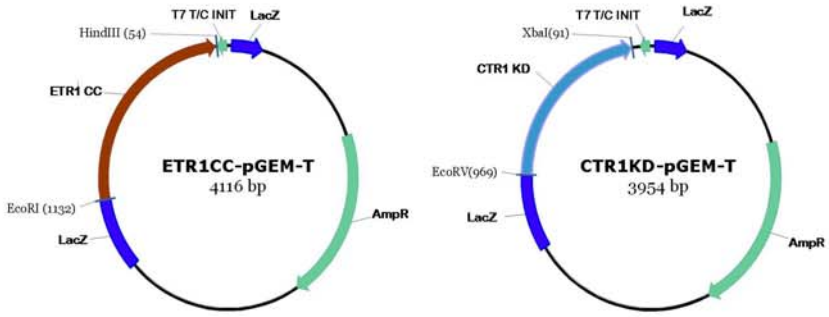
**Plant Transformation Constructs**

For expression of ETR1-CTR1 fusion proteins, both full length ETR1 cDNA and full length CTR1 cDNA each previously cloned into the pGEM-T vector (Promega,

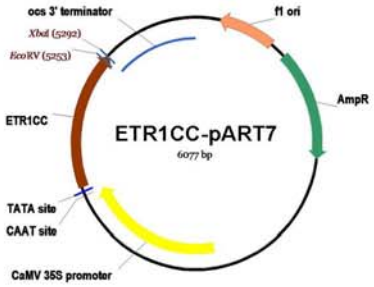
Madison, WI) were used as template. The truncated ETR1$^{1-349}$ was amplified with the following primers: 5' primer-gaattcatggaagtctgcaattgtattgaaccg and 3'primer aagcttgatatcggcacggattgctgtttctgc, which generated *Eco*RI and *Eco*RV-*Hin*dIII restriction site respectively. CTR1 full length was amplified using 5'primer gatatcatggaaatgcccggtaga and 3' primer tctagattacaaatccgagcggttgg, and the CTR1 kinase region$^{533-821}$ using 5' primer gatatcgcaaatagggaacttggac and the same 3' primer as CTR1 full length, generating *Eco*RV and *Xba*I sites, respectively. The ETR1 truncation was cloned into the pART7 vector [108] using *Eco*RI and *Hin*dIII restriction digest. Using the *Eco*RV and *Xba*I restriction sites, the CTR1 fragment (either full length or kinase domain) was cloned into the ETR1$^{1-349}$-pART7 vector. The 35S:ETR1$^{1-349}$-CTR1 fragment was digested out of the pART7 plasmid using *Not*I sites flanking the promoter and MCS and cloned into the pmlBart vector [108] for transformation into agrobacterium strain GV3101. (See figure 3-10 for diagram of cloning procedures). The Quick Change Site-direct Mutagenesis kit (Stratagene, La Jolla, California) was used to introduce the *ctr1-1* mutation into the fusions. The mutagenic primer set used to generate the *ctr1-1* mutation was 5'-acagtcaaggtttgtgaatttggtctctcgcgattgaag and 3'-cttcaatcgcgagagaccaaattcacaaaccttgactgt.

# FIGURE 3-10

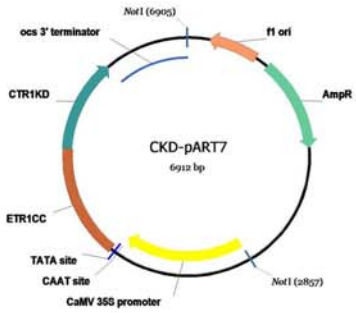
1. Using T/A cloning, ligate ETR1 into pGEM-T and CTR1 into pGEM-T




2. Using EcoRI and HindIII restriction enzymes digest the ETR1CC fragment out of the ETR1CC-pGEM-T vector. Linearize the pART7 vector, carrying the 35SCaMV promoter with EcoRI and HindIII. Ligate the ETR1CC fragment into the pART7 vector.




3. Using EcoRV and XbaI restriction enzymes cut the CTR1KD fragment out of the CTR1KD-pGEM-T vector. Linearize the ETR1CC-pART7 vector with the EcoRV and XbaI enzymes. Ligate the CTR1KD into the ETR1CC-pART7 vector.




4. Using the NotI restriction enzyme cut the CaMV 35S promoter-ETR1CC-CTR1KD fragment out of the CKD-pART7 vector and linearize the plant transformation vector, pmlBart. Ligate the fusion construct into pmlBart (not shown.)

**Figure 3-10:** Cloning scheme used for construction of the ETR1-CTR1 fusions. The ETR1 and CTR1 cDNA templates used for the initial amplification were a gift from Dr. Jason Shockey. In the ETR1CC-containing constructs, "CC" is short for "ETR1 coiled coil" region and represents ETR1 the cDNA coding for ETR1 residues 1-349. In ETR1N-containing constructs (not shown) "N" is short for ETR1 N-terminal region, encoding ETR1 residues 1-106. Both ETR1 truncations were amplified with primers containing a 5'-*Eco*RI and 3'-*Hin*dIII restriction sites for digestion out of pGEM-T and ligation into the pART7 vector, which contains the Cauliflower Mosaic Virus (CaMV) 35S promoter. In addition, an *Eco*RV restriction site was added to the 3' primer, between the ETR1 fragment and the *Hin*dIII site. Both CTR1 KD and CTR1 FL (not shown) were amplified with primers containing *Eco*RV and *Xba*I restriction sites. The CTR1 fragment was digested out of pGEM-T and ligated into both the ETR1CC-pART7 vector and the ETR1N-pART7 vector (not shown). The ETR1-pART7 vectors were linearized with the *Eco*RV enzyme (restriction site contained within the ETR1 primer) and *Xba*I (restriction site within the pART7 MCS.) These four constructs were labeled as follows:

CKD-pART7 = ETR1**C**C$^{(1\text{-}349)}$-CTR1**KD**-pART7

CFL-pART7 = ETR1**C**C$^{(1\text{-}349)}$-CTR1**FL**-pART7

NKD-pART7 = ETR1**N**$^{(1\text{-}106)}$-CTR1**KD**-pART7

NFL-pART7 = ETR1**N**$^{(1\text{-}106)}$-CTR1**FL**-pART7

The constructs (including the CaMV 35S promoter) were then digested out of pART7 with *Not*I enzyme and ligated into the plant transformation vector, pmlBart.

# Chapter 4: Conclusions and Perspectives

When I began this work, the model was that the novel protein AWE1 was involved in facilitating the interaction between ETR1 and CTR1, and that the CTR1 amino-terminal region auto-inhibits CTR1 kinase activity upon ethylene binding. Instead of placing AWE1 in the ETR1 to CTR1 signal relay, my work may have revealed a new trail in what is turning out to be quite a complex signaling pathway.

ETR1's well-documented functions include signaling to CTR1 and the other receptors and perceiving ethylene [17]. My investigation of AWE1 suggests that ETR1 could possibly have a role in a chloroplast-specific pathway through its direct interaction with the AWE1 protein, which localizes to the chloroplast and cytosol. Homologs of AWE1 and the ethylene-binding domain of ETR1 are found in cyanobacteria, mosses, and plants but have not been identified in green algae, which could indicate an ancient and highly conserved interaction. The EBD-homolog in the cyanobacteria *Synechocystis* has been shown to bind ethylene [24], and these results open the possibility that there is a CTR1-independent ethylene signaling pathway in cyanobacteria that includes AWE1 and ETR1. Such a putative pathway might have been retained over time.

Alternatively, AWE1 may function in hypocotyl elongation and cell expansion in an ethylene-independent manner. The *awe1-1* mutant could not suppress the ethylene-insensitive *etr1-1* or *ein2-1* long hypocotyls, and loss of *awe1* did not result in ethylene-induced premature leaf senescence as observed in the ethylene-hypersensitive mutant *etr1*-7. The results collectively suggest AWE1 functions through ethylene-dependent and ethylene-independent pathway.

In the future, confirming the protein interactions between ETR1 and AWE1 in *Arabidopsis* and analyzing the sub-cellular localization of over-expressed *AWE1* in the *etr1-7* background might be useful to elucidate any potential role ETR1 may have in the signaling of AWE1 (which is sub-cellularly localized to the chloroplasts). Testing for protein-protein interactions between AWE1 and other histidine kinases would also be useful in determining if the AWE1 and ETR1 interaction is specific or a consequence of ETR1 being a histidine kinase.

Additionally, looking at ethylene-induced senescence of wild-type lines over-expressing *AWE1*, ETR1-AWE1 and CTR1-AWE1 interactions in *Arabidopsis* cells, and AWE1 protein levels and sub-cellular localization in the presence or absence of ethylene may help to clarify the role of AWE1 in ethylene signal transduction or a lack of a role in ethylene signaling. If the FFC protein modification identified by Wang et al.(2009) through 2-D DIGE analysis is determined to be real, then testing for AWE1 interaction with FFC, FFC interaction with ETR1, and potential localization patterns of FFC in the presence or absence of ethylene compared to AWE1 sub-cellular localization may begin to build a backbone for an ethylene-regulated chloroplast signaling pathway.

Brief literature searches reveal that *awe1* mutants may have a similar rosette phenotype to that of the *ffc* mutant. FFC is part of the chloroplast signal recognition particle that is recognized the FtsY receptor [109]. It will be interesting to determine how ethylene may alter the FFC protein, possibly through AWE1. Alternatively, AWE1 may have ethylene-independent functions in chloroplasts targeting, based on the similar rosette phenotype of the *awe1* and *ffc* mutants.

AWE1 is likely not a component helping ETR1 to regulate CTR1; however, there are likely additional proteins that aid in the regulation of CTR1. I investigated a potential role of the CTR1 amino-terminal region in both CTR1's sub-cellular localization, through interaction with the ETR1 receptor, and in CTR1 kinase regulation based on both the ability of the CTR1 amino-terminal region to associate with the CTR1 kinase domain *in vitro* and models of the regulation of the CTR1-homolog Raf-1. I found that additional proteins are likely required for CTR1 regulation in addition to the potential role of the CTR1 amino-terminal region. This is not completely surprising as there seem to be many unidentified proteins in the ETR1 complex (GE Schaller, personal communication), and many uncharacterized ETR1- and CTR1-interactors have been isolated from yeast-two-hybrid screens (Ding, 2004 thesis).

