ABSTRACT

Title of Dissertation:	THE PHYTOHORMONE ETHYLENE: (I) INVESTIGATING THE MOLECULAR FUNCTION OF RTE1 AND (II) INSIGHTS ON THE EVOLUTION OF THE ETHYLENE BIOSYNTHESIS AND SIGNALING PATHWAYS
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Ethylene is an important phytohormone that regulates growth, development and stress responses in land plants and charophycean green algae. In *Arabidopsis thaliana*, ethylene is perceived by a family of five receptors. One of these five receptors, ETR1, is dependent on *REVERSION-TO-ETHYLENE1 (RTE1)* and *Cytochrome B5 (Cb5)* while the other four receptors are not. We found that RTE1 and Cb5 interact *in planta* and used genetic analyses to place Cb5 upstream of RTE1 in the ethylene signaling pathway. After comparing different ethylene receptors we identified an N-terminally localized proline that is important in determining whether a

receptor is RTE1-dependent. Our results suggest that Cb5 receives electrons from upstream redox molecules, passes these electrons to RTE1; RTE1 is then able to activate the ETR1 receptor possibly by acting a molecular chaperone that refolds the ETR1 receptor into an active conformation.

The ethylene signal transduction pathway is functionally conserved in the charophycean green algae such as *Spirogyra pratensis*, suggesting that this signaling pathway was present in the common ancestor of charophytes and land plants over 450 million years ago. However, it is unclear whether the central regulator of ethylene response, EIN2, was conserved in charophytes. Furthermore, the details of ethylene biosynthesis in charophytes were unresolved. After examining the genomes and transcriptomes of many green algae we are able to report that EIN2 is conserved in most charophytes and even some of the more distantly related chlorophycean green algae. Moreover, the *Spirogyra* EIN2 is functionally conserved and able to activate ethylene responses in *Arabidopsis*.

Ethylene is synthesized via a two-step reaction involving the conversion of S-adenosyl-L-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS), followed by oxidation of ACC to ethylene gas by the enzyme ACC oxidase (ACO). We identified *S. pratensis* ACS homologs and demonstrated that *S. pratensis* can synthesize ACC. *S. pratensis* lacks ACO homologs but we find it is still capable of producing low levels of ethylene. From our results we conclude that the ethylene biosynthesis and signaling pathways were established in early charophytes allowing these algae to establish ethylene as an important signalling molecule.

THE PHYTOHORMONE ETHYLENE: (I) INVESTIGATING THE MOLECULAR FUNCTION OF RTE1 AND (II) INSIGHTS ON THE EVOLUTION OF THE ETHYLENE BIOSYNTHESIS AND SIGNALING PATHWAYS

by

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Foreword

As part of my dissertation, I have made substantial contributions to and am co-first author of the paper Chang *et al.* 2014.

Dedication

I dedicate this thesis to my fantastic family. To my wonderful wife Jessica, thank you for the encouragement necessary to keep going and for always being there for me and our children. To Brayden and Emily, thank you for the smiles and the hugs that always make a bad day good. I love you all.

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First and foremost, I want to acknowledge my mentor Dr. Caren Chang. Caren is a wonderful person who is always there to lend an ear or give sage. As a mentor, she is always willing to give encouragement or sage advice. I am a better scientist because of her and will always try to mimic her example.

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2OGD – 2-oxoglutarate dioxygenase
ACC – 1-aminocyclopropane-1-carboxylic acid
ACO - ACC oxidase
ACS – ACC synthase
AIB – 2-Aminoisobutyric acid
AVG - Aminoethoxyvinylglycine
BI-1 - Bax Inhibitor 1
BLAST – Basic Local Alignment Search Tool
BiFC – Bimolecular Fluorescence Complementation
CaMV – Cauliflower Mosaic Virus
Cb5 – Cytochrome B5
Col-0 – Columbia-0
CDPK - Calcium-dependent Protein Kinase
CTR1 – Constitutive Triple Response1
EIL – EIN3-like
EIN – Ethylene Insensitive
ER - Endoplasmic Reticulum
ERF – Ethylene Response Factor
ERS – Ethylene Response Sensor
ETR – Ethylene Receptor
GAF – cGMP-specific phosphodiesterases, adenyl cyclases and FhlA
LTP - Lipid Transfer Protein
MAPK - Mitogen-activated Protein Kinase
1-MCP – 1-Methylcyclopropane
MET - Methionine
Nramp – Natural Resistance-Associated Macrophage Protein
RAN1 - Responsive to Antagonist1
RTE1 - Reversion-to-Ethylene Sensitivity1
SAM – S-adenosyl Methionine
YFP- Yellow Fluorescent Protein

Chapter 1: Introduction

Significance

Ethylene, although a simple hydrocarbon, is a gaseous phytohormone important for many aspects of plant growth and development (Abeles et al., 1992). Well before scientists understood how ethylene affected plants, people were unknowingly using this hormone to improve agricultural output. In ancient Egypt, people would wound figs in order to make the fruit ripen faster (Galil, 1968). In ancient China, farmers would burn incense (which releases small quantities of ethylene due to partial combustion) in closed rooms to hasten pear ripening (Miller, 1947). In the nineteenth century, people observed that plants growing near lights fueled with coal gas were "damaged" (displayed leaf senescence and abscission). Even today, although the average person may not be aware that ethylene is involved, many people know that putting unripe fruit in a paper bag with a ripe banana will cause the fruit to ripen very quickly.

Modern agriculturists control ethylene responses to help maximize plant production and control food spoilage. Food spoilage during the storage or transport of plant products can be controlled by blocking ethylene production or signaling via many different methods; Air purifiers and scrubbers have been utilized to remove ethylene from the air; Controlled storage facilities with cool temperatures with low oxygen concentrations are utilized to help prevent ethylene production; Hypobaric storage helps ethylene diffuse out of plant tissue (and lower oxygen concentration) to

prevent ethylene response. Ethylene responses can be blocked chemically with SmartFresh^{SM} (1-methylcyclopropene), a molecule that competitively inhibits ethylene response by binding in the binding pocket of the ethylene receptors (Abeles et al., 1992). Ethylene is also used to stimulate desired responses such as promoting citrus degreening, fruit ripening, pineapple flowering, as well as to control seed germination. The chemical Ethephon (2-Chloroethylphosphonic acid) is the most widely used source of ethylene production (Abeles et al., 1992). This "liquid ethylene" is stable at an acidic pH, but at a pH above 5, Ethephon is hydrolyzed to chloride, phosphate and ethylene.

Our understanding of how ethylene affected plants began in 1901, when Dimitry Neljubow identified ethylene as the active component from coal gas that caused pea seedling abnormalities in his greenhouse (Neljubow, 1901). Originally considered an environmental effect, ethylene was found to be a plant hormone when, in 1934, Gane discovered that ripening fruit was capable of producing ethylene. This discovery helped demonstrate why different fruits appeared to be able to affect the ripening speed of other fruits.

Dark-grown *Arabidopsis thaliana* seedlings display a "triple response" phenotype when exposed to ethylene (Guzman and Ecker, 1990). The triple response consists of a shortening and thickening of the hypocotyl, inhibition of root elongation and the formation of an apical hook (Figure 1-1). Screening seedlings based on their hypocotyl size when treated with ethylene has made it easier to identify mutants in

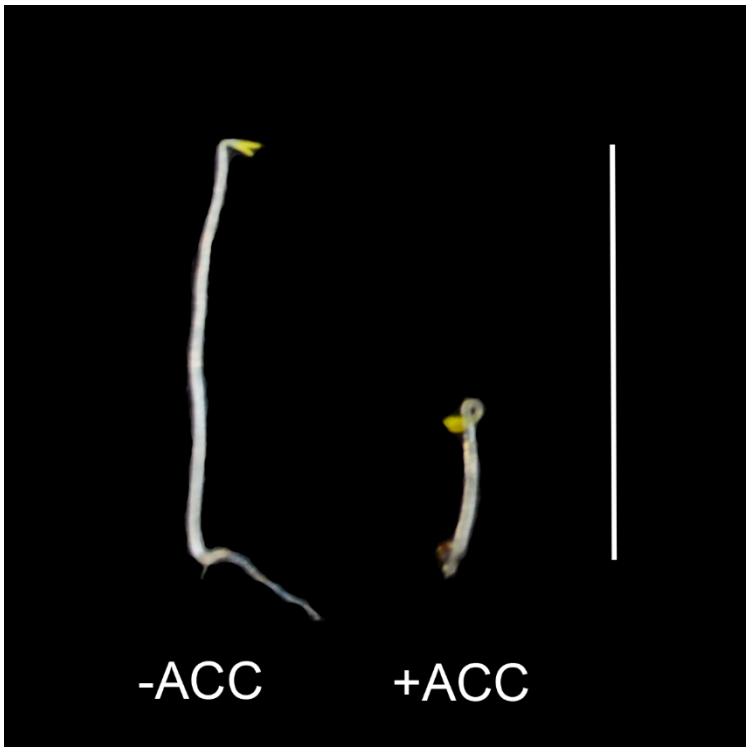


Figure 1-1: The "triple response" phenotype in *Arabidopsis thaliana*.

Dark-grown *Arabidopsis* seedlings grown in the presence of ethylene or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) display a "triple response" phenotype as demonstrated in these 4-day-old wild-type seedlings. The "triple response" phenotype consists of the shortening and thickening of the hypocotyl, formation of an apical hook and inhibition of root elongation. Scale Bar represents 10mm.

ethylene signaling. Seedlings that remain tall when treated with ethylene are insensitive to the ethylene and are easily distinguished from the shorter ethylene-sensitive seedlings. Seedlings that are always short, with or without ethylene, are said to be constitutively responding to ethylene. Additionally, measuring the length of hypocotyls provides an effective means of reporting ethylene sensitivity. Using this phenotype as well as several biochemical assays, most of the components of the ethylene biosynthesis and signal transduction pathways have been identified.

Ethylene Biosynthesis

The ethylene biosynthesis pathway in seed plants is relatively simple and was understood well before the ethylene signal transduction pathway. Ethylene is produced from the amino acid methionine. As part of the methionine salvage or Yang cycle, methionine is converted to S-adenosyl methionine (SAM) by the enzyme SAM SYNTHETASE and ATP (Adams and Yang, 1977). SAM is found in both prokaryotes and eukaryotes and is involved in DNA methylation, transulfuration and polyamine synthesis. SAM is converted back to methionine through a series of enzymes as part of the methionine salvage cycle (Figure 1-2).

The first committed step in ethylene biosynthesis is the conversion of SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC SYNTHASE (ACS) (Yu et al., 1979) (Figure 1-2). ACC is generally thought to serve primarily as a precursor to ethylene. However, the double leucine-rich repeat receptor protein kinase (LRR-RPK) mutant *fei1 fei2* displayed root cell elongation phenotypes that were reversed

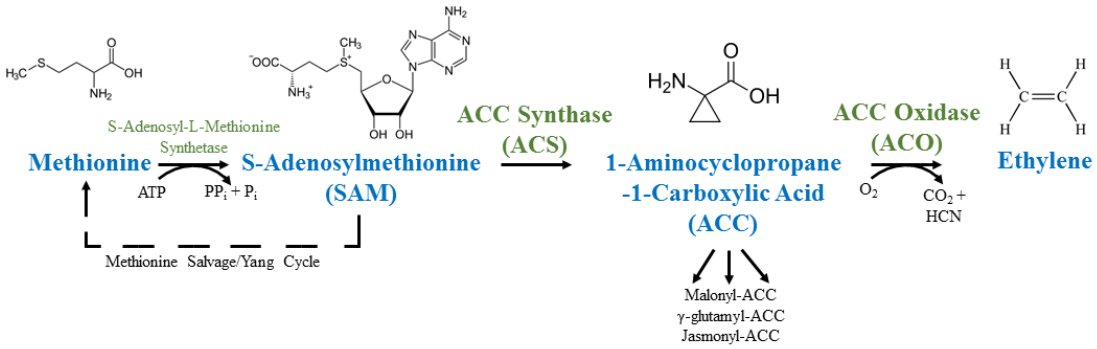


Figure 1-2: Ethylene biosynthesis pathway in Angiosperms

Ethylene is synthesized from the amino acid methionine. As part of the Yang cycle, methionine is converted to SAM, a compound important for methyl transfer reactions. In the first committed step of ethylene biosynthesis, SAM is converted to ACC via the ACS enzyme. ACC can be conjugated to several molecules or can be converted to ethylene via the ACO enzyme.

by an ACC analog and ACS inhibitor but not by ethylene signaling inhibitors (Xu et al., 2008). Additionally, it was reported that cell expansion in the root elongation zone is blocked by the cellulose inhibitor, isoxaben. ACS biosynthesis inhibitors are able to restore cell expansion in roots treated with isoxaben while ethylene signaling inhibitors are not (Tsang et al., 2011). These studies may suggest that ACC has a function outside of ethylene biosynthesis. Several ACC conjugates have been identified but the only suggested function of these conjugates is to regulate ACC pools in order to help regulate ethylene production (Amrhein et al., 1981; Martin et al., 1995; Staswick and Tiryaki, 2004).

The ACS enzyme is a member of the PLP-dependent aminotransferases that use vitamin B6 as a cofactor for their activity. The first ACS enzyme was identified by Satos and Theologis (1989). Since their initial work, twelve ACS enzymes have been identified in *A. thaliana*, eight of which have been shown to have ACC synthase activity (ACS1, ACS10 and ACS12 are inactive for ACC synthase activity *in vitro*; ACS3 is a pseudogene) (Yamagami et al., 2003). ACS enzymes from seed plants are divided into three types based off of conserved carboxyl terminal elements. Type I ACS proteins contain a calcium-dependent protein kinase (CDPK) phosphorylation site as well as three mitogen-activated protein kinase (MAPK) phosphorylation sites (Hyun and Kieber, 2005; Yoon and Kieber, 2013). Type II ACS proteins have only the MAPK phosphorylation sites, while type III ACS proteins do not have any known phosphorylation sites (Hyun and Kieber, 2005; Yoon and Kieber, 2013). Generally, it is believed that phosphorylation at these sites stabilize the ACS protein which

effectively turns on ACC and ethylene production. The ACC synthase reaction is generally considered the rate-limiting step in ethylene production (Yang and Hoffman, 1984) as the tight regulation of ACS enzymes gives plants the ability to efficiently turn ethylene production on and turn off. Besides post-translation regulation, several hormone signaling pathways and other stimuli induce or repress expression of ACS enzymes to control ethylene production (Botella et al., 1995; Cary et al., 1995; Liang et al., 1996; Rodrigues-Pousada et al., 1993; Staswick and Tiryaki, 2004; Woeste et al., 1999).

The second committed step in ethylene biosynthesis is the oxidation of ACC to form ethylene by the ACC OXIDASE enzyme (ACO) (Figure 1-2). ACO is a member of the 2-oxoglutarate-dependent dioxygenase (2OGD) family, which utilizes a non-heme Fe^{2+} to fix two oxygen molecules. The first ACO enzyme was isolated by Ververidis & John (Ververidis and John, 1991) and five ACO enzymes have been identified in *A. thaliana*. ACO has no other known function and little is known about the regulation of ACO.