The PP2A protein phosphatase was isolated in a screen for mutants that display enhanced ethylene sensitivity [57] and has been shown to interact with ETR1 and CTR1 *in vitro*. Various 14-3-3 proteins have been shown to interact with CTR1 *in vivo*. These proteins are good candidates for functioning in the regulation of CTR1, as both phosphatases and 14-3-3 proteins have functions in regulating the CTR1 kinase-homolog, Raf. Our lab is currently collaborating with a lab at USDA to determine what proteins associate with CTR1 through CTR1 immunoprecipitation followed by mass spectrometry analysis. This will hopefully shed some insight on the additional proteins required for CTR1 regulation. Secondly, a split luciferase system has been developed for monitoring changes in protein-protein interactions in *Arabidopsis* [107]. This system will be useful for testing the dynamics of the

interaction between the CTR1 amino-terminal region with the ETR1 receptor *in planta* and add to or detract from a model in which the receptor and CTR1 dissociate upon the receptor binding ethylene.

When my work began, the model was that the novel protein AWE1 was required for helping the interaction between ETR1 and CTR1, and that the CTR1 amino-terminal region auto-inhibits CTR1 kinase activity upon ethylene binding. Through this investigation my work has shown that AWE1's function is likely not to aid in regulation of CTR1, but that there are additional proteins required for CTR1 regulation. This work has opened more questions about ethylene signal transduction, its potential roles in chloroplast development and what proteins are required for proper regulation of CTR1. We are only beginning to learn how complicated ethylene signal transduction truly is.

## Appendix A: Screening for Suppressors of *etr1-2*

Multiple lines of evidence suggest that there are additional, unidentified components of the ethylene signal transduction pathway. However, traditional genetic screens for both *Arabidopsis* ethylene-insensitive and constitutive ethylene response mutants have been saturated. (Over 40 *ein2* ethylene-insensitive mutant alleles and 9 *ctr1* constitutive ethylene response alleles have been identified to date [33, 42].

Alternative genetic approaches must be used to identify additional, novel components of the pathway. The ethylene receptor ETR1 is the receptor thought to play the predominant role in ethylene signaling. Knocking out the ETR1 receptor will yield a mutant plant that is hypersensitive to ethylene. An *ers1* null is the only other known receptor null to have a hypersensitive ethylene-response phenotype [28]. ETR1 and EIN4 are the only *Arabidopsis* receptors whose expression levels are not induced by ethylene but have a constant low-level of expression [16]. We focused on the ETR1 receptor, taking a forward genetics approach to screen for suppressors of the ethylene-insensitive mutant, *etr1-2*. *etr1-2* encodes an A102T amino acid substitution, which impairs the ETR1 receptor's ability to deactivate its signal once ethylene binds and confers ethylene-insensitivity in the *etr1-2* mutant plant [31]. We chose the *etr1-2* mutant over *etr1-1*, the first *etr1* ethylene-insensitive allele isolated [15], because *etr1-2* respond*s* slightly to exogenous ethylene; therefore, we could screen for both enhancers and suppressors of the *etr1-2* ethylene insensitivity. *rte1* (*reversion-to-ethylene sensitivity1*) was the first mutant isolated from an *etr1-2*

suppressor screen and found to encode a novel regulator of the ETR1 receptor *rte1* [31].

In the screens for *etr1-2* suppressors, we would focus on suppressors that behaved similarly to wild type, responding to exogenous ethylene but not displaying constitutive responses. Constitutive response screens have been saturated with *ctr1* mutants and *CTR1* acts downstream of *ETR1* making it likely that any constitutive response mutants isolated from the screen would be new *ctr1* alleles. Because we were interested in a wild-type-like phenotype, avoiding contamination of the seed stock was extremely critical for this screen.

I grew up approximately 5,000 *etr1-2* plants in an isolated green house in order to accumulate a stock of *etr1-2* seeds for mutagenesis while avoiding cross-pollination from other Arabidopsis genotypes. This *etr1-2* parental line carried a recessive *glabrous1* (*glb1*) mutant allele which inhibits trichome development on rosette leaves [110] and would allow for the easy identification of any contaminating seeds within the population. I collected the seeds from these $M_0$ plants and mutagenized approximately 10,000 (200 mg) of the $M_1$ seeds with 0.2% ethyl methanesulfonate (EMS). (See EMS mutagenesis protocol in Figure 1a.) EMS is an alkylating agent that most commonly causes alkylation of guanine's which makes O6-ethylglycine, a molecule that can pair with thymine but not cytosine so after DNA replication a G/C in the DNA is replaced with an A/T transition [111].

The 10,000 $M_1$ mutagenized seeds were planted in 8 flats (approximately 125 seeds per pot) and grown in 20 hours light and 4 hours darkness. Between 50 and 60% of the seeds germinated as expected due to the creation of random lethal

mutations in the seeds. Flat 8 was discarded because some of the plants had trichomes (indicative of seed contamination.) The $M_2$ seeds from each of the remaining 7 flats were collected. (Each flat of seeds was collected separately and labeled as batch 1, 2, 3, 4, 5, 6, and 7.) This system provided a more simplistic version of the optimal assay, collecting and screening seeds from each of the ~5000 $M_1$ plants individually, while at the same time allowing a degree of organization. With this system, we could return to a particular batch of seeds if we lost a mutant and needed to identify a sibling and if two mutants were allelic but from separate batches, we knew that the mutants were the result of independent mutagenesis events. To date, one *etr1* intragenic suppressor, *etr1-10*, and four additional extragenic suppressors have been isolated and mapped (or are currently being mapped.) None of these four candidate suppressors are allelic to *rte1* (personal communication), the first suppressor identified from such a screen, suggesting that this screen is not saturated and additional candidate suppressors may be isolated from these seed stocks.

# FIGURE A-1

A

1. Weigh 200 mg of seeds
2. Wash seeds in 0.1% Tween 20 solution
   A. Add 2 µL Tween 20; 1.998 mL $H_20$ and wash in 15 mL centrifuge tube
   B. Pour off the Tween 20 solution
3. Add 15 mL $H_2O$ (from tap) to the centrifuge tube
4. Add 33 µL 100% EMS to the 15 mL tube
5. Rotate for 12 hours at room temperature in a fume hood
6. Rinse once with tap $H_20$ and pour off the $H_20$
7. Rinse with new tap $H_20$ for 6 hours
8. Discard wash and add the mutagenized seeds to 0.1% agar for planting
9. Soak all EMS-contaminated materials in 0.5 M NaOH for deactivation of EMS activity.

B

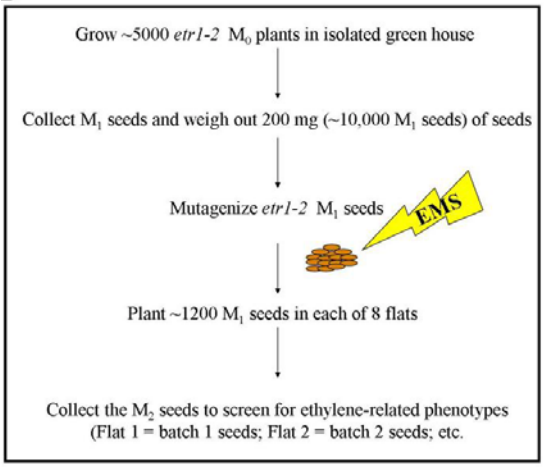



**Figure A-1:** Obtaining a mutagenized *etr1-2* seed stock to screen for suppressors of the ethylene-insensitive *etr1-2* mutant.

**A**. The EMS mutagenesis protocol (adapted from Z. Liu.) used to mutagenize a population of *etr1-2* seeds for a genetic screen to identify *etr1-2* suppressors.

**B.** Growing up and collecting a mutagenized *etr1-2* seed stock.

**Appendix B: Additional Characterization of *AWE1***

# **FIGURE B-1:** Testing for Premature Senescence of *awe1-1*

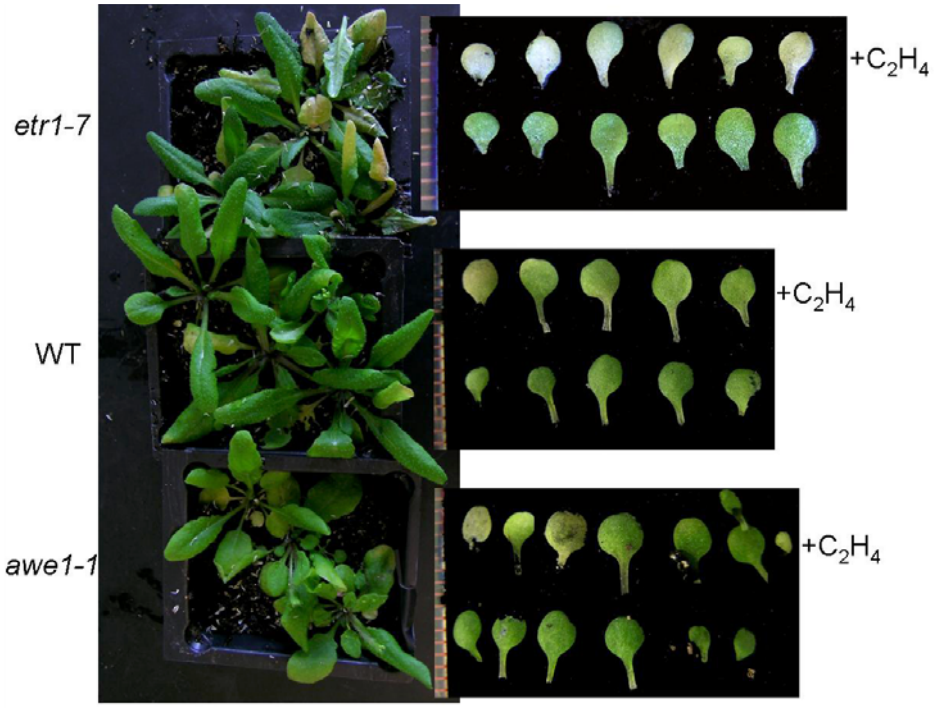



**Figure B-1:** Because the *awe1-1* mutant had a rosette phenotype I tested for hyper-senescence of the mutant plant when grown in the presence of exogenous ethylene. *awe1-1* seedlings were grown alongside *etr1-7* and wild-type seedlings. Fourteen-da old seedlings were treated with ~100 ppm ethylene, and three days later the cotyledo were detached and photographed. Unlike *etr1-7* which displayed complete senescen of the cotyledons as evident by the yellowing, chlorotic tissue, the *awe1-1* mutants d not appear to prematurely senesce (photos on the right). The same was observed of adult plants treated with exogenous ethylene (photos on the left.)