Ethylene Signal Transduction Pathway

Ethylene is perceived at the endoplasmic reticulum (ER) by a family of receptors related to prokaryotic two-component signal transduction proteins (Chang et al., 1993). The receptors interact with CTR1, a Raf-like serine/threonine protein kinase, and together the receptor/CTR1 complex negatively regulates ethylene

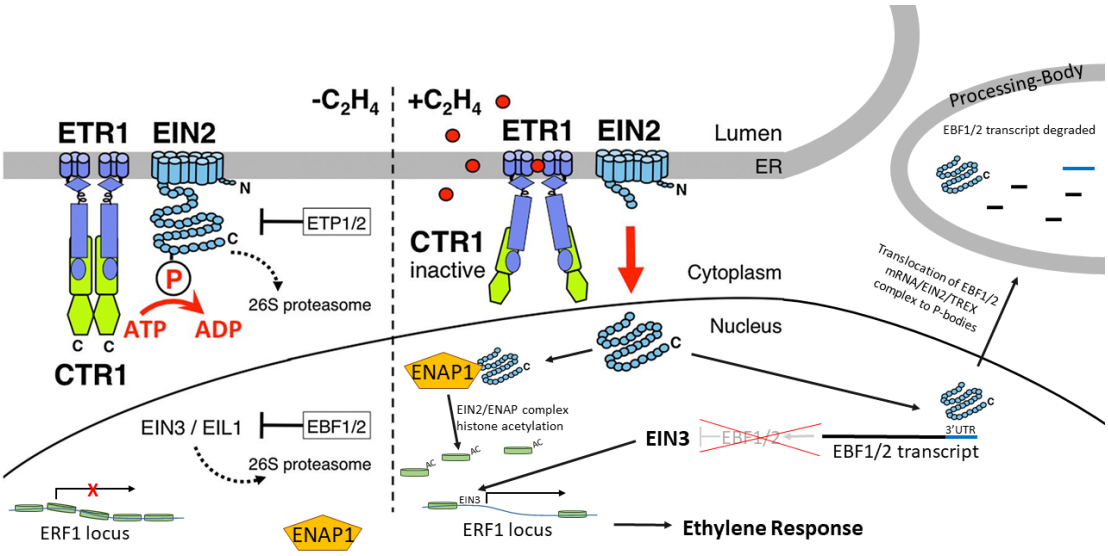


Figure 1-3: Ethylene Signal Transduction Pathway

Ethylene is perceived at the ER membrane by a family of ethylene receptors (represented by ETR1). In the absence of ethylene, a receptor/CTR1 complex phosphorylates EIN2 allowing the F-box proteins EBF1/2 to degrade the central ethylene transcription factors. When ethylene is present, the receptor/CTR1 complex is turned off and no longer phosphorylates EIN2. The carboxyl terminal tail portion of the unphosphorylated EIN2 is cleaved and translocates to the nucleus. The EIN2 C-tail activates ethylene response by: 1.) binding to the 3'UTR of EBF1/2 mRNA and translocating the transcript to processing bodies for degradation, effectively preventing EBF1/2 translation causing EIN3 protein accumulation. 2.) Interacting with ENAP1 forming a complex that interacts with the histones that protect EIN3 target sites. The EIN2 C-tail/ENAP1 complex acetylates the histones allowing EIN3 to bind its targets and initiate transcription of ethylene-responsive genes. Figure is a modified version of a pathway found in Ju et al. 2012.

response (Kieber et al., 1993). The next downstream component of ethylene signaling is EIN2 (Alonso et al., 1999). In the absence of ethylene, CTR1 phosphorylates EIN2 (Ju et al., 2012). When ethylene is present, the receptor/CTR1 complex is inactivated and EIN2 is no longer phosphorylated (Ju et al., 2012). Unphosphorylated EIN2 is cleaved by an unknown protease, allowing the carboxyl-terminal end of EIN2 (EIN2 C-tail) to translocate to the nucleus (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). The carboxyl terminal end of EIN2 (C-tail) can then bind to the transcripts of F-box protein (EIN3 Binding Factor or EBF) and target the transcripts to p-bodies for degradation (Li et al., 2015; Merchante et al., 2015). By eliminating EBF transcripts, the main ethylene signaling transcription factors EIN3 and EIL1 (which are normally targeted for degradation by EBFs) accumulate in the nucleus. Additionally, the carboxyl terminal end of EIN2 forms a complex with EIN2-Nuclear Associated Protein1 (ENAP1) and helps acetylate histones, uncovering targets of the EIN3/EIL1 transcription factors which turn on the ethylene response (Zhang et al., 2017).

The Ethylene Receptors

Ethylene, a small non-polar molecule, easily permeates the cell membrane, which allows for detection of ethylene by ER-localized receptors. ETR1 was the first

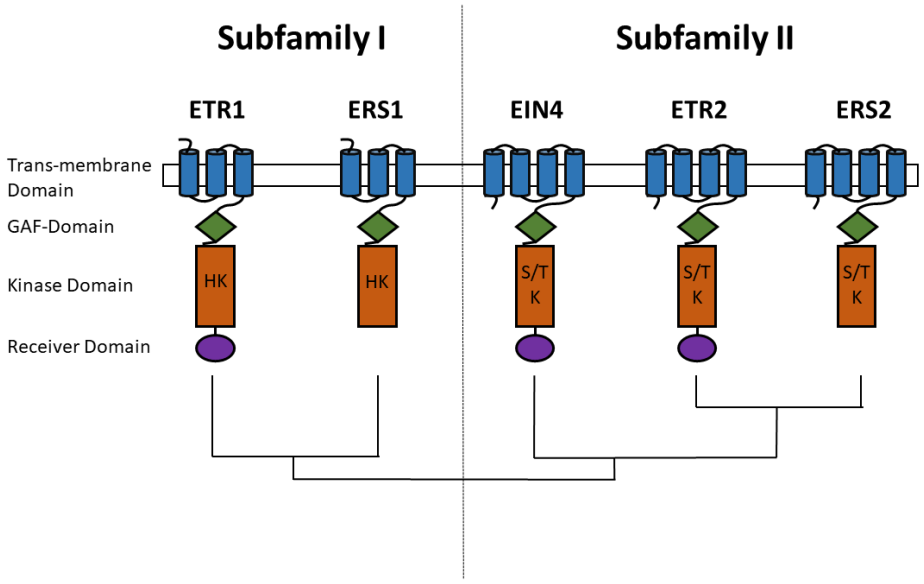


Figure 1-4: The *A. thaliana* Ethylene Receptors

Ethylene receptors are divided into two subfamilies. The subfamily I receptors (ETR1 and ERS1) have three transmembrane domains and histidine kinase (HK) activity. Subfamily II receptors (ETR4, ETR2 and ERS2) have four transmembrane domains and serine/threonine (S/T) kinase activity. ETR/EIN4 receptors have a C-terminal receiver domain while ERS receptors lack a receiver domain. The phylogenetic tree below the receptors indicates that the receptors within a subfamily (i.e. ETR1 and ERS1) are more closely related to each other than receptors in the other subfamily (i.e. ERS1 and ERS2).

plant hormone receptor isolated and cloned (Bleecker et al., 1988; Chang et al., 1993). Four additional ethylene receptors (ERS1, ETR2, ERS2 and EIN4) have beenidentified in *A. thaliana* (Hua et al., 1995, 1998; Sakai et al., 1998). The ethylene receptors are negative regulators of ethylene response and therefore actively repress ethylene response until ethylene is perceived.

Several bacterial proteins have ethylene binding domains but the structure of the ethylene receptors from the cyanobacteria *Synechocystis PCC 6803* and *Anabaena PCC 7120* follow the same general structure as plant ethylene receptors (Mount and Chang, 2002). This led to the hypothesis that ethylene receptors became part of the plant lineage during the cyanobacterial endosymbiosis event that led to the formation of the plastid (Mount and Chang, 2002). The ethylene receptors have evolved into two distinct subfamilies. Subfamily I, consisting of ETR1 and ERS1 in *A. thaliana*, have three transmembrane domains and are histidine kinases. Subfamily II, consisting of ETR2, ERS2 and EIN4 in *A. thaliana*, have four transmembrane domains and have serine/threonine kinase activity. Subfamily I ethylene receptors may be more important as the double loss-of-function mutant *etr1 ers1* result in a lethal phenotype (Hall and Bleecker, 2003; Qu et al., 2007). Triple loss-of-function *etr2 ers2 ein4* mutant plants are hypersensitive to ethylene but are still viable (Hall and Bleecker, 2003). However, this difference may stem from the fact that the subfamily I receptors, ETR1 and ERS1, are thought to make up the majority of the receptors in *A. thaliana* and not necessarily be due to differences in function.

The ethylene receptors show sequence similarity to the prokaryotic two-component signal transduction regulators. Typically, the two-component regulators consist on membrane-bound sensor protein, which contains an amino-terminal ligand binding domain and a carboxyl-terminal histidine kinase domain, and a response regulator protein (Capra and Laub, 2012). The plant ethylene receptors follow the same general structure: An amino-terminal ethylene binding domain with three to four transmembrane domains, followed by a GAF domain and a kinase domain. Several of the ethylene receptors also have a C-terminal receiver domain similar to the response regulator proteins.

The ethylene binding domain consists of the first ~130 amino acids of the ethylene receptor. Within this domain, two ER-lumen localized cysteines (at amino acid positions 4 and 6 in *A. thaliana* ETR1) are necessary for the formation of disulfide-linked receptor dimers. Interestingly however, receptors mutated at these cysteines are still able to function even though they can no longer form disulfide bonds (Chen et al., 2010; Xie et al., 2006). Cys65 and His69 are two conserved amino acids that are necessary for coordinating a copper molecule that is important for ethylene binding (Rodriguez et al., 1999; Schaller and Bleecker, 1995). Mutations in these two conserved amino acids prevents copper and ethylene from binding to the receptor (Rodriguez et al., 1999). The proteins ATX and RAN1 are important for the delivery of the copper cofactor to the ethylene receptors (Hirayama et al., 1999; Li et al., 2017; Woeste and Kieber, 2000).

A cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA (GAF) domain is the follows the ethylene binding domain. GAF domains are known to bind small ligands such as cGMP (Ho et al., 2000; Zoraghi et al., 2004). Generally in GAF domain-containing proteins, the binding of a ligand to the GAF domain causes a conformational change that activates the signaling portion of a protein (Ho et al., 2000). The ethylene receptor GAF domain has been shown to be sufficient for receptor heteromer formation in a yeast two hybrid analysis (Gao et al., 2008).

The exact role of the kinase domain in signal transduction is unknown. In the classic two-component histidine kinase system, after binding of a ligand at the amino-terminal domain (or in some cases in the absence of the ligand), the carboxy-terminal domain autophosphorylates on a conserved histidine. This phosphate is then relayed to an aspartate molecule on the response regulator (which is either attached to the receptor or a separate protein) to begin signal transduction. However, mutations of the ETR1 receptor that inactivate the histidine kinase activity do not significantly affect ethylene signaling (Gamble et al., 2002; Qu and Schaller, 2004) suggesting that the ethylene receptors do not phosphorylate CTR1 the next downstream component of ethylene signaling. This is further supported by the fact that the subfamily II receptors, which are thought to have serine/threonine kinase activity instead of histidine kinase activity, still regulate ethylene signaling. Although kinase activity is not important, the kinase domain is necessary for interaction between the ethylene receptors and CTR1.

Several of the receptors have a receiver domain after the histidine kinase domain. This receiver domain shows sequence similarity to the two-component response regulators. The nomenclature of the ethylene receptors is determined by the presence or absence of the receiver domain. The ETR (ETR1 and ETR2) and EIN4 receptors have the receiver domain, while the ERS (Ethylene Response Sensor) receptors (ERS1 and ERS2) lack this domain. This nomenclature is misleading as the receiver domain has been lost several times and can give false impressions about the origins and perceived functions of the receptors. For example, the two *A. thaliana* subfamily I receptors, ETR1 and ERS1, are more closely related and predicted to function more similarly to each other than either ETR1 is to ETR2 or ERS1 is to ERS2. The receiver domain is not important for ethylene signaling itself but rather is involved in the recovery from the ethylene signal (Binder et al., 2004).

The exact mode of signaling through the ethylene receptor is unknown. It is hypothesized that the binding of ethylene to the copper cofactor in the ethylene binding domain causes a conformational change in the receptor which alters the interaction of the kinase domain with the next downstream component, CTR1 (Wang et al., 2006). The GAF domain, thought to undergo changes in its protein structure after binding to a small molecule, may assist with the conformation change (Aravind and Ponting, 1997). In this model, the receptor is in an "on" state and is turned "off" with the addition of ethylene. Mutations that disrupt the proposed receptor signaling model alter signal output differently. For example, *etr1-1,* an allele pulled out of a triple-response screen, has the C65Y mutation which disrupts copper binding to the

ethylene pocket (Rodriguez et al., 1999). Unable to bind ethylene, this gain-of-function mutant receptor is stuck in an always "on" state, which always negatively regulates ethylene signaling and causes a plant to be insensitive to ethylene. Other receptor mutants, such as *etr1-2* (A102T substitution), are capable of binding ethylene but are still stuck in an always "on" state and cause ethylene insensitivity. *etr1-7* has a nonsense mutation that creates a stop codon at amino acid position 75 (Cancel and Larsen, 2002). This truncated loss-of-function receptor does not contain a kinase domain, cannot interact with CTR1 and is constantly in an "off" state. These loss-of-function receptors cannot turn off the ethylene signal and consequently are hypersensitive to ethylene.

Reversion-To-Ethylene Sensitivity 1 (RTE1) regulates the ETR1 receptor

RTE1 was identified in a screen searching for more components of the ethylene signaling pathway (Resnick et al., 2006). Loss-of-function *rte1* mutants suppress ethylene insensitivity observed in *etr1-2* seedlings suggesting that the etr1-2 receptor being able to signal in the "on" state without a functioning RTE1. This is supported by the fact that loss-of-function *rte1* seedlings are ethylene hypersensitive similar to the *etr1-7* loss-of-function seedlings. Moreover, double loss-of-function *rte1-2 etr1-7* seedlings display ethylene hypersensitivity similar to either *etr1-7* or *rte1-2* seedlings. Overexpression of *RTE1* with the CaMV 35S promoter causes insensitivity, consistent with more ETR1 receptors being in an "on" state. Seedlings

expressing *etr1 (1-349)*, an ETR1 receptor lacking the histidine kinase and receiver domains, is partially functional in an RTE1-dependent manner suggesting that RTE1 acts on the N-terminal portion of ETR1 (Zhou et al., 2007). Together these results suggest that RTE1 acts on the ETR1 N-terminus to help repress downstream ethylene responses.

While the ethylene receptors are generally considered functionally redundant, ETR1 is unique in terms of dependence on RTE1. The other four ethylene receptors, including the other subfamily I receptor, ERS1, are RTE1-independent (Resnick et al., 2008, 2006). Interestingly, not all ethylene insensitive *etr1* alleles are RTE1-dependent (Resnick et al., 2008, 2006). For example, *etr1-1* (C65Y) seedlings are ethylene insensitive with or without a functioning RTE1. This indicates there are differences in the way that mutations cause ethylene insensitivity in the ETR1 receptor. Moreover, there appears to be a connection with the RTE1-dependent ethylene insensitive alleles and their RTE1-dependence. Mutations that cause RTE1-dependent ethylene insensitivity in ETR1 (for example, the A102T mutation) do not cause ethylene insensitivity when found in the other ethylene receptors (*ers1 A102T*) (Rivarola et al., 2009).

RTE1 localizes to the ER-membrane where it interacts with the ETR1 receptor (Dong et al., 2008, 2010). RTE1 is predicted to have two transmembrane domains located near its carboxyl-terminal end. The amino-terminal domain

(predicted to be located in the cytoplasm) has no known function, but has a predicted heme-binding motif (Jianhong Chang thesis, 2011).

RTE1 homologs exist in plants, animals and some protists (Resnick et al., 2006). The RTE1 homolog in *A. thaliana*, RTH, also localizes to the ER membrane and is thought to be indirectly involved in ethylene signaling through interaction with RTE1 (Zheng et al., 2017). In tomato, there are two orthologs of RTE1 (GREEN-RIPE, SlGR and GREEN-RIPE LIKE1, SlGRL1) and one RTH ortholog (GREEN-RIPE LIKE2, SlGRL2) (Barry and Giovannoni, 2006; Barry et al., 2005; Ma et al., 2012). SlGR and SlGRL1 are involved in ethylene signaling in tomato while SlGRL2 is not. Interestingly, animals, which do not have ethylene receptors, have RTE1 homologs. The function of animal RTE1 homologs is unknown but TMEM222 (the human RTE1 homolog) has been shown to be among the top 20 differentially expressed genes in patients with Parkinson's disease (Diao et al., 2013). Additionally, TMEM222 is predicted to interact with the drug Methotrexate, which is used to treat types of uterine cancer and this interaction is thought to be the cause of the Methotrexate's potentially lethal side effects (Zhou et al., 2015). The role of RTE1 homologs outside of plants is unknown and the only protein confirmed to physically interact with any RTE1 homologs is the ETR1 receptor (Dong et al., 2010). It is important to understand RTE1's biochemical function and understand why the ETR1 receptor is dependent on RTE1, not only to better determine what

RTE1 is doing in the ethylene signaling pathway, but to also elucidate what RTE1 homologs may be doing in animal systems as well.