# **FIGURE B-2:** *awe1-2* and *awe1-3* Seedling Dose Response Analysis

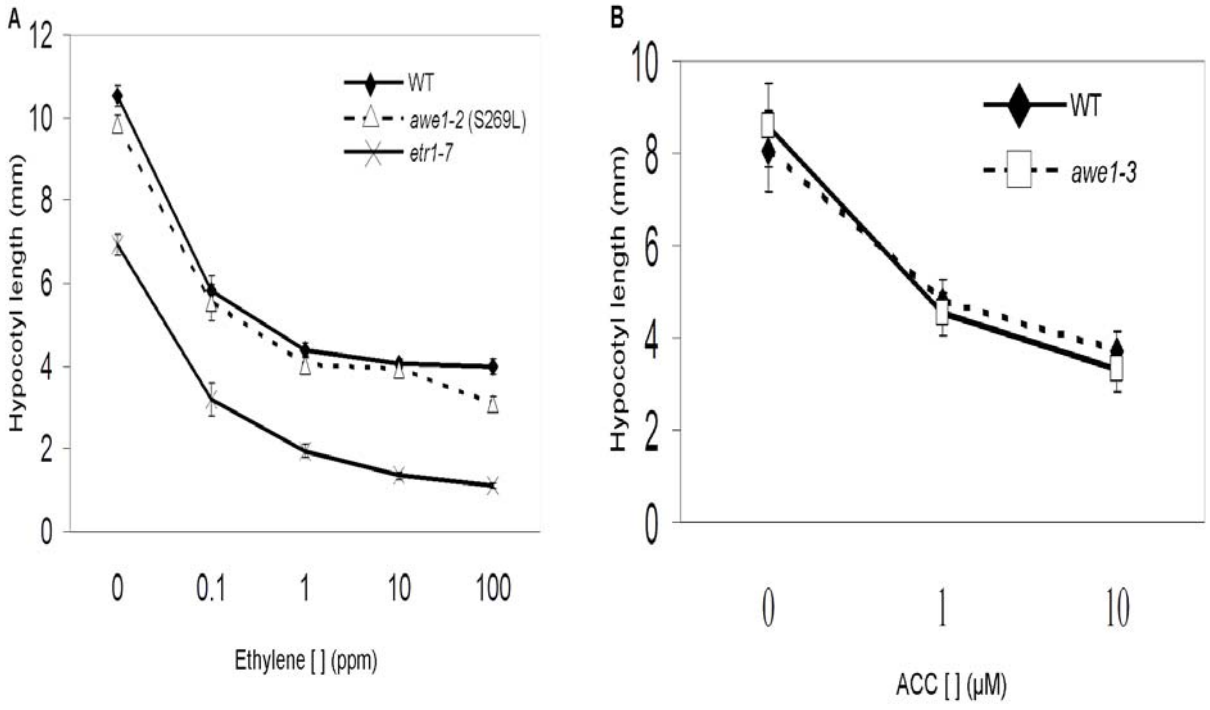



**Figure B-2:** Seedling dose analysis of *awe1-2* and *awe1-3*, both of which were back-crossed once to wild type plants. **A.** *awe1-2* mutant line, which is a tilling line encoding an S269L amino acid change in a conserved serine residue, responded similarly to wild type when grown in the dark in the presence of ethylene. Error bars represent standard error of the mean for n≥22 seedlings. **B.** The *awe1-3* mutant line, which is a SALK T-DNA insertion in the 3-‘ UTR of *AWE1*, responded similarly to wild type when grown on medium containing ACC. Error bars represent standard deviation for n = 15 seedlings.

# **FIGURE B-3:** Over-expression of *AWE1* in Wild-type Backgrounds

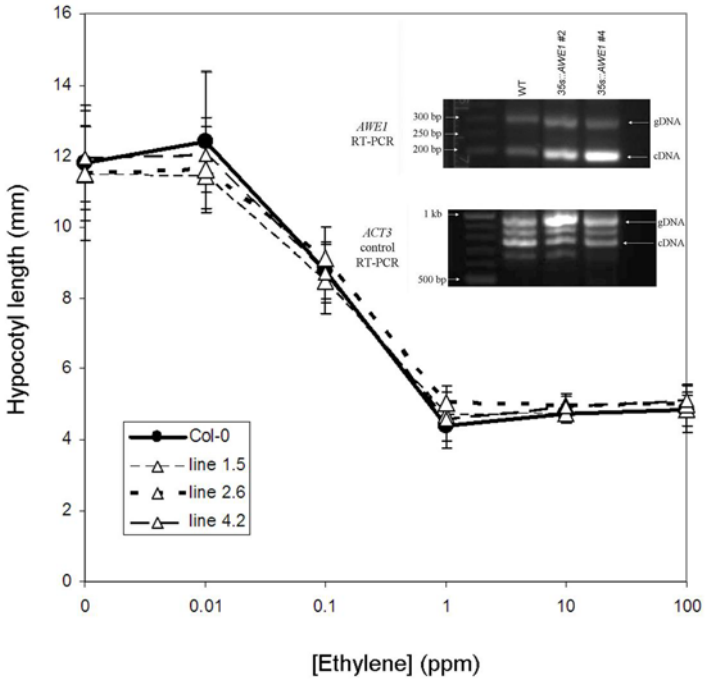



**Figure B-3:** Three independent T3 homozygous wild-type lines over-expressing *AWE1* were measured in a seedling dose response assay over increasing concentrations of exogenous ethylene. The transgenic lines did not appear to be ethylene-insensitive (have longer hypocotyls than the wild-type, untransformed, line.) Multiple RT-PCR's of RNA extracted from single rosettes for lines 2 and 4 showed increased *AWE1* transcript compared to the wild type. However, I could not eliminate the "mystery" band in the *ACTIN3* control, which may be a hybrid forming between genomic and cDNA PCR products. Error bars represent standard deviation of n = 10 seedlings.

# **FIGURE B-4:** Chloroplasts Analysis of *awe1-1* Mutant

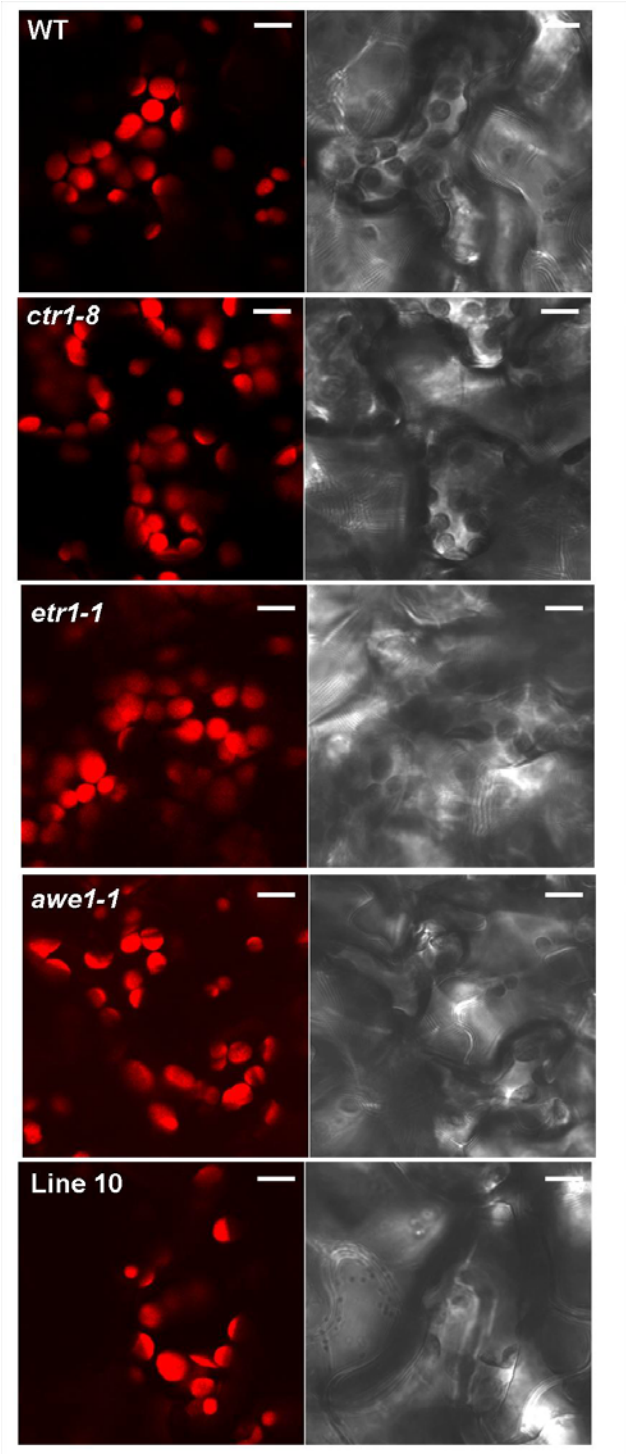



**Figure B-4:** I analyzed the abaxial surface of the most newly formed leaves of *awe1-1* mutants and observed that the chloroplasts of the mutant plants were slightly larger than wild-type or *awe1-1* transgenic lines over-expressing *AWE1*. I then analyzed the abaxial surface of the oldest leaf from the same plants and observed that the chloroplasts of these leaves were similar in the *awe1-1* and wild-type backgrounds.

## Appendix C: Investigating the Nature of the ETR1-CTR1 Interactions

Our lab's current working model of the interaction between the ethylene receptor ETR1 and the protein kinase CTR1 is that once ETR1 binds ethylene, it loses the interaction with CTR1 (possibly due to a conformational change) (Figure C-1.) This allows CTR1 to stop signaling, potentially by freeing the CTR1 amino-terminal region so that it can now associate with the CTR1 kinase domain (Figure C-1). There are a couple of lines of evidence providing support for such a model. Unlike CTR1, the CTR1-8 protein (G354E) cannot interact with the ETR1 ethylene receptor (Figure C-1). CTR1-8 is sub-celluarly localized to the cytosol while wild-type CTR1 peripherally associates with the receptors at the endomembrane system [36]. Although the CTR1-8 protein displays serine/threonine kinase activity similar to wild-type CTR1 *in vitro*, the *ctr1-8* mutant plant displays constitutive ethylene responses [33]. Also, the CTR1-8 amino-terminal region can associate with the CTR1 kinase domain *in vitro* similar to the wild type CTR1 amino-terminal region (Shockey and Chang unpublished.) The constitutive ethylene responses seen in the *ctr1-8* mutant are likely due to the protein's inability to associate with the receptors, and perhaps because the CTR1-8 amino-terminal region is free to constitutively associate with the CTR1 kinase domain. This suggests a model in which the receptors and CTR1 are dissociated when ethylene responses occur in the plant (Figure C-1). Dissociation of the CTR1 amino-terminal region from the receptors could allow the CTR1 amino-terminal to bind to the CTR1 kinase domain, inhibiting kinase activity. The ability of the CTR1 amino-terminal and CTR1 kinase domain to

interact *in vitro* supports such a model. Finally, this model is similar to the model of Raf kinase regulation in mammalian cells.

I wanted to test this proposed model that upon ethylene binding to the ETR1 receptor, the ETR1 and CTR1 physical interaction dissociates. Based on previous studies, yeast seemed like a simple and quick system to use in order to test this model. It was shown previously that the soluble portion of ETR1 can interact with the amino-terminal region of CTR1 in yeast [34] and that the full-length ETR1 receptor can bind ethylene when expressed in yeast [20]. For this experiment I needed to express the full length ETR1 receptor, because the transmembrane region of ETR1 is the ethylene-binding domain and the soluble portion of ETR1 is the region that associates with CTR1. I could not express full length ETR1 in the traditional yeast-two-hybrid assay utilized by Clark et al. because the yeast-two-hybrid assay requires that protein-protein interactions occur in the nucleus in order to activate the reporter [110] which would mean expressing only the soluble portion of ETR1. Therefore I used the yeast split ubiquitin assay which bypasses the nuclear-localization requirement of the yeast-two-hybrid assay [110]. In this split ubiquitin assay if the bait and prey proteins interact in yeast, the yeast cells will grow on drop out plates containing 5-'FOA, which is toxic if taken up by yeast cells. (See Figure C-2 for a description of the system) [109].

To test the ETR1 and CTR1 interaction, I cloned *ETR1* and *CTR1* each into the Gateway destination bait and prey vectors, pMKZ and pMyc-GWY-NubI respectively (Figure C-3a). First I tested expression of each bait in the *Sacchromyce cereviseae* strain JD53. If the bait, which is fused to URA3 is expressed, the

transformed yeast will grow on media lacking uracil. Additionally, expression of the bait is driven by the $P_{MET}$ promoter, which is repressed by increasing concentrations of methionine. I wanted to find a concentration of methionine that would ensure URA3 was being expressed without over-expressing the bait. Over-accumulation of the bait protein relative to the prey can generate false negative results in the screen in the following way: some bait proteins may have no prey-interaction partner. If the URA3 reporter, fused to the carboxy-terminal of the bait and degraded upon bait and prey interaction, remains in the cell because too many bait proteins are present, the yeast cells would die when grown on 5'-FOA, resulting in false negative results. I found that the yeast transformed with the bait plasmid would grow on media lacking uracil and containing 100 μM methionine (Figure C-3b.) To test for protein-protein interactions I used dropout plates that contained 100 μM methionine, 30 μM $CuCl_2$ (the prey is driven by a Cu-inducible promoter), and 5-FOA. Surprisingly we did not see the well-documented ETR1 and CTR1 interactions using this system. Therefore we could not complete the assay. I tested ETR1 as the bait and the CTR1 amino-terminal region (residues 1-550) as the prey and vice versa. In addition, as another positive control, I tested for ETR1-ETR1 interactions. None of the yeast grew well on the selective media (Figures C-3c,d,e and data not shown). Other labs have reported difficulties in expressing ETR1 in this system (personal communication). Having clones that are known to interact in this system would have been useful to ensure that I had set the system up correctly.

Therefore we decided to change approaches and use an *in planta* split YFP technique in plant cells to test the hypothesis that ETR1 and CTR1 lose their

association in the presence of ethylene. The YELLOW FLUORESCENT PROTEIN (YFP) is split into carboxy-terminal and amino-terminal halves (cYFP and nYFP, respectively) [80]. These halves are fused to the proteins of interest. If the proteins interact in the plant, then the YFP halves come together and yellow fluorescence is observed. Again using Invitrogen Gateway cloning, I cloned ETR1 into the pSPYNE-35S vector in which the nYFP is fused to the carboxy-terminal of ETR1. Similarly, I cloned $CTR1^{1-550}$ into the pSPYCE-35S vector in which the cYFP is fused to the carboxy-terminal of CTR1 (Figure 3-4a). I transformed both of these constructs and the p19 helper plasmid into the *C85C1* agrobacterium strain. (Each construct was transformed into the C85C1 strain independently, because all three vectors confer Kanamycin resistance.) Then I infiltrated the abaxial surface of two week old tobacco plants with all 3 transformed agrobacterium lines. (I used tobacco plants instead of *Arabidopsis* for three reasons. Tobacco epidermal cells are larger than *Arabidopsis* epidermal cells. When searching for individual epidermal cells displaying yellow fluorescence I can scan tobacco leaf surfaces using a 10X magnification while I need at least 40X magnification to look at Arabidopsis individual epidermal cells. Secondly infiltration of tobacco leaves take less time and requires no special equipment unlike the gene gun used for particle bombardment in *Arabidopsis,* which is the technique used to transiently express proteins in *Arabidopsis* plants. Finally, the tobacco plants stay intact, unlike making protoplasts from *Arabidopsis,* the other way to transiently express proteins in *Arabidopsis*.) I then looked for YFP expression three days later. A weak YFP signal was detected above background levels (Figure C-4b, 4c). However it turns out that YFP is a stable

protein that is not degraded easily once produced in plants. Therefore, we could not test for dynamic interactions between ETR1 and CTR1 using this system. Recently the split luciferase system has been developed and made available for use in Arabidopsis [105]. This system might be the ideal system to test our model of ETR1 and CTR1 interaction *in vivo*. Visualization in the split luciferase system requires constant presence of the substrate for activation, which is unique to the system and potentially a useful tool for monitoring conditional protein-protein interactions *in planta*.

# FIGURE C-1

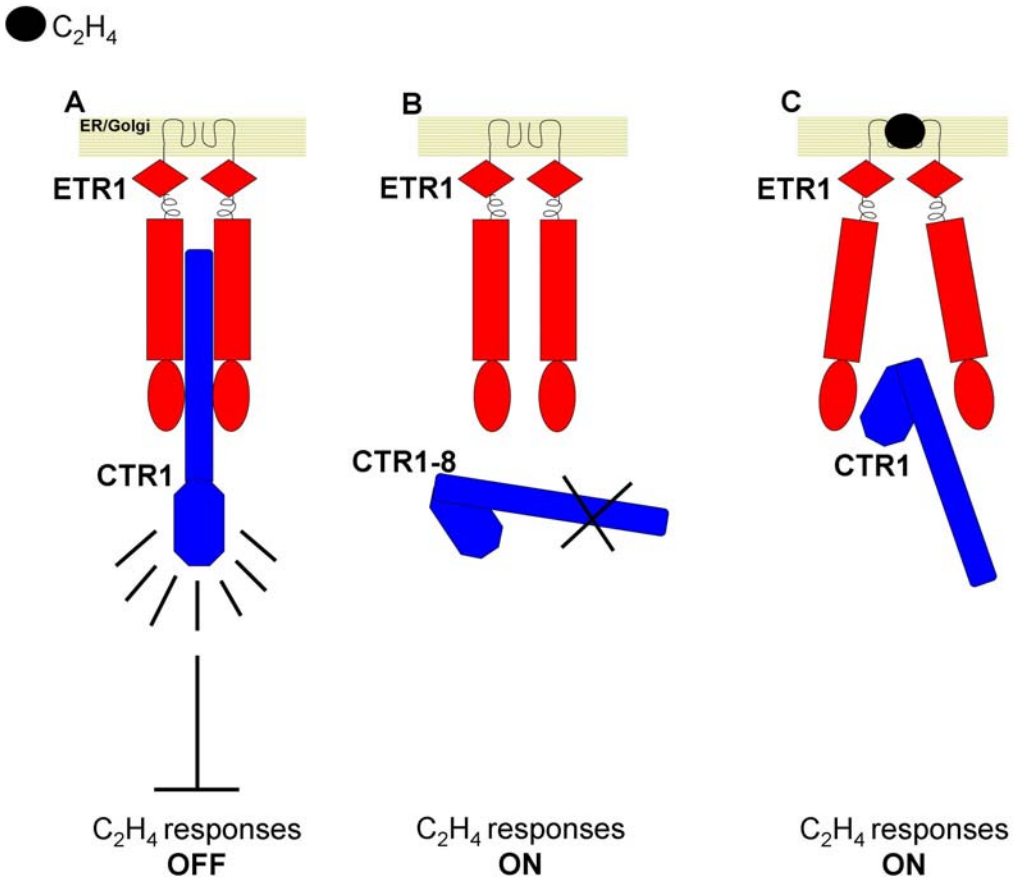
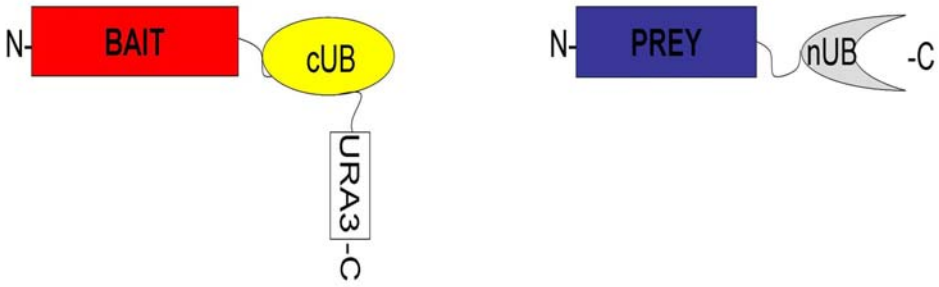



**Figure C-1**: Model of CTR1 regulation. **A**. The ethylene receptors ETR1 and ERS1 (not shown) interact with CTR1. In the absence of ethylene, the receptors and CTR1 actively repress ethylene responses. **B**. The CTR1-8 protein cannot interact with the ETR1 and ERS1 receptors and localizes to the cytosol. This mutation interferes with the ability of CTR1 to repress downstream ethylene responses, causing constitutive ethylene responses even in the absence of ethylene. **C**. Our model of CTR1 regulation once ethylene binds to the receptors. I propose that once the receptors bind ethylene they undergo a conformational change that causes dissociation with CTR1. Furthermore we propose that dissociation with the receptor would allow the CTR1 amino-terminal region to interact with the CTR1 kinase domain, inhibiting kinase activity.