CTR1

After the ethylene receptors, the next downstream component of the ethylene signal transduction pathway is CONSTITUTIVE TRIPLE RESPONSE1 (CTR1). CTR1 was identified in a screen that identified mutants that always displayed ethylene phenotypes with or without the ethylene signal (Kieber et al., 1993). Mutants from this screen included ACS mutants that overproduced ethylene and mutants that were part of the signaling pathway (CTR1). Epistasis analysis suggested that CTR1 mutants were downstream of the ETR1 but upstream of EIN2 (Kieber et al., 1993). Identification of the *CTR1* gene indicated that CTR1 has sequence similarity with the RAF-like mitogen activated kinase kinase kinase (MAPKKK) (Kieber et al., 1993).

CTR1 is a soluble protein that is divided into two portions, an amino terminal regulatory portion and a carboxyl terminal serine-threonine kinase domain. The amino terminal portion contains a conserved EDR1 domain that is only found in CTR1 and several closely related genes. This amino-terminal portion interacts with the kinase domain of the ethylene receptors and helps form a receptor/CTR1 complex at the ER membrane (Clark et al., 1998; Gao et al., 2003). The carboxyl-terminal serine-threonine kinase domain phosphorylates the next downstream component of

the ethylene signaling pathway, EIN2, in the absence of ethylene (Chen et al., 2011; Ju et al., 2012). In the presence of ethylene, the phosphorylation of EIN2 by the ethylene receptor/CTR1 complex is reversed and ethylene signal transduction continues (Ju et al., 2012).

EIN2 and downstream ethylene response

After CTR1, ETHYLENE INSENSITIVE2 (EIN2) is the next component of the ethylene signaling pathway. *EIN2* was originally isolated using the triple response screen for ethylene insensitive mutants (Guzman and Ecker, 1990). *EIN2* is a positive regulator of ethylene response (Alonso et al., 1999). Because mutations that disrupt EIN2 function display the strongest ethylene insensitive phenotypes, EIN2 is often considered a central regulator of ethylene response.

The EIN2 protein is generally divided into two portions, an amino-terminal Nramp domain and a carboxyl terminal signaling portion. The N-terminal Nramp domain has sequence similarity to the Nramp family of metal ion transporters (Alonso et al., 1999). Nramp proteins transport divalent metals such as Cu^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+} (Nevo and Nelson, 2006). Although this domain is well-conserved, researchers have been unable to show that the EIN2 Nramp domain is capable of transporting a metal. The EIN2 Nramp domain contains twelve predicted transmembrane domains that span the ER membrane. The carboxyl terminal domain is located in the cytoplasm and is unique to EIN2 proteins.

In the absence of ethylene, CTR1 phosphorylates several EIN2 serines in the carboxyl-terminal portion and a threonine (Chen et al., 2011; Ju et al., 2012). When ethylene is present, CTR1 no longer phosphorylates these amino acids and a portion of the carboxyl terminal end (EIN2 c-tail) is cleaved and translocates to the nucleus (Ju et al., 2012). A serine at position 924 and a serine at position 645 have both been reported to be important for controlling cleavage of the EIN2-ctail (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). EIN2 is thought to be cleaved immediately after serine 645 (Qiao et al., 2012). The predicted nuclear localization signal at the carboxyl terminus of EIN2 is necessary for translocation and activation of ethylene response (Li et al., 2015). This cleavage and translocation of the EIN2 c-tail relays the signal from the ER membrane where ethylene is perceived to the nucleus to activate response.

Once the EIN2 c-tail translocates to the nucleus, EIN2 activates ethylene responses via two functions. First, the EIN2 c-tail protein associates with the 3'UTR of the mRNA of the F-box proteins EBF1 and EBF2 (Li et al., 2015; Merchante et al., 2015). As part of a protein complex, EIN2 directs the EBF1/2 mRNA to p-bodies where the transcripts are degraded preventing the synthesis of new EBF1/2 proteins (Li et al., 2015; Merchante et al., 2015). Without EBF1/2, the central ethylene transcription factors, EIN3 and EIL1 accumulate. Second, inside the nucleus, the EIN2 c-tail associates with ENAP1 (EIN2 NUCLEAR-ASSOCIATED PROTEIN1), a histone binding protein (Wang et al., 2017; Zhang et al., 2016, 2017). Together as part of a complex, EIN2/ENAP1 promote histone acetylation of EIN3 target areas,

allowing EIN3 to bind to the promoters of ethylene responsive genes and activate expression of these genes.

Ethylene biosynthesis and signaling in *Spirogyra pratensis*

Lands plants evolved from charophycean green algae approximately 450 million years ago (Sanderson et al., 2004). Studying extant charophytes, such as *Spirogyra pratensis*, provides insight to the transition of life in an aquatic environment to terrestrial life. *S. pratensis* is a filamentous freshwater alga of the zygnematales order and represents some of the most closely related extant species of algae to land plants. When treated with high doses of the ethylene precursor ACC, *S. pratensis* is capable of producing very low levels of ethylene (Ju et al., 2015). *S. pratensis* elongates in response to ethylene treatment (Ju et al., 2015). Furthermore, several abiotic stresses induce ethylene-mediated cell elongation (Van de Poel et al., 2016).

Homologs of most of the ethylene biosynthesis and signaling pathways deduced from angiosperms are conserved in charophytes with the major exception of EIN2 (Ju et al., 2015). However, no full-length *EIN2* sequences had been identified in the only available charophyte genome of *Klebsormidium nitens* (Hori et al., 2014) nor in several charophyte transcriptomes (Ju et al., 2015); Partial EIN2 C-terminus sequences were identified in *S. pratensis* and *Coleochaete orbicularis,* which is thought to belong to a sister order of the zygnematales (Ju et al., 2015).

Outline of dissertation

We continue to advance our knowledge about function and origins of the components of ethylene biosynthesis and signaling pathways. In this thesis, I begin by building upon previous findings in pursuit of the biochemical function of RTE1. In chapter 2, I continue to examine the role that cytochrome B5 and RTE1 play in the ethylene signaling pathway. In chapter 3, I compare the subfamily I ethylene receptors from *A. thaliana* and *Oryza sativa* and determine why some receptors are RTE1-dependent while others are not. I then switch gears to better understand the origins of ethylene biosynthesis and signaling. In chapter 4, I scrutinize the ethylene biosynthesis pathway in *S. pratensis* and report that while *S. pratensis* can make the ethylene precursor ACC, it lacks the final enzyme in the angiosperm ethylene biosynthesis pathway. In chapter 5, I search full-length EIN2 proteins in green algae and find a functional EIN2 in *S. pratensis* as well as EIN2 homologs in many chlorophycean and charophycean green algae.

Chapter 2: Cytochrome b5 affects ethylene signaling upstream of RTE1 and ETR1

Introduction

RTE1 was identified as part of the ethylene signaling pathway in a genetic screen for mutants that could suppress the weak ethylene insensitivity ethylene receptor mutant *etr1-2* (Resnick et al., 2006). Similar to loss-of-function *etr1* mutant *A. thaliana* plants, loss-of-function *rte1* mutants are hypersensitive to ethylene while overexpression of *RTE1* results in ethylene insensitive phenotypes (Resnick et al., 2006). RTE1 affects ethylene signaling through only the ETR1 receptor, the other four ethylene receptors in *A. thaliana* are RTE1-independent (Resnick et al., 2006; Rivarola et al., 2009). The molecular function of RTE1 and its homologs in plants, animals and protists is unknown. There are no known targets of RTE1 function in animals, and animals lack ethylene receptors. By elucidating how RTE1 is affecting the ETR1 receptor, we can better understand what RTE1 is doing in other organisms such as animals, which do not have ethylene receptors, as well.

To gain insights into the molecular function of RTE1, a previous graduate student, Dr. Jianhong Chang, screened for RTE1-interacting proteins in an *A. thaliana* cDNA library using a split-ubiquitin assay. In this screen, Jianhong identified two potential RTE1 interacting proteins, Lipid Transfer Protein 1 (LTP1) and Cytochrome b5 Isoform D (Cb5D).

Cytochrome b5s are membrane-bound hemoproteins conserved in eukaryotes and purple proteobacteria. Cb5s function as electron carriers accepting electrons

from upstream reductases and passing the electrons to downstream acceptors. In humans, Cb5 mutations can cause methemoglobinemia, a disease caused when methemeglobin is not reduced into the biologically active hemoglobin (Giordano S et al., 1994). In plants, Cb5 are important for electron transport to Fatty Acid Desaturases (FAD) (Kumar et al., 2012) and Fatty Acid Hydroxylases (FAH) (Nagano et al., 2009a). Cb5s bridge calcium signaling and fatty acid hydroxylation through an interaction with Bax Inhibitor1 (BI-1) (Nagano et al., 2009a). *A. thaliana* has five isoforms of Cb5 (lettered A through E). Cb5A localizes to the chloroplast while Cb5B, Cb5B, Cb5D and Cb5E localize to the ER membrane. For the most part Cb5s B-E appear to be functionally redundant.

After identifying Cb5D as a potential RTE1-interacting protein, Jianhong tested to see if Cb5s were important for ethylene signaling. Jianhong found that loss-of-function mutations in *cb5b, cb5c* and *cb5d,* were capable of partially suppressing the ethylene insensitivity exhibited by *etr1-2* seedlings, similar to *rte1* loss-of-function mutants. Furthermore, she found that the double loss-of-function *cb5b cb5c* or *cb5b cb5d* plants were hypersensitive to ethylene while overexpression of *CB5D (*driven by the CaMV 35S promoter) caused ethylene insensitivity in *A. thaliana* seedlings, again similar to *rte1* mutants. Moreover, Jianhong found that the loss-of-function mutations in *cb5d* repressed RTE1-dependent ethylene insensitive *etr1* alleles but not a RTE1-independent ethylene insensitive allele. These results suggest that RTE1 and Cb5 function together to regulate ethylene signaling through the ETR1 receptor.

As I took over this project, several questions remained about the function of Cb5 and RTE1. Do the Cb5s and RTE1 interact *in planta?* A direct interaction may help suggest that RTE1 requires redox potential from Cb5. Does Cb5 affect the ethylene signal transduction pathway upstream or downstream of RTE1? Is the function of Cb5 in other pathways, such as fatty acid desaturation or hydroxylation, dependent on RTE1? RTE1, like Cb5, may be necessary to pass electrons to fatty acid modifying enzymes and any effect on the ETR receptor may be the result of altered fatty acid composition of the ER membrane. Is the interaction between Cb5 and RTE1 conserved in a non-plant species?

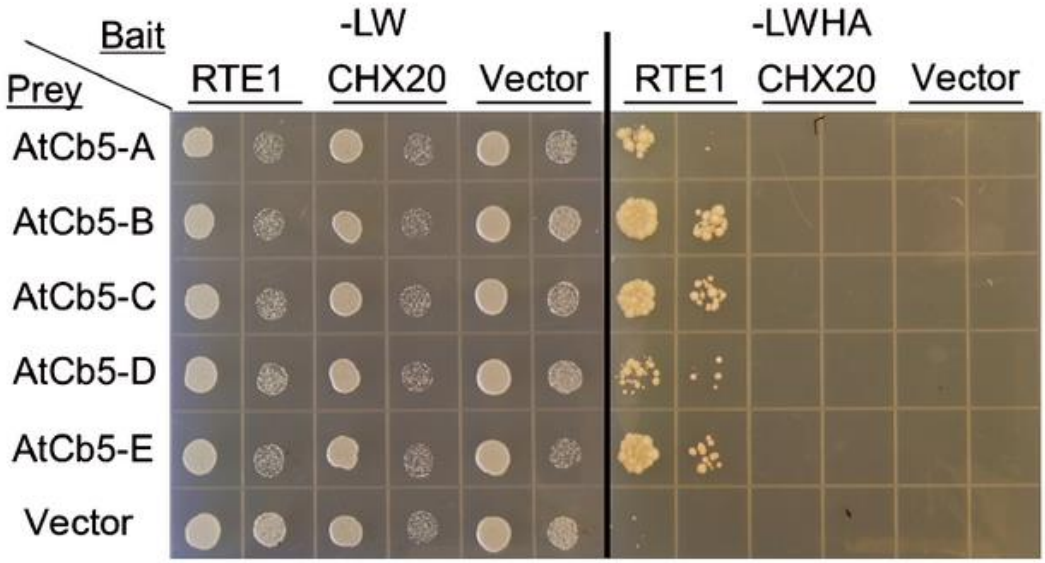
Results

Cytochrome b5 interacts with RTE1

Previously, Cb5D was identified as a potential RTE1-interacting protein using a split-ubiquitin library screen. A preliminary split-ubiquitin assay suggests that RTE1 may be able to interact with all five cytochrome b5 isoforms from *A. thaliana*. In short, the split-ubiquitin assay relies on the interaction of a prey protein fused to half of an ubiquitin molecule and a bait protein fused to the other half of ubiquitin as well as a reporter gene. The split-ubiquitin assay is susceptible to false positives if either the bait protein is able to self-activate or if the prey protein is "sticky" causing it to bind to and activate any bait protein. To confirm that the RTE1 (bait) and Cb5 (prey) interaction is a true positive, I tested the five *A. thaliana* Cb5s for interaction

with RTE1, CHX20 (an ER-localized cation transporter used as a negative control) and the empty bait vector (negative control). A growth plate confirmed equal growth of all yeast transformants when not selecting for interaction (Figure 1-1 Upper). Yeast cotransformed with a Cb5 and CHX20, Cb5 and empty bait vector or empty prey vector and RTE1 did not grow on interaction-selective plates (indicating no false positive interactions). Growth of yeast coexpressing Cb5 and RTE1 on interaction selective plates confirms that Cb5 and RTE1 in this assay. Cb5A, the normally chloroplast-localized Cb5 displayed the weakest interaction with RTE1 while isoforms B, C and E showed the strongest.

Interaction between Cb5 and RTE1 in the split-ubiquitin assay is a good preliminary indicator of interaction but does not prove that Cb5 and RTE1 interact *in planta*. To test for Cb5 and RTE1 interaction *in planta*, I utilized the bimolecular fluorescence complementation (BiFC) assay. The coding sequence of half of YFP was fused to the coding sequence of the Cb5s. The coding sequence for the other half of YFP was fused to the coding sequence of RTE1. YFP fluorescence was detected in tobacco leaf epidermal cells transiently transformed with cYFP-RTE1 and nYFP-Cb5B,C,D,E suggesting RTE1 and several isoforms of Cb5 interact (Figure 1-1 Lower). No fluorescence could be detected in cells transformed with cYFP-RTE1 and the chloroplast-localized nYFP-Cb5A. Additionally, we tested for interaction between Cb5D and the ETR1 receptor but no interaction could be detected by BiFC.