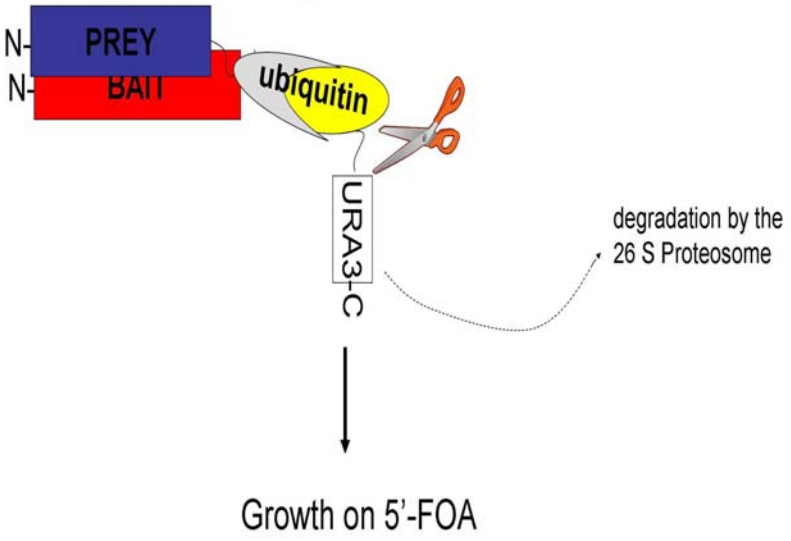
# FIGURE C-2

(The split ubiquitin system modified from Reichel and Johnsson. [111])

1. The bait protein has a C-terminal fusion consisting of the C-terminal portion of a ubiquitin molecule followed by the URA3 marker. The prey protein is fused to the N-terminal half of ubiquitin.




2. After co-transformation and selection of yeast carrying the bait and prey vectors, the yeast are plated on dropout media containing 5-'FOA. If the bait and prey protein interact in the cell, the two halves of the ubiquitin protein can come together. The newly formed ubiquitin molecule recruits a ubiquitin-specific protease, which cleave the 3'-URA3 and targets URA3 for degradation via the 26S Proteosome.




3. Only cells lacking the URA3 protein can survive when plated on 5'-FOA. URA3 converts 5-'FOA into 5-fluorouracil, which is toxic to yeast .

# FIGURE C-3

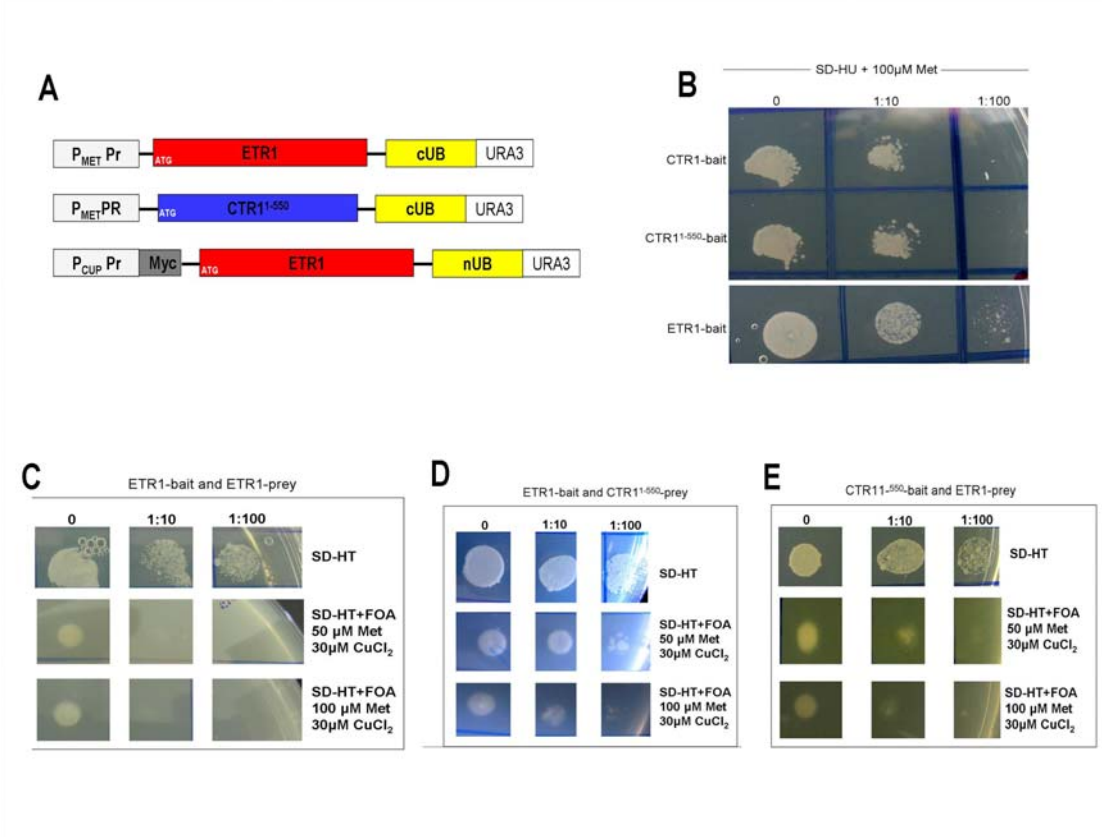

A
P MET Pr
ATG
ETR1
cUB
URA3
P MET PR
ATG
CTR1 1-550
cUB
URA3
P CUP Pr
Myc
ATG
ETR1
nUB
URA3
B
SD-HU + 100µM Met
0
1:10
1:100
CTR1-bait
CTR1 1-550-bait
ETR1-bait
C
ETR1-bait and ETR1-prey
0
1:10
1:100
SD-HT
SD-HT+FOA
50 µM Met
30µM CuCl2
SD-HT+FOA
100 µM Met
30µM CuCl2
D
ETR1-bait and CTR1 1-550-prey
0
1:10
1:100
SD-HT
SD-HT+FOA
50 µM Met
30µM CuCl2
SD-HT+FOA
100 µM Met
30µM CuCl2
E
CTR11-550-bait and ETR1-prey
0
1:10
1:100
SD-HT
SD-HT+FOA
50 µM Met
30µM CuCl2
SD-HT+FOA
100 µM Met
30µM CuCl2

**Figure C-3:** Confirming ETR1 and CTR1 interaction in yeast. **A.** Full-length ETR1 and $CTR1^{1-550}$ were cloned into the Gateway destination vectors, pMKZ (bait vector), and Myc-Gwy-NubI (prey vector) using homologous recombination. ETR1 was previously cloned into the Gateway entry vector pDONR221, and I cloned CTR1 (residues 1-550) into the pDONR221 entry vector using the Invitrogen BP clonase II enzyme and the following primers: 5' primer – acaagtttgtacaaaaaagcaggctctccaaccaccatggaaatgcccggtagaag and 3' primer – tccgccaccaccaaccactttgtacaagaaagctgggtacaaatccgagcggttggggc. The recombination site within each primer that is essential for homologous recombination, attB1 and attB2 respectively, is underlined. After cloning into the entry, pDONR221 vector, I used the Invitrogen LR clonase II enzyme to clone each into the final (destination) vector, the pMKZ bait vector or the Myc-GWY-NubI prey vector. In the figure, the L/R homologous recombination sites are represented by the black lines, one after the promoter and one prior to the carboxy terminal cUB/nUB fusion. After obtaining these clones I transformed the bait constructs directly into the JD53 yeast strain using the 1-step yeast transformation protocol. **B.** To test expression of the bait vectors in yeast I made dilutions of two-day old overnight yeast cultures (grown in SD-H liquid media for selection of the bait plasmid), spotted 5 μL of each dilution on plates containing SD-H, -U, + 100 μM Met (which reduces the expression of the bait) and tested for growth. All baits grew, suggesting that the URA3-fusions were being expressed in the yeast. **C.** Unlike in other systems, the ETR1 bait could not interact with ETR1 prey. I transformed the ETR1-nUB prey vector into the JD53 yeast expressing the ETR1 bait and selected for transformants on SD –H –T media. Then I screened for interaction on SD –H –T + FOA + Met plates. **D and E.** Interactions between ETR1 and CTR1 were not above background levels (not shown). The system could not be used for testing our model in Figure C-1.