Bait
Prey
-LW
-LWHA
RTE1
CHX20
Vector
RTE1
CHX20
Vector
AtCb5-A
AtCb5-B
AtCb5-C
AtCb5-D
AtCb5-E
Vector

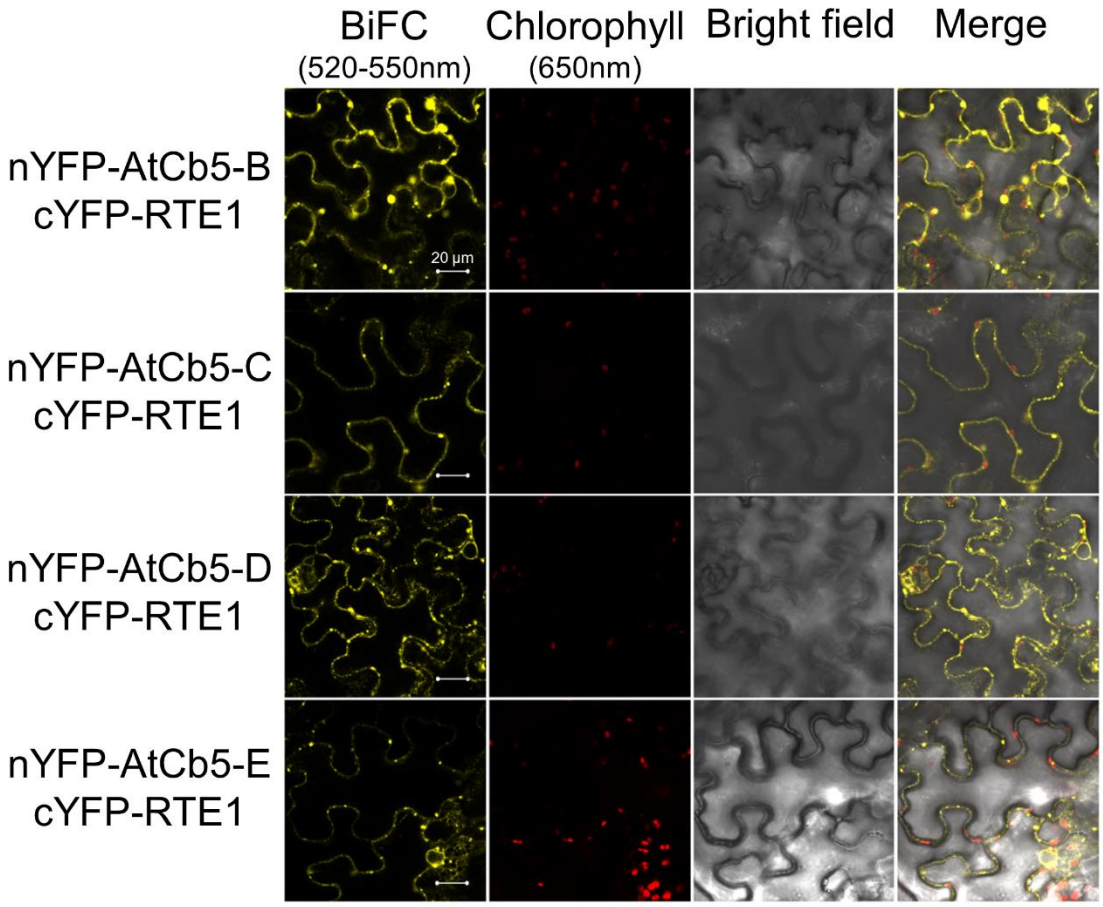

BiFC
(520-550nm)
Chlorophyll
(650nm)
Bright field
Merge
nYFP-AtCb5-B
cYFP-RTE1
20 µm
nYFP-AtCb5-C
cYFP-RTE1
nYFP-AtCb5-D
cYFP-RTE1
nYFP-AtCb5-E
cYFP-RTE1

Figure 2-1: Cb5 interacts with RTE1

Upper: Interaction of RTE1 and full-length AtCb5 isoforms in the yeast split-ubiquitin assay. Bait proteins RTE1, CHX20 (an ER-localized cation transporter used as a negative control) and the empty bait vector (negative control) were paired with each of the five AtCb5 isoforms or an empty prey vector. Yeast viability is shown on medium lacking leucine and tryptophan (–LW), while interaction is indicated by growth on medium lacking leucine, tryptophan, histidine and ala- nine (–LWHA). Undiluted and 1:10 diluted liquid cultures were spotted on the indicated plates and incubated for 3 days (–LW) or 5 days (–LWHA) at 30°C.

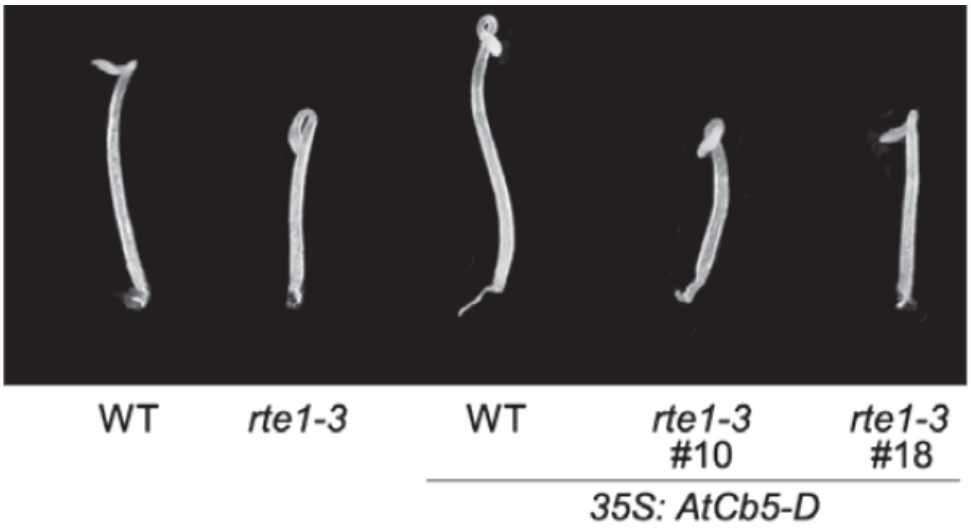
Lower: Interaction of RTE1 and full-length AtCb5 isoforms in tobacco leaf epider-mal cells shown by BiFC. Constructs expressing the N- and C-terminal halves of YFP fused to the N-terminus of the AtCb5 proteins and RTE1, respectively, were co-infiltrated into leaves of tobacco plants. YFP and chlorophyll signals were detected by laser scanning confocal microscopy at 520–550 nm and 650 nm,respectively. Scale bar = 20 μm.

Cb5 functions upstream of RTE1 in the ethylene signaling pathway

Jianhong demonstrated that RTE and CB5 work together to affect ethylene signaling through the ETR1 receptor in an apparent linear pathway but it was unclear whether Cb5 was upstream of RTE1 or vise-versa. To determine whether RTE1 or Cb5 was upstream, we crossed plants that overexpressed *Cb5D* (driven by the CaMV 35S promoter) with the loss-of-function *rte1-3* mutant and selected for double homozygous plants. Overexpression of *Cb5D* confers ethylene insensitivity while *rte1-3* seedlings are hypersensitive to ethylene (figure 2-2). *OE-Cb5D rte1-3* seedlings were hypersensitive to ethylene, similar to *rte1-3* plants. Typical epistasis analysis of signaling pathways suggests that RTE1 functions downstream of Cb5 consistent with previous observations that ETR1 interacts with RTE1 but not Cb5 and that ethylene insensitivity conferred by overexpression of RTE1 is not suppressed by a *cb5d* loss-of-function mutation.

RTE1 and RTH1 are not necessary for Cb5-linked fatty acid modifications

Cb5s transfer electrons from upstream reductases to a wide range of molecules. In plants, Cb5s are known to transfer electrons to Fatty Acid Desaturases (FAD) and Fatty Acid Hydroxylases (FAH). We hypothesized that RTE1 may also be involved in electron transfer to these fatty acid modifying enzymes. Moreover, the observed effects that Cb5 and RTE1 have on the ETR1 receptor may be the result of an altered fatty acid composition of the ER membrane rather than a direct effect.


WT
rte1-3
WT
rte1-3 #10
rte1-3 #18
35S: AtCb5-D

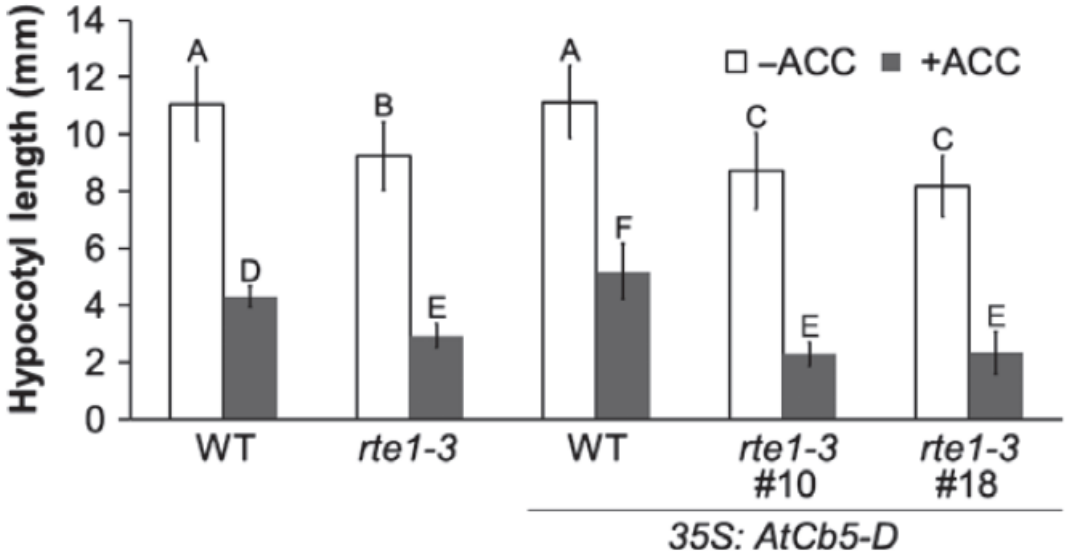

Hypocotyl length (mm)
14
12
10
8
6
4
2
0
A
D
B
E
A
F
C
E
C
E
–ACC
+ACC
WT
rte1-3
WT
rte1-3 #10
rte1-3 #18
35S: AtCb5-D

Figure 2-2: Loss-of-function rte1-3 suppresses ethylene insensitivity conferred by 35S:Cb5D

Upper: Representative 4-day-old etiolated seedlings of the wild-type and *rte1-3* mutant before and after transformation with the *35S:AtCb5-D* transgene are shown. Two independent transgenic lines (#10 and #18) are shown for the *rte1-3* background. Seedlings were germinated on 1 μM ACC.

Lower: The hypocotyl lengths of 4-day-old etiolated seedlings treated with or without 1 μM ACC indicate that rte1-3 blocks the reduced ethylene sensitivity conferred by *AtCb5-D* over-expression. Values are means ± SD for 22–31 seedlings

Loss-of-function *cb5* and *rte1* mutants may cause increased levels of fatty acid saturation which may in turn cause the ETR1 receptor to not fold correctly. This hypothesis could explain the observed ethylene hypersensitivity observed in *cb5* and *rte1* loss- of-function mutants. This hypothesis is also consistent with the observations that fatty acid saturation of membranes is dependent on temperature (Falcone et al., 2004) and that RTE1-dependence of the ETR1 receptor may be temperature dependent (Jianhong Chang Thesis, 2011).

To test the general hypothesis that RTE1 was involved in fatty acid saturation and hydroxylation, we collaborated with Dr. Maki Kawai-Yamada from Saitama University and compared the fatty acid content of *A. thaliana* wild-type plants with plants that were either overexpressing *RTE1*, null for *rte1* or double null *rte1 rth* (an *A. thaliana* RTE1 homolog). A preliminary examination of fatty acid hydroxylation indicated a significant difference in the levels of the 16h:0 fatty acid in the wild-type control compared to *rte1-2* plants, however, double null *rte1 rth* plants as well as plants overexpressing *RTE1* had 16h:0 levels similar to wild-type plants (figure 2-3). If RTE1 and/or RTH are involved in fatty acid hydroxylation then we would expect the double *rte1 rth* to display either similar or more severe differences in fatty acid hydroxylation compared to wild-type. Furthermore, we would expect overexpression of *RTE1* to have the opposite effect of the *rte1-2* mutation. No significant difference in fatty acid hydroxylation were observed between *rte1 rth* and *RTE1* overexpression lines. No difference was detected in the level of desaturation of several fatty acids

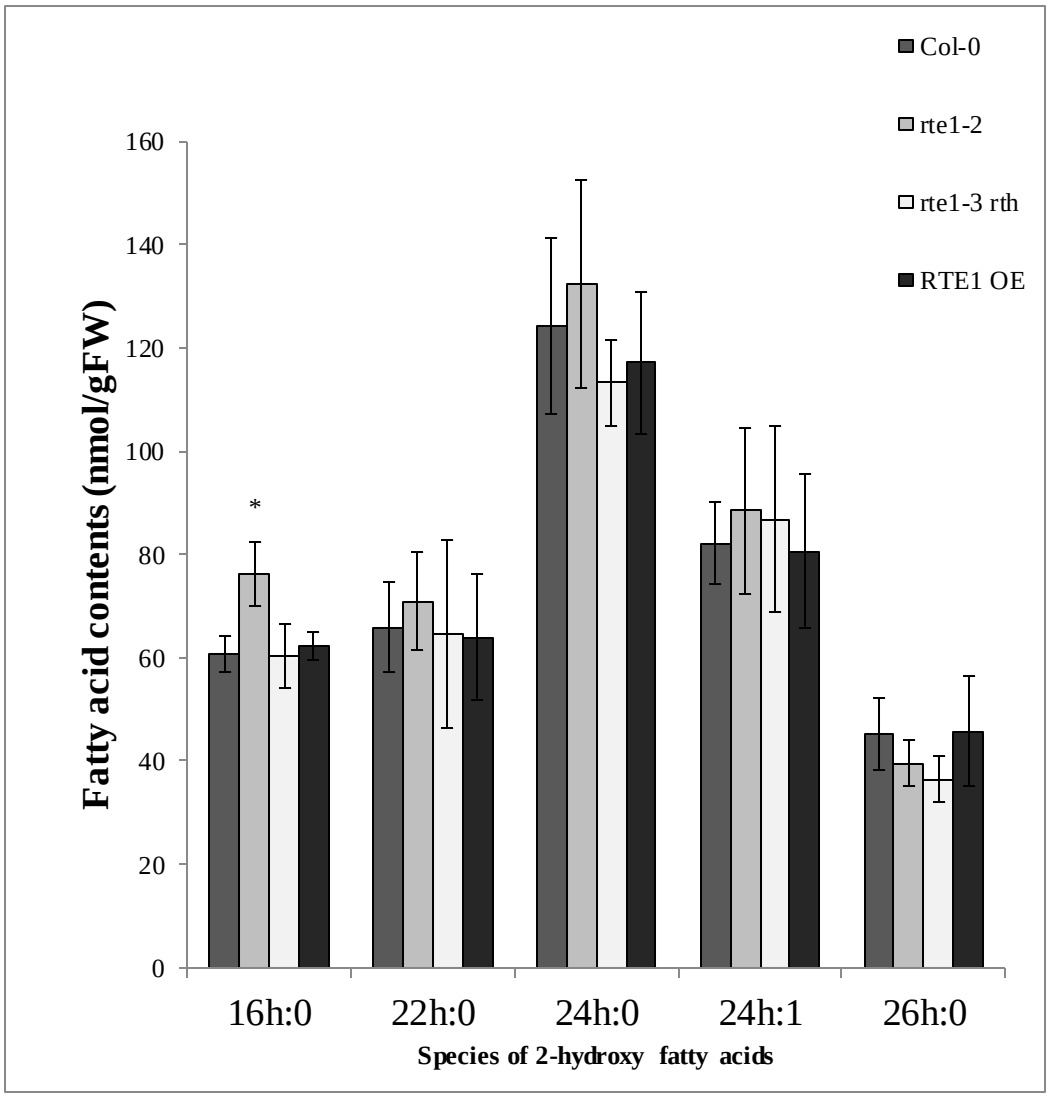


Figure 2-3: RTE1/RTH are not involved in fatty acid hydroxylation

Analysis of fatty acid hydroxylation in the rosettes of wild-type (Col-0), *rte1-2*, *rte1-3 rth*,and 35S:*RTE1 Arabidopsis* plants. Concentration of five 2-hydrox fatty acid were measured by GC-MS as indicated below bars. Values indicate means ± standard deviation. Asterisk indicates value that is significantly different from wild-type control as determined by the Students's T-test.

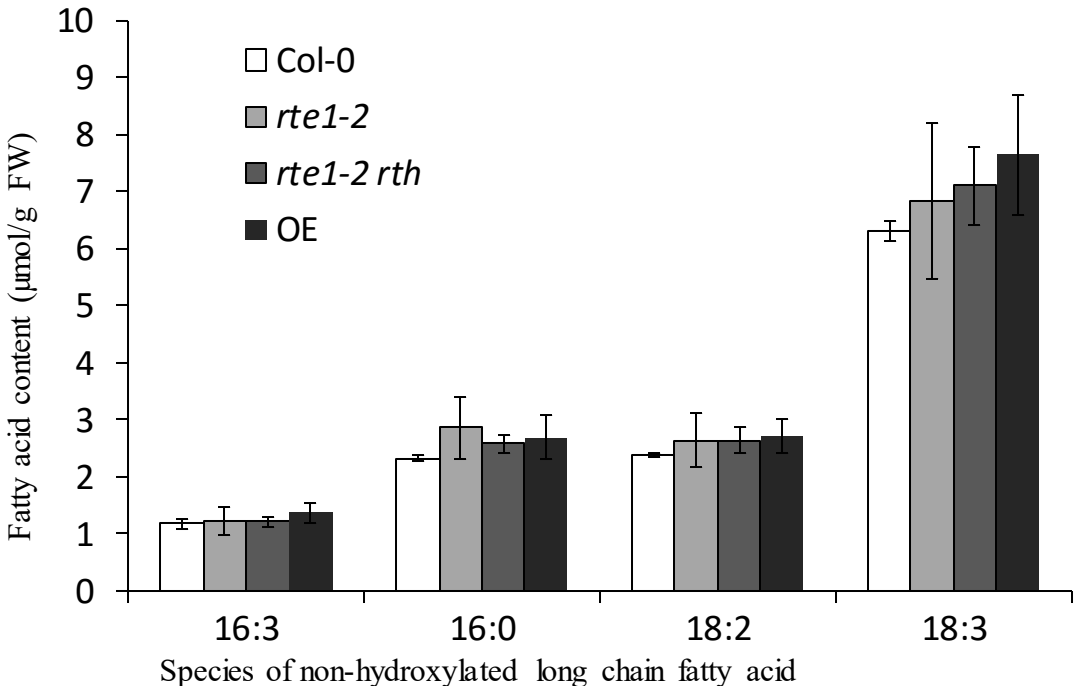


Figure 2-4: RTE1/RTH is not involved in fatty acid hydroxylation

Concentration of four non-hydroxylated fatty acids from the rosettes of wild-type (Col-0), *rte1-2*, *rte1-2 rth*, and 35S-*RTE1 Arabidopsis* plants determined by GC-MS. Values indicate means ± standard deviation.

(figure 2-4). Several differences in the concentrations of very long chain fatty acids were detected between our wild-type Col-0 control and our experimental samples (figure 2-5). However again, we expected the *RTE1* overexpression and *rte1/rte1 rth* null lines to exhibit opposite effects on fatty acid formation but no significant differences between these samples were detected. Because we could not detect significant differences between the *RTE1* overexpression and *rte1* or *rte1 rth* null lines we conclude that RTE1 is likely not involved fatty acid modification reactions.

Cb5 and RTE1 are not oxygen sensors for the ethylene signaling pathway

An *in vitro* study from our laboratory has suggested that RTE1, like Cb5, may bind heme (Jianhong Chang's thesis 2011). Heme proteins are well known for their ability to serve as electron transport molecules as well as their ability to bind oxygen. Low oxygen (hypoxic) environments are known to affect ethylene response in plants and are often utilized in agriculture for long term storage of fruit and vegetables. Much of this is due to decreased ethylene production but there is evidence that oxygen is important for ethylene signal transduction as well (personal communication Mark Tucker, USDA; Xie et al., 2015). Given this possibility we hypothesized that Cb5 and RTE1 may be involved in oxygen sensing. Cb5 could bind an oxygen molecule and then pass the oxygen to RTE1. Oxygen binding to RTE1 could activate RTE1 which then activates the ETR1 receptor. To test this hypothesis I examined the "triple response" of *A. thaliana* seedlings in air, in ethylene, in 1.5% oxygen and in

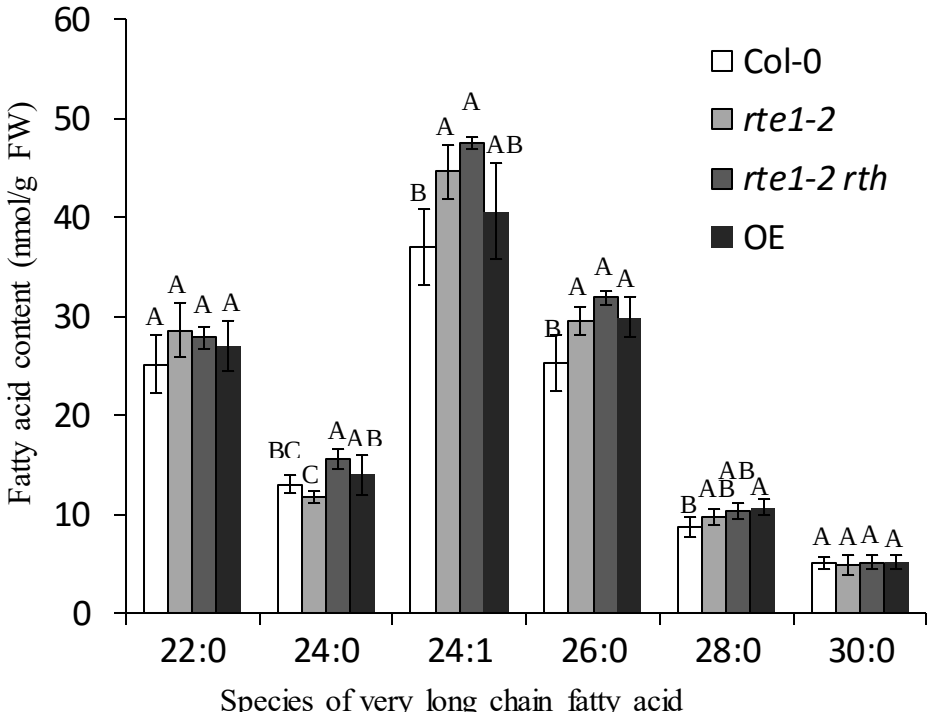


Figure 2-5: RTE1/RTH mutants do not alter very long chain fatty acid levels

Concentration of four six species of very long chain of fatty acids from the rosettes of wild-type (Col-0), *rte1-2*, *rte1-2 rth*, and *RTE1* overexpression (OE) *Arabidopsis* plants determined by GC-MS. Values indicate means ± standard deviation. Concentrations within a single fatty acid species were compared and samples with significantly different concentrations as determined by Tukey's test is indicated by different letters.

1.5% oxygen plus ethylene. We initially encounter a problem with the hypoxic conditions causing inconsistent seed germination making it impossible to accurately measure and compare hypocotyl lengths for the triple response assay. To overcome this problem, all seeds were allowed to first germinate in atmospheric oxygen. Seeds were then observed under a light microscope to confirm germination, and moved to hypoxic conditions. We hypothesized that if Cb5 and RTE1 were involved in oxygen sensing, then we should be able to observe a difference in ethylene sensitivity in seedlings wild-type for *Cb5* and *RTE1* versus loss-of-function *cb5* and *rte1* seedlings. Additionally we believed that the RTE1-independent ethylene insensitive *etr1-1* allele could not be affected by hypoxia while the RTE1-dependent ethylene insensitive *etr1-2* allele would be affected by hypoxia conditions. Our results however indicate that there was no difference in the ethylene insensitivity of any of our seedlings in hypoxic conditions versus normal oxygen environment (figure 2-6). These results suggest that Cb5 and RTE1 do not act as oxygen sensors.

C. elegans Cb5 and RTE1 interact in the split ubiquitin assay

Little is known about the animal RTE1 homolog. The only known target of RTE1, the ETR1 receptor, is not found in animals. Cb5, however, is conserved in both plants and animals suggesting that the RTE1/Cb5 interaction is also conserved in

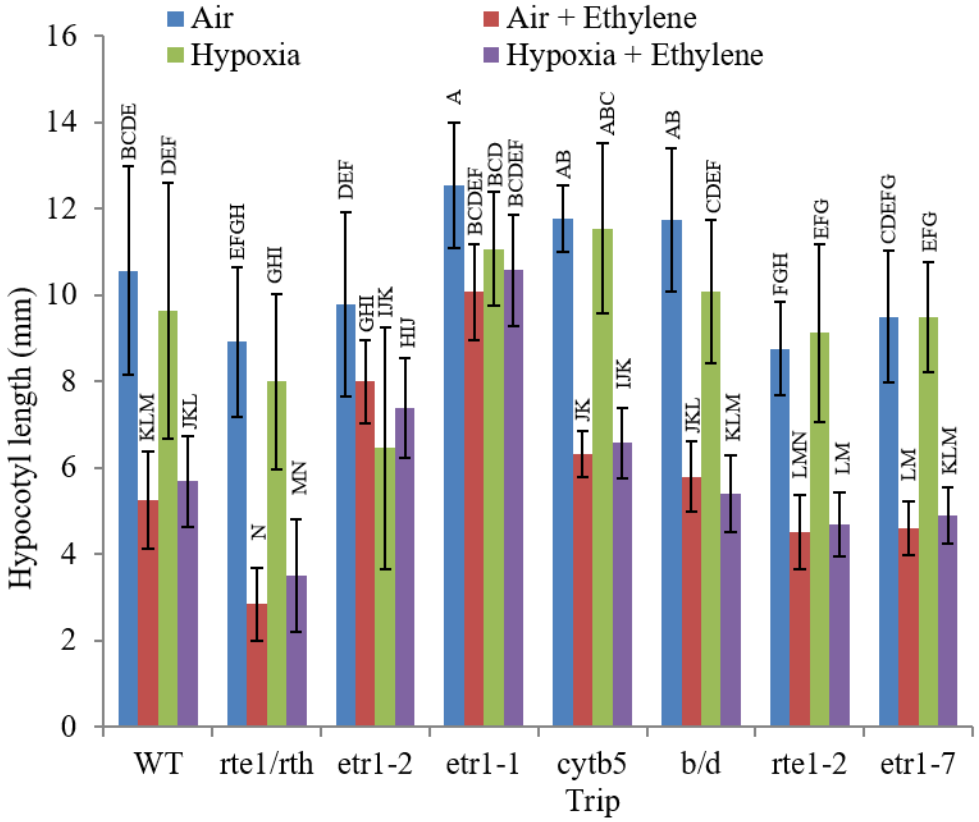


Figure 2-6: RTE1/Cb5s are not involved in oxygen signaling/ethylene signaling cross-talk

Hypocotyl lengths of 4-day-old etiolated germinated in air (40 hrs) then grown in either air, air + 10ppm ethylene, 2% oxygen (hypoxia) or 2% oxygen + 10ppm ethylene. Bars indicate hypocotyl lengths means. Error bars indicate standard deviation.

animals. To test the interaction of the *Caernorhabditis elegans* RTE1 homolog (H0J04.6) and several *C. elegans* Cb5s (W02D3, a mitochondria-localized Cb5; C31E10.7, an ER-localized Cb5; D2023.1 isoforms e and f, two ER-localized Cb5-like proteins; C25A1.5, an ER-localized FAH with a Cb5 domain), I used the split ubiquitin assay. The *C. elegans* RTE1 was cloned into the bait vector pMetYCGate, the *C. elegans* Cb5s were cloned into the prey vector pNX35 and bait and prey plasmids were cotransformed into yeast. A growth plate was used to confirm viability of yeast. Yeast cotransformed with RTE1 and empty prey or empty bait and a Cb5 did not grown on interaction selective plates. Yeast cotransformed with RTE1 and the mitochondrial Cb5 (W02D3) also did not grow on interaction selective plates. Yeast cotransformed with RTE1 and either C31E10.7, D2023.1e, D2023.1f or

animals. To test the interaction of the *Caernorhabditis elegans* RTE1 homolog (H0J04.6) and several *C. elegans* Cb5s (W02D3, a mitochondria-localized Cb5; C31E10.7, an ER-localized Cb5; D2023.1 isoforms e and f, two ER-localized Cb5-like proteins; C25A1.5, an ER-localized FAH with a Cb5 domain), I used the split ubiquitin assay. The *C. elegans* RTE1 was cloned into the bait vector pMetYCGate, the *C. elegans* Cb5s were cloned into the prey vector pNX35 and bait and prey plasmids were cotransformed into yeast. A growth plate was used to confirm viability of yeast. Yeast cotransformed with RTE1 and empty prey or empty bait and a Cb5 did not grown on interaction selective plates. Yeast cotransformed with RTE1 and the mitochondrial Cb5 (W02D3) also did not grow on interaction selective plates. Yeast cotransformed with RTE1 and either C31E10.7, D2023.1e, D2023.1f or

C25A1.5 grew on interaction selective plates suggesting that the *C. elegans* RTE1 homolog interacts with the four ER-localized *C. elegans* Cb5s (figure 2-7).

Discussion

RTE1 interacts with the electron transport protein Cb5, and together, RTE1 and Cb5 affect ethylene signaling through the ETR1 receptor. Cb5 proteins are involved in electron transfer through a bound heme molecule in plants, animals, fungi and some proteobacteria (Schenkman and Jansson, 2003). Interestingly, RTE1 was also shown to bind heme in an *in vitro* assay (Jianhong Chang's thesis, 2013). Together, these observations led us to the hypothesis that RTE1, like Cb5, was somehow involved in electron transport.

We considered the possibilities that Cb5 and RTE1 may be indirectly affecting the ETR1 receptor by altering the lipid membrane. Interestingly, the second protein pulled out of Jianhong's library screen, Lipid Transfer Protein1 (LTP1) is also necessary for ethylene response through ETR1 (Wang et al., 2016), further suggesting that RTE1's function involves lipids. This hypothesis is particularly exciting as it would suggest that RTE1 homologs in animals are also involved in lipid

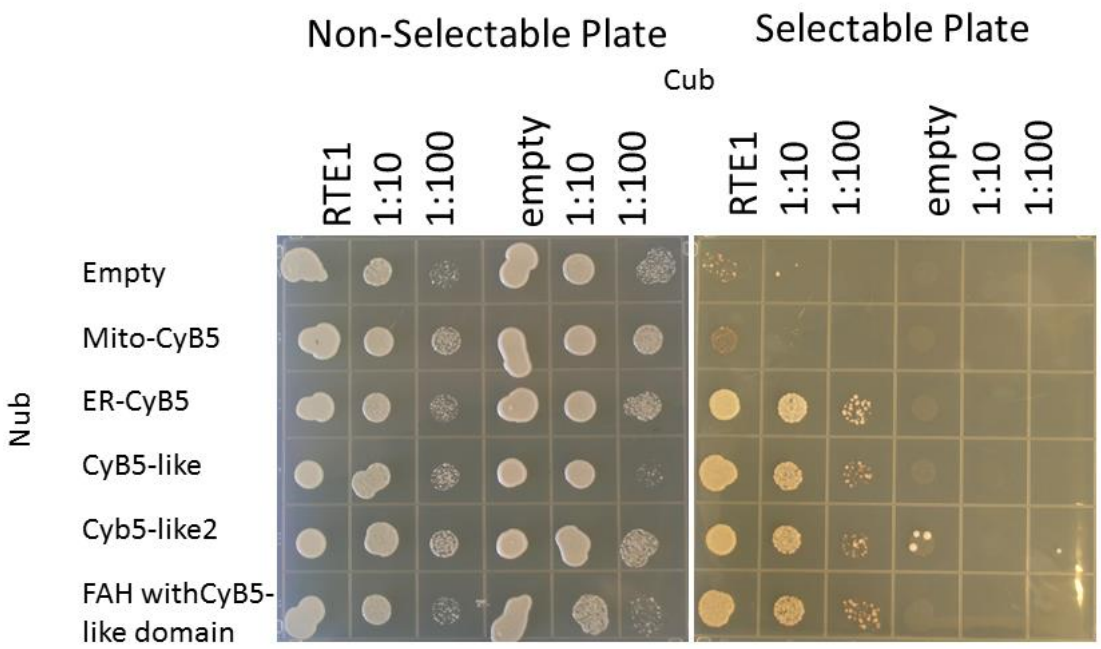


Figure 2-7: C. elegans RTE1 interacts with ER-localized C. elegans Cb5

Bait protein C. elegans RTE1 (H0J04.6) or empty bait plasmid were both paired with a mitochondria-localized C. elegans Cb5 (W02D3), ER-localized C. elegans Cb5 (C31E10.7), Cb5-like1 (D2023.1 isoform e), Cb5-like2 (D2023.1 isoform f), an ER-localized FAH with conjugated Cb5 (C25A1.5) or empty prey plasmid. Yeast growth without selection for interaction is indicated on plates lacking leucine and tryptophan (-LW, left). Interaction is indicated by growth on plates lacking leucine, tryptophan and alanine (-LWHA, right). Undiluted, 1:10 diluted and 1:100 diluted liquid cultures were spotted on plates and incubated for 3 days (–LW) or 5 days (-LWHA) at 30°C.

modification. However, we did not detect any significant difference in the levels of saturated, hydroxylated or very long chain fatty acids in double *rte1 rth* null and RTE1-overexpression *A. thaliana* plants. Importantly, these results do indicate that RTE1 is not necessary for Cb5 function outside of ethylene signaling and suggest that Cb5 and RTE1 may be affecting the receptor directly.