# FIGURE C-4

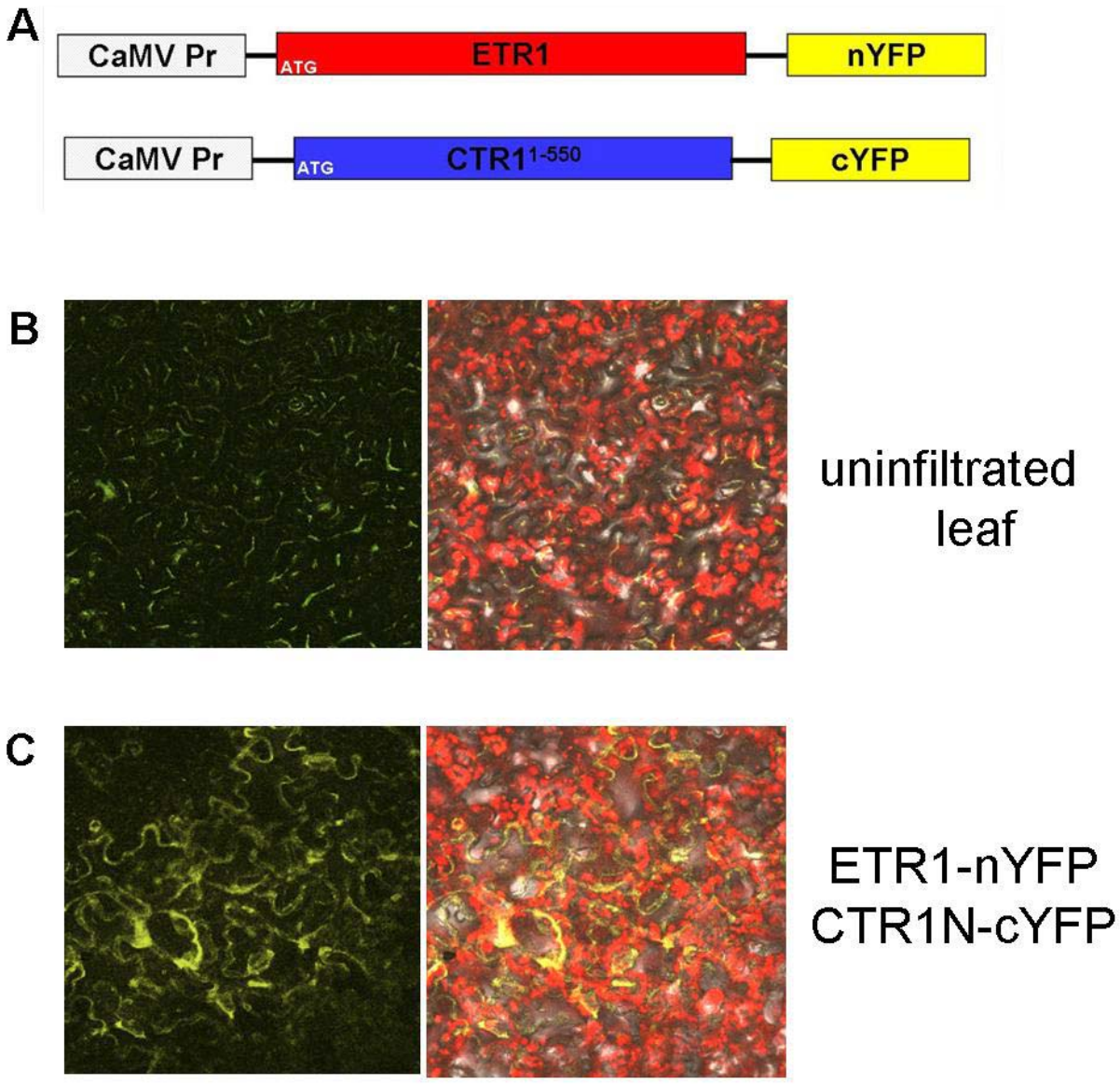

A
CaMV Pr
ATG
ETR1
nYFP
CaMV Pr
ATG
CTR1 1-550
cYFP
B
uninfiltrated leaf
C
ETR1-nYFP
CTR1N-cYFP

**Figure C-4**: ETR1 and CTR1 interact *in vivo*. **A.** ETR1 and CTR1$^{1-550}$ were cloned into the pSPYNE-35S and pSPYCE35S Gateway destination vectors respectively, using the gateway cloning system. The homologous recombination sites, between the promoter and gene and between the gene and YFP fusions are indicated by black lines. nYFP = the amino-terminal region of YFP and cYFP = the caboxy-terminal region of YFP. **B and C.** The ETR1-pSPYNE35S and CTR1$^{1-550}$-pSPYCE35S constructs, along with the p19 helper plasmid, were infilitrated in the leaves of 2 week old tobacco plants. The photos above, taken with the 10X objective lens, are of abaxial leaf surfaces three days post infiltration. The left panel is the YFP channel, and the right panel is overlay of the YFP channel, chlorophyll autofluorescence channel (red), and bright field image. **B.** uninfiltrated leaf showing background levels of yellow fluorescence. **C.** Tobacco leaves infiltrated with ETR1-nFYP and CTR1-cYFP.

# Bibliography


1. Cook JP. (1882) *Principles of Chemical Philosophy.* Oxford: J Allyn, p. 623.

2. Worrell E, Phylipsen D, Einstein D, and N Martin. (2000) *Energy Use and Energy Intensity of the U.S. Chemical Industry.* University of California Energy Analysis Department, Environmental Energy Technology Division: Berkley. pp. 1-40.

3. Galil J. (1968) An Ancient Technique for Ripening Sycomore Fruit in East-Mediterranean Countries. *Economic Botany* **22**(2): 178-190.

4. Saltviet M, Yang S, and W Kim. (1998) *Discoveries in Plant Biology*, ed: Y.S. Kung, Singapore: World Scientific Publishing Co. **1**: 371.

5. Foster J, and D Lehoux. (2007) The Delphic Oracle and the ethylene-intoxication hypothesis. *Clinical Toxicology* **45**(1): 85-89.

6. Denny F. (1924) Hastening the Coloration of Lemons. *Journal of Agricultural Research* **27**: 757-769.

7. Wallace R. (1926) The Production of Intumescences Upon Apple Twigs by Ethylene Gas. *Bulletin of the Torrey Botanical Club* **53**(6): 385-401.

8. Gane R. (1934) Production of Ethylene by Some Ripening Fruits. *Nature* **134:** 1008.

9. McGlasson WB, and HK Pratt. (1964) Effects of Wounding on Respiration and Ethylene Production by Cantaloupe Fruit Tissue. *Plant Physiology* **39**(1): 128-132.

10. Abeles FB, Morgan PW, and ME Saltveit Jr. (2004) *Ethylene in Plant Biology*. 2nd ed: Academic Press.

11. Wang K, Li H, and JR Ecker. (2002) Ethylene Biosynthesis and Signaling Networks. *Plant Cell* **14**: s131-s151.

12. Chung M, Chou S, Kuang L, Charng Y, and S Yang. (2002) Subcellular Localization of 1-Aminocyclopropane-1-Carboxylic Acid Oxidase in Apple Fruit. *Plant and Cell Physiology* **43**(5): 549-554.

13. Yang S, and N Hoffman. (1984) Ethylene Biosynthesis and Its Regulation in Higher Plants. *Annual Review of Plant Physiology* **35**: 155-189.

14. Bleecker AB, Estelle MA, Somerville C, and S Kende. (1988) Insensitivity to Ethylene Conferred by a Dominant Mutation in *Arabidopsis thaliana*. *Science* **241**: 1086-1089.

15. Chang C, Kwok SF, Bleecker AB, and EM Meyerowitz. (1993) *Arabidopsis* Ethylene-response Gene *ETR1*: Similarity of Product to Two-component Regulators. *Science* **262**(5133): 539-544.

16. Hua J, and E Meyerowitz. (1998) Ethylene Responses are Negatively Regulated by a Receptor Gene Family in *Arabidopsis thaliana*. *Cell* **94**: 261-271.

17. Kendrick MD and C Chang. (2008) Ethylene Signaling: New Levels of Complexity and Regulation. *Current Opinion in Plant Biology* **11**: 479-485.

18. Hirayama T, Kieber JJ, Hirayama N, Kogan M, Guzman P, Nourizadeh S, Alonso JM, Dailey WP, Dancis A, and JR Ecker. (1999) RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson Disease-Related Copper Transporter, is Required for Ethylene Signaling in *Arabidopsis*. *Cell* **97**: 383-393.

19. Rodriguez F, Esch J, Hall A, Binder B, Schaller GE, and AB Bleecker. (1999) A Copper Cofactor for the ETR1 Receptor From *Arabidopsis*. *Science* **283**: 996-998.

20. Schaller GE and AB Bleecker. (1995) Ethylene-Binding Sites Generated in Yeast Expressing the *Arabidopsis ETR1* Gene. *Science* **270**(5243): 1809-1811.

21. Dong CH, Rivarola M, Resnick JS, Maggin BD, and C Chang. (2008) Subcellular Co-localization of *Arabidopsis* RTE1 and ETR1 Supports a Regulatory Role for RTE1 in ETR1 Ethylene Signaling. *Plant Journal* **53**: 275-286.

22. Xie F, Liu Q, and CK Wen. (2006) Receptor Signal Output Mediated by the ETR1 N-terminus is Primarily Subfamily I Receptor Dependent *Plant Physiology* **142**: 492-508.

23. Hall B, Shakeel S, and GE Schaller. (2007) Ethylene Receptors: Ethylene Perception and Signal Transduction. *Journal of Plant Growth Regulation* **26**(2): 118-130.

24. Wang W, Esch JJ, Shiu SH, Agula H, Binder BM, Chang C, Patterson SE, and AB Bleecker. (2006) Identification of Important Regions for Ethylene Binding and Signaling in the Transmembrane Domain of the ETR1 Ethylene Receptor of *Arabidopsis*. *Plant Cell* **18**: 3429-3442.

25. Anantharaman V, Koonin E, and L Aravind. (2001) Regulatory Potential, Phyletic Distribution and Evolution of Ancient, Intracellular Small-Molecule-Binding-Domains. *Journal of Molecular Biology* **307**: 1271-1292.

26. Grefen C, Stadele K, Ruzicka K, Obrdlik P, Harter J, and J Horak. (2007) Subcellular Localization and *In Vivo* Interactions of the *Arabidopsis thaliana* Ethylene Receptor Family members. *Molecular Plant* **1**: 308-320.

27. Moussatche P, and H Klee. (2004) Autophosphorylation Activity of the *Arabidopsis* Ethylene Receptor Multigene Family. *Journal of Biological Chemistry* **279**(47): 48734-48741.

28. Qu X, Hall B, Gao Z, and GE Schaller. (2007) A Strong Constitutive Ethylene-response Phenotype Conferred on *Arabidopsis* Plants Containing Null Mutations in the Ethylene Receptors ETR1 and ERS1. *BMC Plant Biology* **7**: 3-18.

29. Chen YF, Shakeel Sm, Bowers J, Zhao XC, Etheridge N, and GE Schaller. (2007) Ligand-induced Degradation of the Ethylene Receptor ETR2 Through a Proteosome-dependent Pathway in *Arabidopsis*. *Journal of Biological Chemistry* **282**: 24752-24758.

30. Kevany BM, Tieman DM, Taylor MG, Dal Cin V, and HJ Klee. (2008) Ethylene Receptor Degradation Controls the Timing of Ripening in Tomato Fruit. *Plant Journal* **51**: 458-467.

31. Resnick JS, Wen CK, Shockey JA, and C Chang. (2006) *REVERSION-TO-ETHYLENE SENSITIVITY 1*, a Conserved Gene That Regulates Ethylene Receptor Function in *Arabidopsis*. *Proceedings of the National Academy of Sciences* **103**: 7917-7922.

32. Kieber J, Rothenberg M, Roman G, Feldmann A, and JR Ecker. (1993) CTR1, a Negative Regulator of the Ethylene Response Pathway in *Arabidopsis,* Encodes a Member of the Raf Family of Protein Kinases. *Cell* **72**: 427-441.

33. Huang Y, Li H, Hutchison C, Laskey J, and J Kieber. (2003) Biochemical and Functional Analysis of CTR1, a Protein Kinase that Negatively Regulates Ethylene Signaling in *Arabidopsis*. *The Plant Journal* **33**(2): 221-233.

34. Clark KL, Larsen PB, Wang X, and C Chang. (1998) Association of the *Arabidopsis* CTR1 Raf-like Kinase with the ETR1 and ERS Ethylene Receptors. *Proceedings of the National Academy of Sciences* **95**: 5401-5406.

35. Zhong S, Lin Z, and D Grierson. (2008) Tomato Ethylene Receptor-CTR1 Interactions: Visualization of NEVER-RIPE Interactions with Multiple

CTR's at the Endoplasmic Reticulum. *Journal of Experimental Botany* **59**: 965-972.

36. Gao Z, Chen YF, Randlett MD, Zhao XC, Fendell JL, Kieber JJ, and GE Schaller. (2003) Localization of the Raf-like Kinase CTR1 to the Endoplasmic Reticulum of *Arabidopsis* Through Participation in Ethylene Receptor Signaling Complexes. *Journal of Biological Chemistry* **278**: 34725-34732.

37. Ghosh S, Moore S, Bell R, and M Dush. (2003) Functional Analysis of a Phosphatidic Acid Binding Domain in Human Raf-1 Kinase: Mutations in the Phosphatidate Binding Domain Lead to Tail and Trunk Abnormalities in Developing Zebrafish Embryos. *Journal of Biological Chemistry* **278**: 45690-45696.

38. Testerink C, and T Munnik. (2005) Phosphatidic Acid: A Multifunctional Stress Signaling Lipid in Plants. *Trends in Plant Science* **10**: 368-375.

39. Testerink C, Larsen PB, van der Does D, van Himbergen J, and T Munnik. (2007) Phosphatidic Acid Binds to and Inhibits the Activity of CTR1. *Journal of Experimental Botany* **58**(14): 3905-3914.

40. Oaked F, Rozhon W, Lecourieux D, and H Hirt. (2003) MAPK Pathway Mediates Ethylene Signaling in Plants. *EMBO Journal* **22**: 1282-1288.

41. Yoo S, Cho Y, Tena G, Xiong Y, and J Sheen. (2008) Dual Control of Nuclear EIN3 by Bifurcate MAPK Cascades in C2H4 Signaling. *Nature* **451**: 789-795.

42. Alonso J, Hirayama T, Roman G, Nourizadeh S, and JR Ecker. (1999) EIN2, a Bifunctional Transducer of Ethylene and Stress Responses in *Arabidopsis*. *Science* **284**: 2148-2152.

43. Wang Y, Liu C, Li K, Sun F, Hu H, Li X, Zhao Y, Han C, Zhang W, Duan Y, Liu M, and X Li. (2007) *Arabidopsis* EIN2 Modulates Stress Response Through Abscisic Acid Response Pathway. *Plant Molecular Biology* **64**(6): 633-644.

44. Leon P, and J Sheen. (2003) Sugar and Hormone Connections. *Trends in Plant Science* **8**: 110-116.

45. Qiao H, Chang KN, Yazaki J, and JR Ecker. (2009) Interplay Between Ethylene, ETP1/ETP2 F-box Proteins, and Degradation of EIN2 Triggers Ethylene Responses in *Arabidopsis*. *Genes and Development* **23**: 512-521.

46. Solano R, Stepanova A, Chao Q, and JR Ecker. (1998) Nuclear Events in Ethylene Signaling: a Transcriptional Cascade Mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE FACTOR1. *Genes and Development* **12**: 3703-3714.

47. Potuschak T, Lechner E, Parmentier Y, Yanagisawa S, Grava S, Koncz C, and P Genschik. (2003) EIN3-depedent Regulation of Plant Ethylene Hormone Signaling by Two *Arabidopsis* F-box Proteins: EBF1 and EBF2. *Cell* **115**:679-689.

48. Guo H, and JR Ecker. (2003) Plant Responses to Ethylene Gas are Mediated by $SCF^{EBF1/EBF2}$-Dependent Proteolysis of EIN3 Transcription Factor. *Cell* **115**: 667-677.

49. Gagne J, Smalle J, Gingerich D, Walker J, Yoo S, Yanagisawa S, and R Viestra. (2004) *Arabidopsis* EIN3-Binding F-Box 1 and 2 Form Ubiquitin-protein Ligases That Repress Ethylene Action and Promote Growth by Directing EIN3 Degradation. *Proceedings of the National Academy of Sciences* **101**: 6803-6808.,

50. Binder B, Walker J, Gagne J, Emborg T, Hemmann G, Bleecker AB, and R Viestra. (2007) The *Arabidopsis* EIN3 Binding F-box Proteins EBF1 and EBF2 Have Distinct But Overlapping Roles in Ethylene Signaling. *Plant Cell* **19**: 509-523.

51. Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, and JR Ecker. (1997) Activation of the Ethylene Gas Response Pathway in *Arabidopsis* by Nuclear Protein ETHYLENE-INSENSITIVE3 and Related Proteins. *Cell* **89**: 1133-1144.

52. Lee JH, Deng XW, and WT Kim. (2006) Possible Role of Light in the Maintenance of EIN3/EIl1 Stability in *Arabidopsis* Seedlings. *Biochemical and Biophysical Research Communications* **350**: 484-491.

53. Yanagisawa S, Yoo SD, and J Sheen. (2003) Differential Regulation of EIN3 Stability by Glucose and Ethylene Signaling in Plants. *Nature* **425**: 521-525.

54. Olmedo G, Guo H, Gregory B, Nourizadeh S, Aguilar-Henonin L, Li H, An F, Guzman P, and JR Ecker. (2006) ETHYLENE-INSENSITIVE5 Encodes a 5' to 3' Exoribonuclease Required for Regulation of the EIN3-targeting F-box Proteins EBF1/2. *PNAS* **103**: 13286-13293.

55. Potuschak T, Vansiri A, Binder B, Lechner E, Vierstra R, and P Genschik. (2006) The Exoribonuclease XRN4 is a Component of the Ethylene Response Pathway in *Arabidopsis*. *Plant Cell* **18**: 3047-3057.

56. Larsen PB, and C Chang. (2001) The *Arabidopsis eer1* Mutant Has Enhanced Ethylene Response in the Hypocotyl and Stem. *Plant Physiology* **125**: 1061-1073.

57. Larsen PB, and JD Cancel. (2003) Enhanced Ethylene Responsiveness in the *Arabidopsis eer1* Mutant Results From a Loss-of-function Mutation in the Protein Phosphatase 2A a Regulatory Subunit, *RCN1*. *The Plant Journal* **34**: 709-718.

58. Robles L, Wampole J, Christians M, and PB Larsen. (2007) *Arabidopsis ENHANCED ETHYLENE RESPONSE4* Encodes an EIN3-interacting TFIID Transcription Factor Required for Proper Ethylene Response, Including ERF1 Induction. *Journal of Experimental Botany* **58**: 2627-2639.

59. Christians MJ and PB Larsen. (2007) Mutational Loss of the Prohibitin AtPHB3 Results in an Extreme Constitutive Ethylene Response Phenotype Coupled with Partial Loss of Ethylene-Inducible Gene Expression in *Arabidopsis* Seedlings. *Journal of Experimental Botany* **58**: 2237-2248.

60. Christians MJ, Robles LM, Zeller SM, and PB Larsen. (2008) The *eer5* Mutation, Which Affects a Novel Proteosome-related Subunit, Indicates a Prominent Role for the COP9 Signalosome in Resetting the Ethylene Signaling Pathway in *Arabidopsis*. *The Plant Journal* **55**(3): 467-477.

61. Wilkinson JQ, Lanahan MB, Clark DG, Bleecker AB, Chang C, Meyerowitz EM, and HJ Klee. (1997) A Dominant Mutant Receptor from *Arabidopsis* Confers Ethylene Insensitivity in Heterologous Plants. *Nature Biotechnology* **15**:447-447.

62. Arora A. (2005) Ethylene Receptors and Molecular Mechanisms of Ethylene Sensitivity in Plants. *Current Science* **89**(8): 1348-1361.

63. Mao C, Wang S, Qiaojun J, and W Ping. (2006) *OsEIL1,* a Rice Homolog of the *Arabidopsis EIN3* Regulates the Ethylene Response as a Positive Component. *Plant Molecular Biology* **61**(1-2): 141-152.

64. Dharmasiri N, Dharmasiri S, and M Estelle. (2005) The F-box Protein TIR1 is an Auxin Receptor. *Nature* **435**: 441-445.

65. Kepinski S, and O Leyser. 2005 The *Arabidopsis* F-box Protein TIR1 is an Ausin Receptor. *Nature* **435**: 446-451.

66. Thines B, Kastir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, and J Browse. (2007) JAZ Repressor Proteins are Targets of the $SCF^{COI1}$ Complex During Jasmonate Signaling. *Nature* **448**: 661-665.

67. Griffits J, Murase K, Rieu I, Zentella R, Zhang Z, Powers S, Gong F, Phillips A, Hedden P, Sun T, and S Thomas. (2006) Genetic Characterization and Functional Analysis of the GID1 Gibberelin Receptors in *Arabidopsis*. *The Plant Cell* **18**: 3399-34141.

68. Shen YY, Wang X, Wu F, Du S, Cao Z, Shang Y, Wang X, Peng C, Yu X, Zhu S, Fan R, Xu Y, and D Zhang. (2006) The Mg-chelatase H Subunit is an Abscisic Acid Receptor. *Nature* **443**: 823-826.

69. Liu X, Yue Y, Li B, Nie Y, Li W, Wu W, and L Ma. (2007) A G Protein-Coupled Receptor is a Plasma Membrane Receptor for the Plant Hormone Abscisic Acid. *Science* **315**: 1712-1716.