Our results indicate that Cb5 functions upstream of RTE1 in the ethylene signaling pathway consistent with previous observations that RTE1 interacts with ETR1 and our observations that Cb5 did not interact with ETR1. Expanding on our RTE1 redox hypothesis, Cb5 and RTE1 may be involved in redox folding of the ETR1 receptor. Upstream redox molecules such as Cytochrome B5 reducatase pass electrons to Cb5, Cb5 transfers the electrons to the heme molecule bound to RTE1, RTE1 then serves as a molecular chaperone for the ETR1 receptor by potentially reducing an incorrect disulfide bond on ETR1, allowing ETR1 to fold into an active conformation.

The function of RTE1 homologs in animals, which lack ethylene receptors, is unknown. By examining the function of the *A. thaliana* RTE1 (AtRTE1), we hope to understand the function of RTE1 homologs in animals as well. We initially found that AtRTE1 interacted with and required Cb5 to function in *A. thaliana*. We used this knowledge gained in this model plant system, applied it to animal homologs and found that the *C. elegans* RTE1 interacted with several *C. elegans* Cb5 in the split-ubiquitin yeast system. The conservation of the Cb5/RTE1 interaction suggests that this interaction may have originated before the most recent common ancestor of

plants and animals, an organism that existed 1.6 billion years ago (Hedges et al., 2004).

Materials and Methods

Plant materials and growth conditions

The Arabidopsis thaliana Columbia ecotype (Col–0) was used as the wild-type in all experiments. Nicotiana benthamiana was used for BiFC. Plants were grown in soil under a 16 h light per 8 h dark regimen in controlled environment chambers at 22°C under white fluorescent light. The triple response assay using ACC was performed as described previously (Resnick et al., 2006). Statistical analyses on hypocotyl lengths were performed either by Student's t test or by one-way ANOVA with a 95% confidence interval in conjunction with Tukey's test, using GraphPad's Prism software (http://www.graphpad.com/) to analyze samples and determine significant differences.

Yeast split-ubiquitin assays

To generate the CHX20 negative control, pDONR-CHX20 (a gift from Dr. Heven Sze) was transferred to the bait plasmid pNXgate33-3HA (C. Cappellaro and E. Boles, Institute for Microbiology, University of Frankfurt, Germany) using the Gateway cloning system (Invitrogen, http://www.lifetechnologies. com). Yeast plasmids were co-transformed with one of the Cb5 prey constructs (originally made

by Jianhong Chang) and either an empty bait, pNXgate33-RTE1 or pNXgate33-CHX20 (constructs described in Chang et al. 2013) into yeast strain THY.AP4.

To test interaction between *C. elegans* RTE1 and Cyb5, the *C. elegans* homologs were amplified with primers in Table 2-1. Amplified PCR products were cloned into pDONR221 via Gateway cloning. The *C. elegans* RTE1 homolog (H0J04.6) was transferred into pMetYCgate and the *C. elegans* Cb5 homologs were transferred into pNX35 (gift from Christopher Gefen).

All interactions were tested on agar medium lacking leucine, tryptophan, adenine and histidine. Yeast cells were maintained on enriched yeast extract/peptone/ dextrose agar plates or liquid medium at 30°C. Solid and liquid synthetic complete media comprised 0.17% yeast nitrogen base (US Biological, http://www.usbio.net/), 2% dextrose, 0.5% (NH4)2SO4 and amino acids.

BiFC: constructs, infiltration and microscopy

All plasmid clones described below were constructed using the Gateway cloning system (Invitrogen), except where noted, and all plasmid constructs were verified by DNA sequencing. PCR primer sequences are shown in Table S1. cYFP–RTE1 and ETR1–nYFP were described previously by Dong et al. (2010). The cYFP–RTE1 clone was tested for interaction with each nYFP–AtCb5 clone (encoding the N–terminal portion of YFP fused to the N–terminus of each AtCb5 isoform), which were all generated in the same way as follows. We first fused the nYFP fragment (encoding residues 1–155) to the 5′ end of the AtCb5 coding sequence by replacing a

fragment of ETR1 with the AtCb5 coding sequence in the existing nYFP–ETR1 plasmid (Dong et al. (2010). To accomplish this, we PCR-amplified the coding sequences using forward and reverse primers carrying the restriction sites AscI and XhoI, and ligated the resulting product using T4 DNA ligase into the AscI- and XhoI-digested nYFP–ETR1 plasmid. Using the resulting plasmid as a template, the fused sequences of nYFP–AtCb5 were then PCR-amplified and cloned into pDONR221, verified by DNA sequencing, and finally transferred into binary plant transformation vector pEarleygate100 (Earley et al., 2006) containing the CaMV 35S promoter. nYFP–RTE1 was constructed in exactly the same way, but using a primer carrying the restriction site SpeI in place of XhoI. To construct the plasmid expressing cYFP–AtCb5–D, the full-length AtCb5–D coding sequence with a 5′ linker sequence and the C–terminal portion of the YFP molecule (amino acids 156–239) with a complementary 3′ linker sequence were PCR-amplified from Cytochrome b5 affects ethylene receptor signaling 565 an AtCb5–D cDNA clone and the pSPYCE vector (Walter et al., 2004), respectively. Purified PCR products were then combined in a fusion PCR using a forward primer with an attB1 site complementary to the 5′ end of the cYFP coding sequence and a reverse primer with an attB2 site complementary to the 3′ end of the AtCb5–D coding sequence. The amplified product was cloned into pDONR221, verified by DNA sequencing, and then transferred into the binary plant transformation vector pH2GW7 (Karimi et al., 2002) containing the CaMV 35S promoter.

H20J04.6attB1F- GGGGACAAGTTTGTACAAAAAAGCAGCCTTCATGATGCCGCTCACCGACC
H20J04.6attB1R- GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAAAAAGCCCACAGCGCAAC
C25A1.5 attB1F- GGGGACAAGTTTGTACAAAAAAGCAGCCTTCATGGGGATAGGAGCAGATGAGAAG
C25A1.5 attB2R- GGGGACCACTTTGTACAAGAAAGCTGGGTTTAATGGGCCCATTCCGAGTGTG
C31E10.7attB1F- GGGGACAAGTTTGTACAAAAAAGCAGCCTTCATGGCCGATCTTAAGCAAATC
C31E10.7attB2R- GGGGACCACTTTGTACAAGAAAGCTGGGTTCGCAGCGATAAGATAATAAACAAG
D2023.1battB1F- GGGGACAAGTTTGTACAAAAAAGCAGCCTTCATGTCTGACTCCATCATCACCC
D2023.1battB2R- GGGGACCACTTTGTACAAGAAAGCTGGGTTATGAGCCATTGGAAGAGAGATC
D2023.1fattB1F- GGGGACAAGTTTGTACAAAAAAGCAGCCTTCATGGCCAATGAGAGTTCAGG

D2023.1fattB2R- GGGGACCACTTTGTACAAGAAAGCTGGGTTATGAGCCATTGGAAGAGAGAATC
W02D3attB1F- GGGGACAAGTTTGTACAAAAAAGCAGCCTTCATGTCGGAGCTACGTGTCATTTC
W02D3attB2R- GGGGACCACTTTGTACAAGAAAGCTGGGTTATTGAACATGCACTTGTACACGGC

Table 2-1: Primers used to clone *C. elegans RTE1* and *Cb5* homologs

The above nYFP and cYFP constructs were co-transformed into Agrobacterium tumefaciens strain C58C1, and selected on the basis of kanamycin and spectinomycin resistance, respectively. For tobacco infiltration, liquid cultures of the agrobacteria were prepared as described by Voinnet et al. (2003), and each culture was combined with a separate liquid culture of Agrobacterium carrying the p19 plasmid (encoding the RNAi silencing inhibitor from the Cymbidium ringspot virus) in a 1:1 ratio, and the resulting mixtures were used to infiltrate leaves of 3-week old tobacco plants as described by Voinnet et al. (2003). Tobacco leaf pieces were directly mounted on glass slides in a drop of water, and YFP was observed using laser scanning confocal microscopy (Zeiss LSM710, http://corporate.zeiss.com/gateway/en_de/home.html). For ETR1–nYFP + cYFP–AtCb5–D, we examined 30 plants (one or two leaves per plant). For CTR1–nYFP + cYFP–AtCb5–D, we examined nine plants (one or two leaves per plant).

Oxygen sensing experiments

Arabidopsis seedlings were germinated on MS agar plates for 40 hours in the dark at 22°C. Seedlings were then transferred to desiccation chambers with a flow-through system that continuously circulated either normal or hypoxic air (2.8% oxygen) with or without ethylene (10ppm).

Fatty Acid Analysis

Analysis of fatty acid saturation and hydroxylation was performed by Dr. Toshiki Ishikawa in collaboration with Dr.Maki Kawai-Yamada lab at Saitame University. Samples were analyzed via gas-chromatography as previously described (Nagano et al., 2009b).

Chapter 3: Proline 9 is important for RTE1-dependence in Arabidopsis and Oryza ERS1 receptors

Introduction

Ethylene is a gaseous plant hormone that affects growth, development and responses to biotic and abiotic stress (Abeles et al., 1992). Dark-grown, ethylene treated, 4-day-old *Arabidopsis thaliana* seedlings exhibit a "triple response" phenotype consisting of the formation of an apical hook, inhibition of root elongation and, shortening and thickening of the hypocotyl (Bleecker et al., 1988; Guzman and Ecker, 1990). The isolation of mutants with altered triple response phenotypes has been an effective tool for deciphering components of the ethylene signaling pathway.

Ethylene is perceived at the endoplasmic reticulum in *Arabidopsis* by five ethylene receptors (Chen et al., 2002). These receptors interact with and activate the serine/threonine protein kinase CTR1 (Clark et al., 1998). In the absence of ethylene, CTR1 phosphorylates the next downstream component EIN2, thereby blocking the ethylene signaling cascade (Chen et al., 2011; Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). With the addition of ethylene, the receptor/CTR1 complex is inactivated allowing EIN2 to now activate downstream ethylene responses (Li et al., 2015; Merchante et al., 2015).

The plant ethylene receptors show homology to bacterial two-component histidine protein kinase receptors and possibly were introduced to the plant lineage during plastid endosymbiosis (Mount and Chang, 2002). The N-terminus of the ethylene receptors contains 3 or 4 transmembrane domains that bind a copper cofactor

necessary for ethylene binding (Rodriguez et al., 1999). Proceeding the ethylene binding domain is a GAF domain that is necessary for dimerization of the ethylene receptors (Gao et al., 2008; Grefen et al., 2008; Xie et al., 2006). The GAF domain is followed by a protein kinase domain. The presence of this domain is important for ethylene signaling through the receptor, however, the kinase activity itself is less important (Gamble et al., 2002; Qu and Schaller, 2004). In bacterial two-component systems, a histidine kinase phosphorylates the receiver domain of a regulator protein in response to a signal. Several of the ethylene receptors have this receiver domain fused to the c-end of the kinase domain while other receptors lack this domain. The receiver domain has been suggested as having a role in ethylene response recovery but appears unnecessary for ethylene signaling (Binder et al., 2004).

Gene duplication of ethylene receptors in an ancient algal ancestor has resulted in the receptors being divided into two receptor subfamilies (Hua et al., 1998; Ju et al., 2015). In *Arabidopsis,* the subfamily I receptors, ETR1 and ERS1, have three predicted transmembrane domains and histidine kinase activity. Much of what is known about the plant ethylene receptors comes from studies on the ETR1 receptor from *Arabidopsis*. Less is known about the ERS1 receptors especially from other organisms. The high level of sequence similarity between ETR1 and ERS1 (76% identity, 84% similarity across the transmembrane domains of these receptors) allows us to effectively compare these receptors to better understand subtle differences in the functions of these receptors. The subfamily II receptors, ETR2, EIN4 and ERS2 have four predicted transmembrane domains and have serine/threonine kinase activity (Xie

et al., 2003). The subfamily I and II receptors are largely redundant, as gain-of-function mutations in any of the five receptors confer dominant ethylene-insensitivity in Arabidopsis.

A major structural difference between ethylene receptors involves the presence or absence of a receiver domain. ETR and EIN4 ethylene receptors are true hybrid kinase sensors in that they have a receiver domain fused to the c-end of the kinase domain. Ethylene receptors are annotated as ERS (Ethylene Response Sensor) if they have lost this receiver domain. At least two separate evolutionary events have resulted in the loss of the receiver domain and the formation of an ERS receptor (Gallie, 2015). ERS2 is found only in Brassicas and is derived from an Subfamily II ETR2-like receptor that has lost the receiver domain (Gallie, 2015). ERS1 receptors are more widespread having evolved from Subfamily I ETR1-like receptor in an early Angiosperm. It is important to consider that even though these ERS1 receptors are annotated as ERS due to the absence of a receiver domain, this annotation tells us little about the actual function of the receptors (Gallie, 2015). In fact, several early Angiosperms and Monocots lack ETR1 receptors altogether and instead have one or more ERS1 receptors potentially serving as the primary ethylene receptor in replace of the missing ETR1 receptors (Gallie, 2015)

Many gain-of-function mutations have been identified in the transmembrane domain of the ethylene receptors in *Arabidopsis* (Wang et al., 2006). These gain-of-function mutations switch the receptors into an always on conformation, repressing ethylene responses and conferring ethylene insensitivity. Typically, a mutation that

causes ethylene insensitivity in one receptor will also cause insensitivity in the other receptors (Wang et al., 2006). However, a subset of ETR1 gain-of-function mutations do not confer ethylene insensitivity in the other receptors (Rivarola et al., 2009). For example, *etr1-2* (*etr1 A102T*, alanine 102 substituted for a threonine) plants are ethylene insensitive while plants expressing the ERS receptor with the analogous amino acid substitution (*ers1* A102T) remain sensitive to ethylene. These ETR1-specific mutations distinguish ETR1 from ERS1 and are important in understanding the differences between the receptors.

REVERSION-TO-ETHYLENE-SENSITIVITY1 (RTE1) was identified in a screen for mutants that could suppress the ethylene-insensitive *etr1-2* allele (Resnick et al., 2006). RTE1 is important for ethylene signaling through the ETR1 receptor. Unlike ETR1, the other four ethylene receptors, including the other subfamily I receptor, ERS1, do not require RTE1 to function (Resnick et al., 2006; Rivarola et al., 2009). Furthermore, the ETR1-specific ethylene insensitive mutations (such as *etr1 A102T* mentioned above) are RTE1-dependent while mutations that confer ethylene insensitivity in both ETR1 and ERS1 are not RTE1-specific (Resnick et al., 2008). These observations suggests that there may be a connection between RTE1-dependence and the ETR1-specific insensitive mutations.

RTE1 homologs have been identified and studied in several different species of plants outside of *Arabidopsis*. The RTE1 homolog in tomato, GREEN-RIPE, is important for fruit ripening (Barry and Giovannoni, 2006; Barry et al., 2005; Ma et al., 2012). RTE1 homologs have also been identified and have been shown to be

important for ethylene response in several dicot flowers (Yang et al., 2015; Yu et al., 2010, 2011). The rice RTE1 homolog in the monocot *Oryza sativa*, OsRTH1, is capable of rescuing an *Arabidopsis rte1* mutant (Wei Zhang, Xin Zhou, 2012). Overexpression of *OsRTH1* in *Oryza* confers ethylene insensitivity, despite the fact that *Oryza* lacks ETR1 receptors suggesting that OsRTH1 affecting ethylene signaling through a different ethylene receptor.