70. Wang X, and D Zhang. (2008) Abscisic Acid Receptors: Multiple Signal-perception Sites. *Annals of Botany* **101**: 311-317.

71. Lorenzo O, and R Solano. (2005) Molecular Players Regulating the Jasomonate Signaling Network. *Current Opinion in Plant Biology* **8**(5): 532-540.

72. Lorenzo O, Piqueras R, Sanchez-Serrano J, and R Solano. (2003) ETHYLENE RESPONSE FACTOR1 Integrates Signals from Ethylene and Jasmonate Pathways in Plant Defense. *The Plant Cell* **15**: 165-178.

73. Tang X, Chang L, Wu S, Li P, Liu G, and N Wang. (2008) Auto-regulation of the Promoter Activities of *Arabidopsis* 1-Aminocyclopropane-1-carboxylate Synthase Genes AtACS4, AtACS5, and AtACS7 in Response to Different Plant Hormones. *Plant Science* **175**(2): 161-167.

74. Vandenbussche F, and D van der Straeten. (2007) One for All and All for One: Cross-talk of Multiple Signals Controlling the Plant Phenotype. *Journal of Plant Growth Regulation* **26**: 178-187.

75. Gao Z, Wen Ck, Binder B, Chen YF, Chang J, Chiang YH, Kerris RJ, Chang C, and GE Schaller. (2008) Heteromeric Interactions Among Ethylene Receptors Mediate Signaling in *Arabidopsis. Journal of Biological Chemistry* **283**(35): 23801-23810.

76. Emanuelsson O, Nielsen H, and G von Heijne. (1999) ChloroP, a Neural Network-based Method for Predicting Chloroplast Transit Peptides and their Cleavage Sites. *Protein Science* **8**: 978-984.

77. Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, and NJ Provart. (2007) An "Electronic Fluorescent Pictograph" Browser for Exploring and Analyzing Large-Scale Biological Data Sets. *PLOS ONE* **2**(8): e718.

78. Kelley LA, and MJE Sternberg. (2009) Protein Structure Prediction on the Web: a Case Study Using the Phyre Server. *Nature Protocols* **4**: 363-371.

79. Schleuhuber S, and A Skerra. (2005) Lipocalins in Drug Discovery: From Nature Ligand-binding Proteins to "Anticalins." *Drug Discovery Today* **10**(1): 23-33.

80. Walter M, Chaban C, Schutze K, Batistic O, Weckermann K, Nake C, Blazevic D, Grefen C, Schumacher K, Oecking C, Harter K, and J Kudla. (2004) Visualization of Protein Interactions in Living Plant Cells Using Bimolecular Fluorescence Complimentation. *The Plant Journal* **40**:428-439.

81. Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland M, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, and JR Ecker. (2003) Genome-Wide Insertional Mutagenesis of Arabidopsis thaliana. *Science* **301**: 653-657.

82. Henikoff S, Till BJ, and L Comai. (2004) TILLING. Traditional Mutagenesis Meets Functional Genomics. *Plant Physiology* **135**: 630-636.

83. Lieberman M, Kunishsi AT, and LD Owens. (1975) Specific Inhibitors of Ethylene Production as Retardants of the Ripening Process in Fruits. *Colloquim Int Cent Natl Rech Sci* **238**: 161-170.

84. Beyer EM. (1976) Silver Ion, a Potent Inhibitor of Ethylene Action in Plants. *Plant Physiology* **58**: 268-271.

85. Gepstein S, and KV Thimann. (1981) The Role of Ethylene in the Senescence of Oat Leaves. *Plant Physiology* **68**: 349-354.

86. Singh ND, Li M, Lee SB, Schnell D, and H Daniell. (2008) *Arabidopsis* Tic40 Expression in Tobacco Chloroplasts Results in Massive Proliferation of the Inner Envelope Membrane and Upregulation of Associated Proteins. *The Plant Cell* **20**: 3405-3417.

87. Hua J, Chang C, Sun Q, and EM Meyerowitz. (1995) Ethylene Insensitivity Conferred by *Arabidopsis ERS* Gene. *Science* **269**(5231): 1712-1714.

88. Shimokawa K, Sakanoshita A, and K Horiba. (1978) Ethylene-induced Changes of Chloroplast Structure in *Satsuma mandarin*. *Plant and Cell Physiology* **19**(2): 229-236.

89. Kleffmann T, Russenberger D, von Zychlinski CW, Sjolander K, Gruissem W, and S Baginsky. (2004) The *Arabidopsis thaliana* Chloroplast Proteome Reveals Pathway Abundance and Novel Protein Functions. *Current Biology* **14**: 354-362.

90. Franklin AE, and NE Hoffman. (1993) Characterization of chloroplast homologue of the 54-kDA subunit of the signal recognition particle. *Journal of Biological Chemistry* **268**: 22175-22180.

91. Ferro M, Salvi D, Brugiere S, Miras S, Kowalski S, Louwagie M, Garin J, Joyard J, and N Rolland. (2003) Proteomics of the Chloroplast Envelope Membranes from *Arabidopsis thaliana. Molecular Cell Proteomics* **2**(5): 325-345.

92. Kiessling J, Martin A, Gremillon L, Rensing SA, Nick P, Sarnighausen E, Decker EL, and R Reski. (2004) Dual targeting of plastid division protein FtsZ to chloroplasts and the cytoplasm. *EMBO* **5**: 889-894.

93. Schwab R, Ossowski S, Riester M, Warthmann N, and D Weigel. (2006) Highly Specific Gene Silencing by Artificial MicroRNA's in *Arabidopsis*. *Plant Cell* **18**: 1121-1133.

94. Oreb M, Tews I, and E Schleiff. (2007) Policing Tic'n'Toc, the Doorway to Chloroplasts. *Trends in Cell Biology* **18**(1): 19-27.

95. Cho YH, and SD Yoo. (2007) ETHYLENE RESPONSE 1 Histidine Kinase Activity of *Arabidopsis* Promotes Plant Growth. *Plant Physiology* **143**(2): 612-616.

96. Neff MM, Turk K, and M Kalishman. (2002) Web-based Primer Design for Single Nucleotide Polymorphism Analysis. *Trends in Genetics* **18:** 613-615.

97. Clough SJ, and AF Bent. (1998) Floral Dip: a Simplified Method for *Agrobacterium*-mediated Transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**(6): 735-743.

98. Gamble RL, Qu X, and GE Schaller. (2002) Mutational Analysis of the Ethylene Receptor ETR1. Role of the Histidine Kinase Domain in Dominant Ethylene Insensitivity. *Plant Physiology* **128**: 142-1438.

99. Hall A, and AB Bleecker. (2003) Analysis of Combinatorial Loss-of-Function Mutants in the *Arabidopsis* Ethylene Receptors Reveals that the *ers1 etr1* Double Mutant Has Severe Developmental Defects that are EIN2 Dependent. *The Plant Cell* **15**(9): 2032-2041.

100. Cvetkovska M, Rampitsch C, Bykova N, and T Xing. (2005) Genomic Analysis of MAP Kinase Cascades in *Arabidopsis* Defense Responses. *Plant Molecular Biology Reporter* **23**: 331-343.

101. Cutler RE, Stephens RM, Saracino MR, and DK Morrison. (1998) Autoregulation of the Raf-1 Serine/threonine Kinase. *Proceedings of the National Academy of Sciences* **95**(16): 9214-9219.

102. Marias R, Light Y, Paterson HF, and CJ Marshall. (1995) Ras Recruits Raf-1 to the Plasma Membrane for Activation by Tyrosine Phosphorylation. *EMBO Journal* **14**(13): 3136-3145.

100. Mueller-Dieckmann HJ, Grantz AA, and S Kim. (1999) The Structure of the Signal Receiver Domain of the *Arabidopsis thaliana* Ethylene Receptor ETR1. *Structure* **7**(12): 1547-1556.

101. Qu X, and GE Schaller. (2004) Requirement of the Histidine Kinase Domain for Signal Transduction by the Ethylene Receptor ETR1. *Plant Physiology* **136**: 2961-2970.

102. Jaumot M, and JF Hankcock. (2001) Protein Phosphatases 1 and 2A Promote Raf-1 Activation by Regulating 14-3-3 Interactions. *Oncogene* **20**: 3949-3958.

103. Chang C, Larsen PB, Wen CK, Ding W, Shockey JA, and Z Pan. (1999) Protein-protein Interactions in Ethylene Signal Transduction in *Arabidopsis*. *Biology and Biotechnology of the Plant Hormone Ethylene* **II**: 65-70.

104. Fujikawa Y, and N Kato. (2007) Split Luciferase Complimentation Assay to Study Protein-Protein Interactions in *Arabidopsis* Protoplasts. *Plant Journal* **52**(1): 185-195.

105. Gleave AP. (1992) A Versatile Binary Vector System with a T-DNA Organisational Structure Conducive to Efficient Integration of Cloned DNA into the Plant Genome. *Plant Molecular Biology* **20**: 1203-1207.

106. Tzvetkova-Chevolleau T, Hutin C, Noel LD, Goforth R, Care J, Caffarri S, Sinning I, Groves M, Teulon J, Hoffman NE, Henry R, Havaux M, and L Nussaume. (2007) Canonical Signal Recognition Particle Components Can be Bypassed for Posttranslational Protein Targeting in Chloroplasts. *The Plant Cell* **19**: 1635-1648.

107. Oppenheimer DG, Herman PL, Sivakumaran S, Esch J, and MD Marks. (1991) A *myb* Gene Required for Leaf Trichome Differentiation in *Arabidopsis* is Expressed in Stipules. *Current Biology* **67**(3): 483-493.

108. Kim Y, Schumaker KS, and JK Zhu. (2006) EMS Mutagenesis of *Arabidopsis. Methods in Molecular Biology* **323**: 101-103.

109. Wang B, Pelletier J, Massaad MJ, Herscovics A, and GC Shore. (2004) The Yeast Split-ubiquitin Membrane Protein Two-hybrid Screen Identifies BAP31 as a Regulator of the Turnover of Endoplasmic Reticulum-Associated Protein Tyrosine Phosphatase-Like B. *Molecular and Cellular Biology* **24**: 2767-2778.

110. Reichel C, and N Johnsson. (2005) The Split-Ubiquitin Sensor: Measuring Interactions an Conformational Alterations of Proteins *In Vivo*. *Methods in Enzymology* **399**: 757-776.