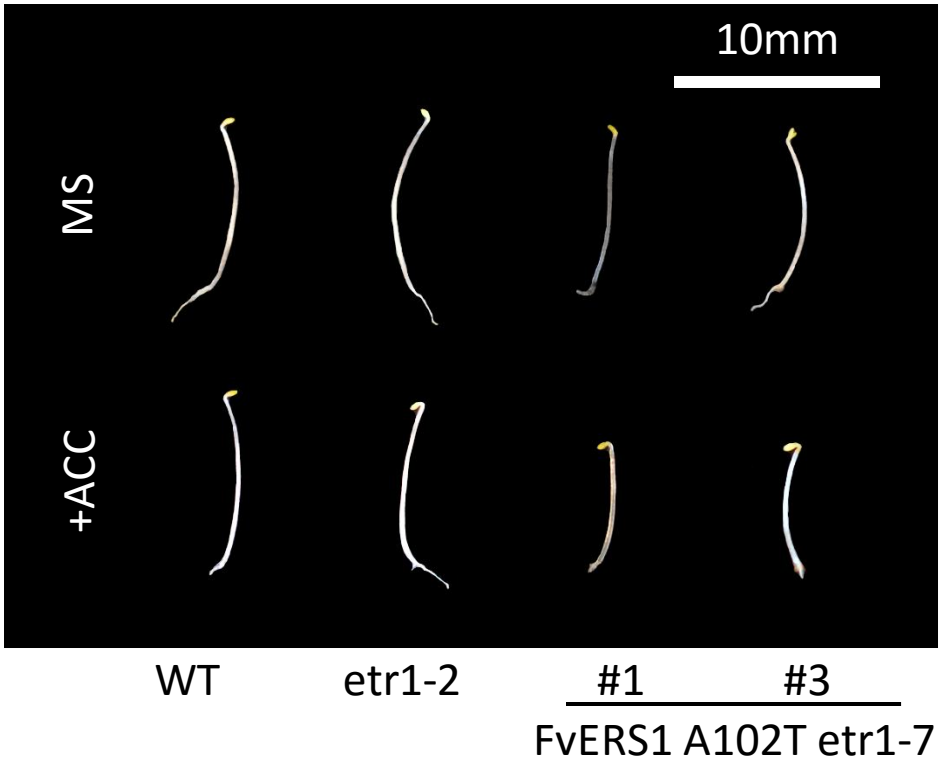
Although the ETR1 and ERS1 receptors have very conserved sequences, ETR1 is RTE1-dependent while ERS1 is not. We do not understand what makes a receptor RTE1-dependent. In this chapter, I examine ETR1 and ERS1 protein sequences and identify an N-terminally located proline that is conserved in ETR1 receptors but not dicot ERS1 receptors. We then test if we can convert ERS1 into a RTE1-dependent receptor by substituting in the proline conserved in ETR1 receptors. Furthermore, we find that ERS receptors from monocots such *Oryza sativa* have this conserved proline. We then examine whether the OsERS receptors are RTE1-dependent. Our findings suggests that this proline is partially responsible for RTE1-dependence in ethylene receptors and that RTE1-dependence in the subfamily I receptors originated during or before early angiosperms.

Results

AtERS1 is not a unique ERS1 receptor

Before making any assumptions about whether ERS1 receptors were RTE1-independent, we considered it important to first confirm other dicot ERS1 receptors

function similarly to AtERS1. This was particularly important as an alignment of the AtERS1 sequence with other dicot ERS1s reveals that AtERS1 has slightly diverged amino acid sequence compared to the other dicot ERS1s particularly in the first 15 amino acids (Figure 3-2). It is therefore plausible that AtERS1 is unique and therefore not necessarily a good model for dicot ERS1s. Recall, a key functional difference that distinguishes the highly similar subfamily I receptors AtETR1 and AtERS1 is whether ETR1-specific mutations, such as the A102T mutation, confers ethylene insensitivity. Moreover, we believe that there is correlation between these ETR1-specific mutations and RTE1-dependence. The mutation encoding A102T, was introduced into an FvERS1 transgene expressed under the control of a 35S promoter (Figure 3-1). We assayed the seedling triple response in four independent lines, and found that like AtERS1(A102T), FvERS1(A102T) did not confer ethylene insensitivity even using a low dose of ACC. These results suggests that FvERS1 is similar to AtERS1 and that the AtERS1 receptor is not unique.


10mm
MS
+ACC
WT
etr1-2
#1
#3
FvERS1 A102T etr1-7

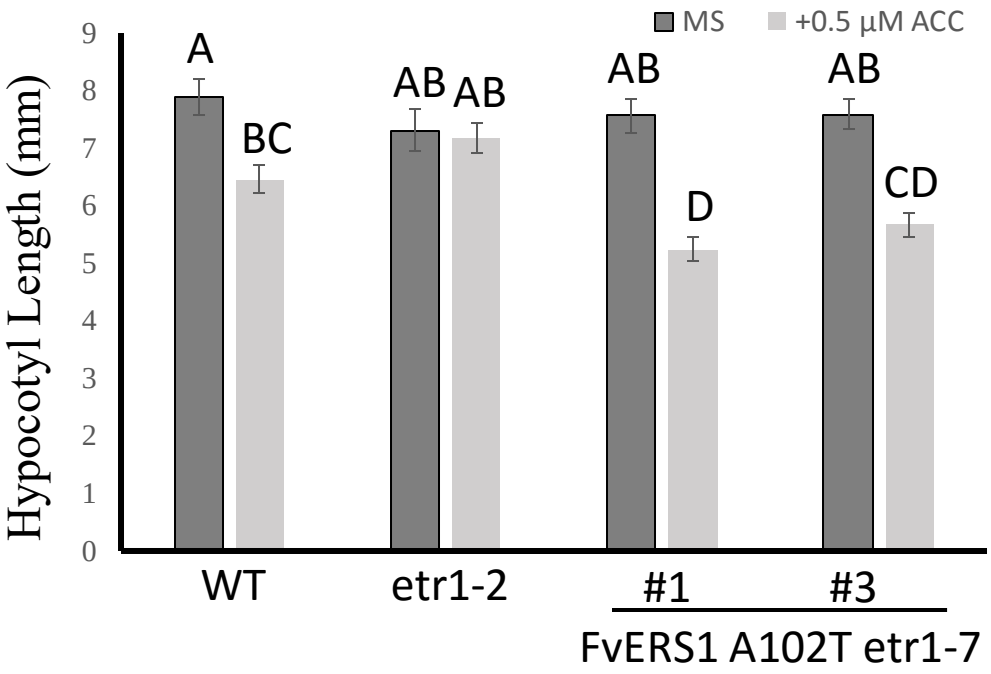

MS
+0.5 µM ACC
Hypocotyl Length (mm)
0
1
2
3
4
5
6
7
8
9
A
BC
AB
AB
AB
D
AB
CD
WT
etr1-2
#1
#3
FvERS1 A102T etr1-7

Figure 3-1: Fvers1 A102T, similarly to Aters1 A102T does not confer ethylene insensitivity.

Upper- Representative 4-day-old etiolated Arabidopsis seedlings grown on MS plates or MS plates + 0.5 μM ACC. Two independent transgenic lines (#1 and #3) of Fvers1 A102T etr-7 are sensitive to ethylene as compared to etr1-2 (A102T) and WT (Col-0) controls.

Lower- Measurements of hypocotyl lengths of 4-day-old seedlings described above on either MS (dark grey) or MS + 0.5 μM ACC (light grey). Values indicate means ± standard error. Different letters above bars indicate samples that are significantly different.

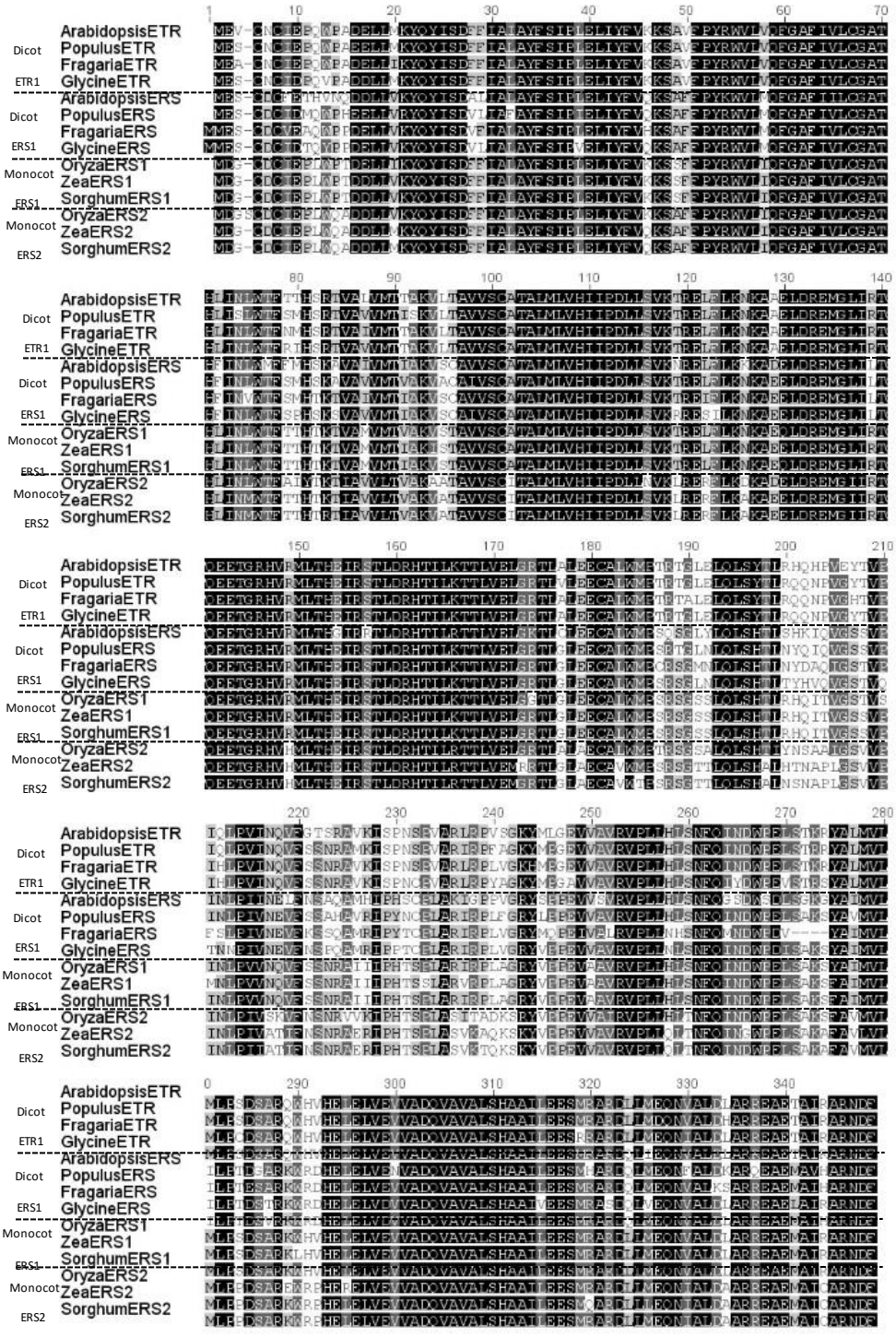

ArabidopsisETR
PopulusETR
FragariaETR
GlycineETR
ArabidopsisERS
PopulusERS
FragariaERS
GlycineERS
OryzaERS1
ZeaERS1
SorghumERS1
OryzaERS2
ZeaERS2
SorghumERS2
Dicot
ETR1
Dicot
ERS1
Monocot
ERS1
Monocot
ERS2

Figure 3-2: Proline 9 is conserved in dicot ETR1 receptors and monocot ERS1 receptor but not dicot ERS1.

Amino acid alignment of subfamily I ethylene receptors from several representative dicot and monocot species. Dicot and monocot ERS receptors share a more recent ancestor than dicot ETR receptors. Arrows indicate amino acids examined in this paper, cysteine 4, cysteine 6, and proline 9. Black boxes indicated identity among all receptors in alignment, grey boxes indicate similarity among most of the receptors in alignment.

Sequence Comparison of Subfamily I Ethylene Receptors

To better understand differences in subfamily I ethylene receptors that could cause a receptor to be RTE1-dependent or independent, we aligned the amino acid sequences of several angiosperm subfamily I ethylene receptors and compared their sequences (Figure 3-2). The ethylene binding domain of these receptors is highly conserved and yielded few conserved differences between ETR1 and ERS1 receptors. One exception, however, was a proline at amino acid 9. Proline 9 is conserved in all ETR1 sequences examined but is absent in all dicot ERS1 sequences examined. Interestingly, monocots lack ETR1 receptors but instead have ERS1 receptors with this conserved proline (Gallie, 2015, Figure 3-3). It is possible that these monocot ERS1 receptors are functionally more similar to dicot ETR1 receptors than the dicot ERS1 receptors and therefore could be RTE1-dependent. The conservation of proline 9 is particularly noteworthy due to proline's unique structure that causes bends and protein rigidity. The proximity of this proline to cysteines 4 and 6, which are responsible for forming intermolecular disulfide bonds with another receptor monomer to form the active receptor dimer, is also potentially important (Chen et al., 2010; Schaller et al., 1995). Therefore we considered proline 9 a potentially significant difference between dicot ERS1 and ETR1 receptors.

AtERS1 T9P is RTE1-dependent

Jianhong Chang, a former graduate student, tested if she could make AtERS1 RTE1-dependent by substituting proline for the threonine at position 9. In order to

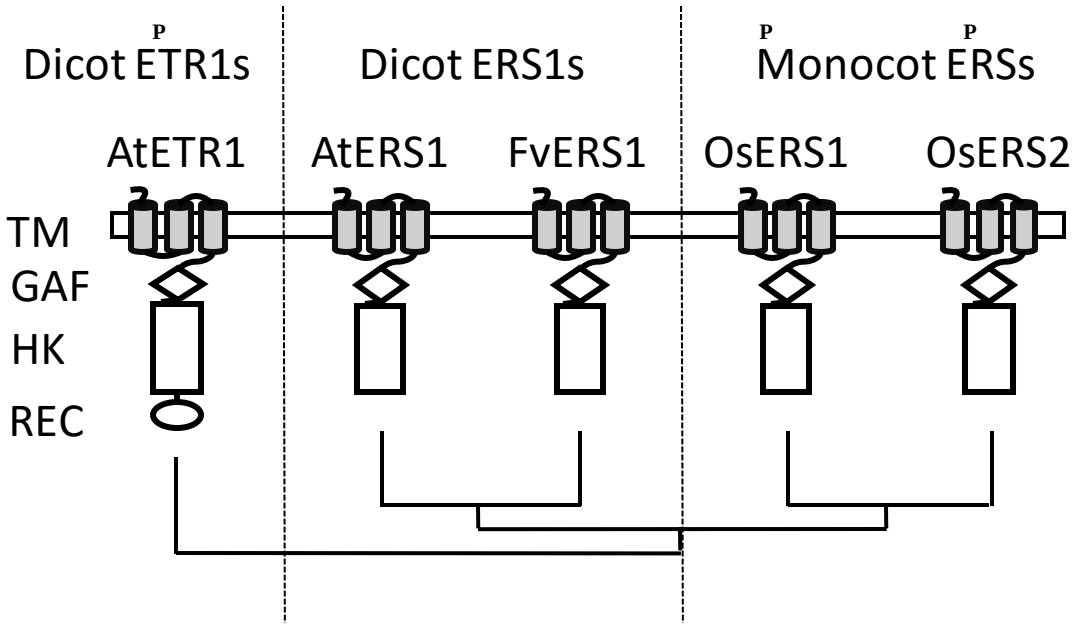


Figure 3-3: Ethylene receptors discussed in this chapter.

Upper: Dicot ETR1s are represented by Arabidopsis ETR1. Dicot ERS1s are represented by *F. vesca* and *A. thaliana* ERS1. Monocot ERSs are represented by OsERS1 and OsERS2. The relative positions of the ethylene receptor domains indicated; TM (transmembrane domain), GAF, HK (histidine kinase domain) and REC (receiver domain;ETRs only). Receptors with the conserved N-terminal proline are indicated with a P.

Lower: A simplified gene tree indicating that the ERS receptors from dicot and monocot are more closely related to each other than to the dicot ETR1s.

more easily distinguish any differences this proline substitution may cause, she introduced the T9P substitution in the T*ers1(A102T)* transgene and expressed in *Arabidopsis* seedlings under control of the ETR1 promoter. As previously shown, the A102T mutation is ETR1-specific as plants expressing T*ers1(A102T)* did not confer ethylene insensitivity in wild-type plants (Rivarola et al., 2009). However, plants transgenically expressing an *ers1* receptor with both the T9P and A102T substitutions (T*ers1 T9P A102T*) were partially ethylene insensitive (Figure 3*)*. These results indicate that the T9P substitution makes the AtERS1 receptor functionally more like AtETR1 suggesting the proline 9 is an important difference in the function of these two receptors.

To determine if ers1(T9P A102T) was RTE1-dependent, Jianhong crossed T*ers1(T9P A102T)* plants with plants containing the loss-of-function allele *rte1-3*. The resulting line, T*ers1(T9P A102T) rte1-3*, was hypersensitivity to ethylene similar to *rte1-3* plants (Figure 3-4). This confirms that ers1(T9P A102T) requires RTE1 to be functional further indicating that ers1(T9P) is functionally more similar to an AtETR1 receptor than an ERS1 receptor and suggests proline 9 is an important distinction between dicot ERS and ETR1 receptors.

I then tested if the reciprocal substitution could make AtETR1 RTE1-independent. We again took advantage of the ETR1-specific RTE1-dependent ethylene insensitive mutation A102T and this time substituted out the proline of the AtETR1 receptor to generate Tetr1(P9T A102T), which we expressed in *Arabidopsis* controlled by the ETR1 promoter. Although adding this proline to Ters1(A102T) had

resulted in ethylene insensitivity, removing the proline from Tetr1(A102T) did not alter ethylene sensitivity; *Tetr1(P9T A102T)* still conferred insensitivity to ethylene (Figure 3-5). *Tetr1(P9T A102T)* plants were then crossed with *rte1-2* loss-of-function plants to test for RTE1-dependence. *Tetr1(P9T A102T) rte1-2* plants were no longer insensitive to ethylene, indicating that Tetr1(P9T) is still dependent on RTE1.

AtETR1 4A6A is still RTE1-dependent

Having confirmed that proline 9 has some importance in determining if a receptor is RTE1-dependent, we considered the possibility that the unique properties of proline, especially its propensity to cause rigidness and bends in the structure of a protein and the effect it may have on the intermolecular disulfide bonds formed by cysteines 4 and 6. In a potentially analogous example, the yeast SOD1 protein requires a chaperone to form correct disulfide bonds that is blocked by a proline two amino acids away (Leitch et al., 2009). The human SOD1 protein lacks this proline and does not require the chaperone to fold correctly (Leitch et al., 2009). Furthermore, Cytochrome B5, a known redox molecule, interacts with RTE1 and is important for ethylene signaling through ETR1 (Chang et al., 2014) suggesting the possibility that RTE1 may serve as a chaperone of ETR1 to help the receptor form the intermolecular disulfide bonds.

The ETR1 receptor with cysteines 4 and 6 substituted for alanines, etr1(4A6A), is unable to form disulfide bond-linked receptor dimers but can rescue

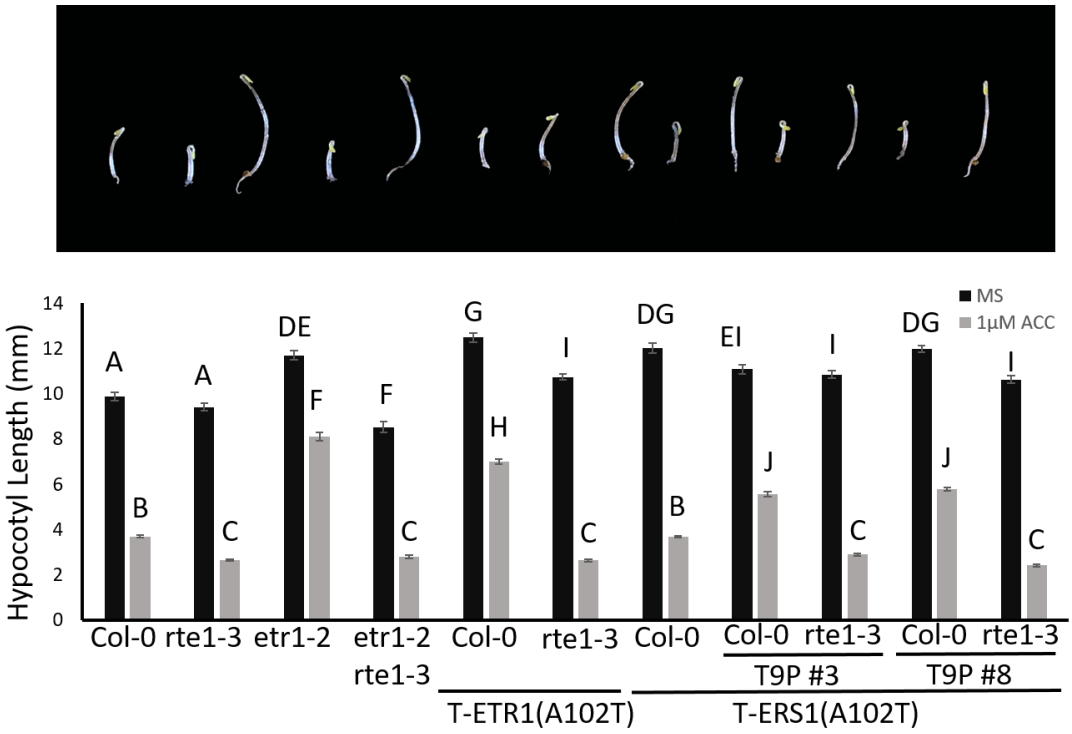


Figure 3-4: Aters1 T9P A102T confers ethylene insensitivity and is RTE1-dependent.

Upper- Representative 4-day-old etiolated Arabidopsis seedlings grown on 1µM ACC. WT (Col-0), the loss-of-function rte1-3 allele, etr1-2, etr1-2 rte1-3, transgenic etr1 A102T, etr1 A102T rte1-3, and transgenic ers1 A102T are previously published controls. Two independent lines (#3 and #8) of transgenic Aters1 T9P A102T display ethylene insensitivity unlike Aters1 A102T. These two lines crossed with rte1-3 are no longer insensitive to ethylene indicating a dependence on RTE1 for signaling. **Lower-** Measurements of hypocotyl lengths of 4-day-old etiolated Arabidopsis seedlings from part (a) grown on MS plates (black) or MS plates with 1µM ACC

(light grey). Values indicate means ± standard error. Different letters above bars indicate samples that are significantly different.

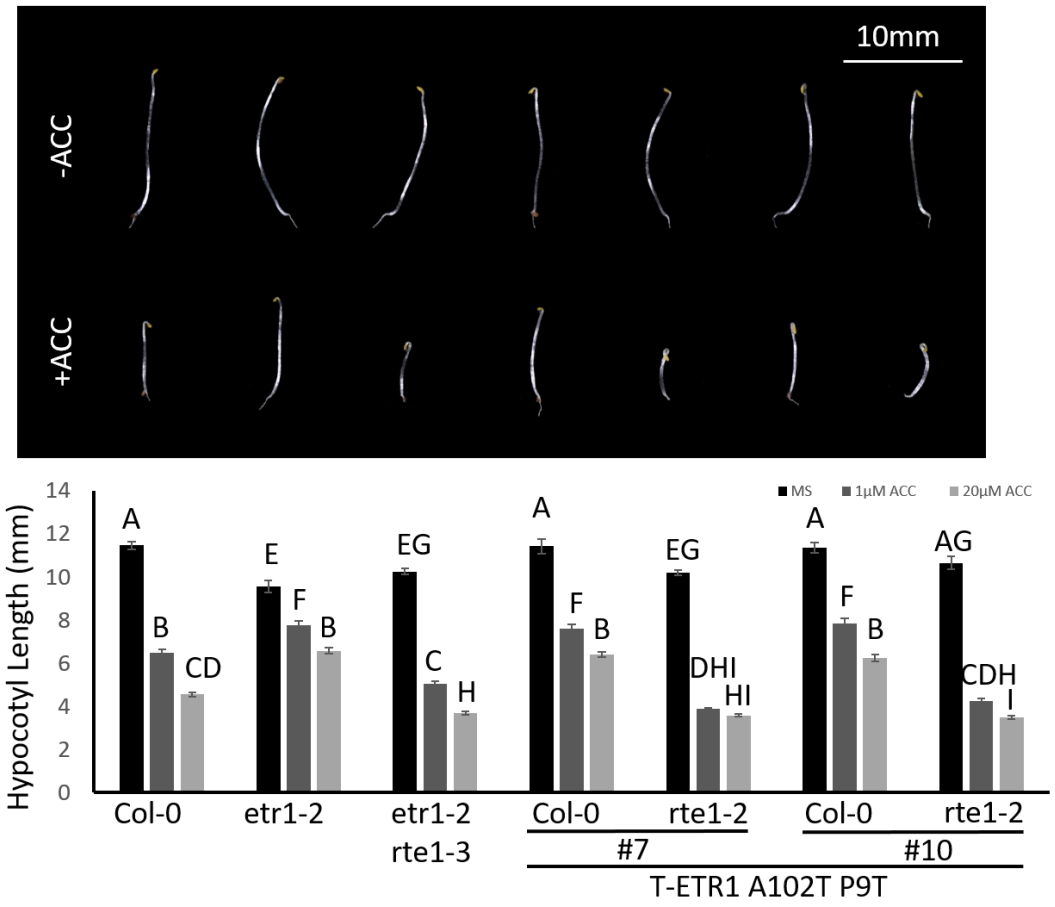


Figure 3-5: Atetr1 P9T A102T still confers ethylene insensitivity and is RTE1-dependent.

Upper- Representative 4-day-old etiolated Arabidopsis seedlings grown on MS with and without 1µM ACC. Two independent lines (#7 and #10) of transgenic Atetr1 P9T A102T display ethylene insensitivity similarly to etr1-2 (A102T). These two lines crossed with rte1-2 are no longer insensitive to ethylene indicating that Atetr1 P9T is still RTE1-dependent.

Lower- Measurements of hypocotyl lengths of 4-day-old etiolated Arabidopsis seedlings from part (a) grown on MS plates (Black), MS + 1µM ACC (dark grey) or

MS + 20μM ACC (light grey). Values indicate means ± standard error. Different letters above bars indicate samples that are significantly different.

the normally infertile *etr1-7 ers1-2* double receptor loss-of-function mutant (Chen et al., 2010; Xie et al., 2006). If RTE1 functioned as a chaperone that assisted in the formation of correct disulfide bonds created by these cysteines, then we would predict that the etr1(4A6A) receptor would not require RTE1 for its function. T*etr1(4A6A) etr1-7 ers1-2* plants were crossed with *etr1-7 rte1-2* to obtain the quadruple homozygote T*etr1(4A6A) etr1-7 ers1-2 rte1-2*. T*etr1(4A6A) etr1-7 ers1-2 rte1-2* plants were hypersensitive to ethylene compared to T*etr1(4A6A) etr1-7 ers1-2* (with wild-type *RTE1*) (Figure 3-6). These results indicate that the etr1(4A6A) receptor is still RTE1-dependent suggesting that RTE1 does not function on these cysteines.

OsERS1 requires AtRTE1 to be functional in Arabidopsis

Even though *O. sativa* lacks ETR1 receptors, the rice RTE1 homolog is important for ethylene signaling (Wei Zhang, Xin Zhou, 2012). Interestingly, the two ERS receptors *O. sativa*, OsERS1 and OsERS2, have the conserved proline we previously identified as partially important for determining RTE1-dependence. Together these observations suggest that the *O. sativa* ERS receptors may be RTE1-dependent. To confirm this hypothesis, we stably expressed *OsERS1* and *OsERS2* under the control of the native AtETR1 promoter in *Arabidopsis* seedlings. *T-OsERS1* and *T-OsERS2* were both able to rescue the ethylene hypersensitive phenotype of *etr1-7* seedlings. *T-OsERS1* in the *etr1-7 rte1-2* background however

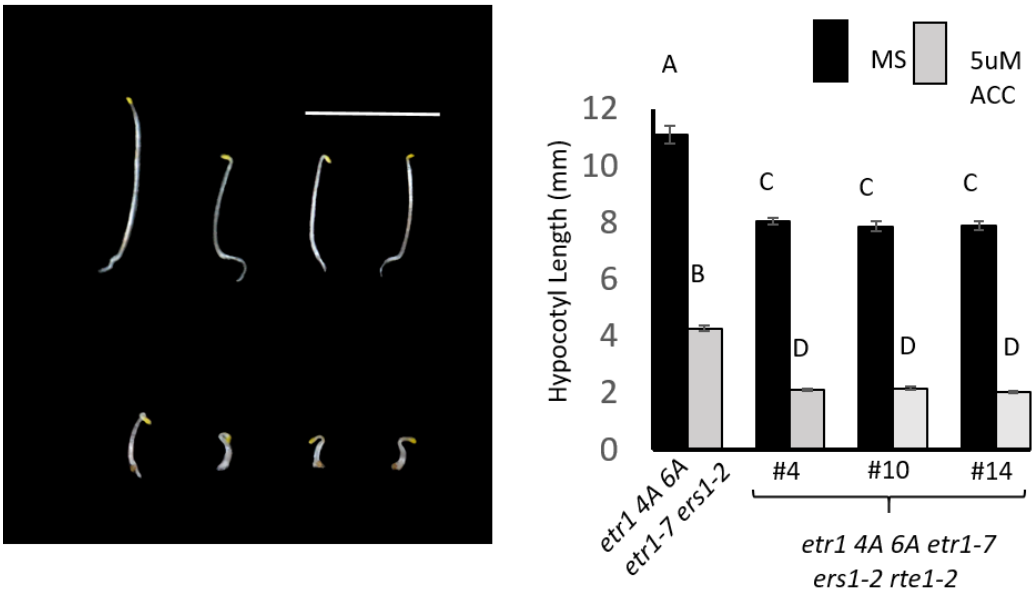


Figure 3-6: etr1 4A 6A is RTE1-dependent.

Upper-Representative 4-day-old etiolated Arabidopsis seedlings grown on MS with and without 5µM ACC. etr1 4A 6A etr1-7 ers1-2 was crossed with etr1-7 rte1-2 to generate etr1 4A 6A etr1-7 ers1-2 rte1-2. etr1 4A 6A etr1-7 ers1-2 rte1-2 is hypersensitive compared to etr1 4A 6A etr1-7 ers1-2 confirming that etr1 4A 6A is still RTE1-dependent.

Lower-Measurements of hypocotyl lengths of 4-day-old etiolated Arabidopsis seedlings from (a) grown on MS (black) or 5µM ACC (light grey). Values indicate means ± standard error. Different letters above bars indicate samples that are significantly different.

displayed ethylene hypersensitivity confirming that *T-OsERS1*, like ETR1, is dependent on RTE1 (Figure 3-7). *T-OsERS2* in the etr1-7 rte1-2 background may indicate partial dependence on RTE1 (Figure 3-7) but no conclusion could be drawn from these results.

There is a correlation between ETR1-specific mutations, like the A102T mutation, and RTE1-dependence (Resnick et al., 2006; Rivarola et al., 2009). If plants expressing *Osers1 A102T* and *Osers2 A103T* are insensitive to ethylene then this would suggest that both of these receptors are RTE1-dependent. Furthermore, if *Osers2 A103T* plants are ethylene insensitive, then determining whether the receptor is RTE1-dependent should be easier as the difference in ethylene insensitive and ethylene sensitive should be exaggerated. Like before, we stably expressed *T-Osers1(A102T)* and *T-Osers2(A103T)* under the control of the native AtETR1 promoter in *Arabidopsis* seedlings. *T-Osers1(A102T)* in the *etr1-7* background conferred ethylene insensitivity at a level similar to Arabidopsis *etr1-2* (Figure 3-8). *T-Osers2(A103T)* conferred a stronger ethylene insensitive phenotype than *etr1-2* (Figure 3-9). The same constructs were transformed into the *etr1-7 rte1-2* double loss-of-function mutant. Insensitivity conferred by T-*Osers1(A102T)* was greatly suppressed in this mutant (Figure 3-8) suggesting that OsERS1 is similar to AtETR1 in that the A102T mutation confers ethylene insensitivity and is RTE1-dependent. *T-Osers2(A103T)* ethylene insensitivity appeared to only be partially suppressed in *etr1-7 rte1-2* seedlings (Figure 3-9). These results indicate that while OsERS1 is dependent on RTE1, OsERS2 is only partially dependent on RTE1 and would explain

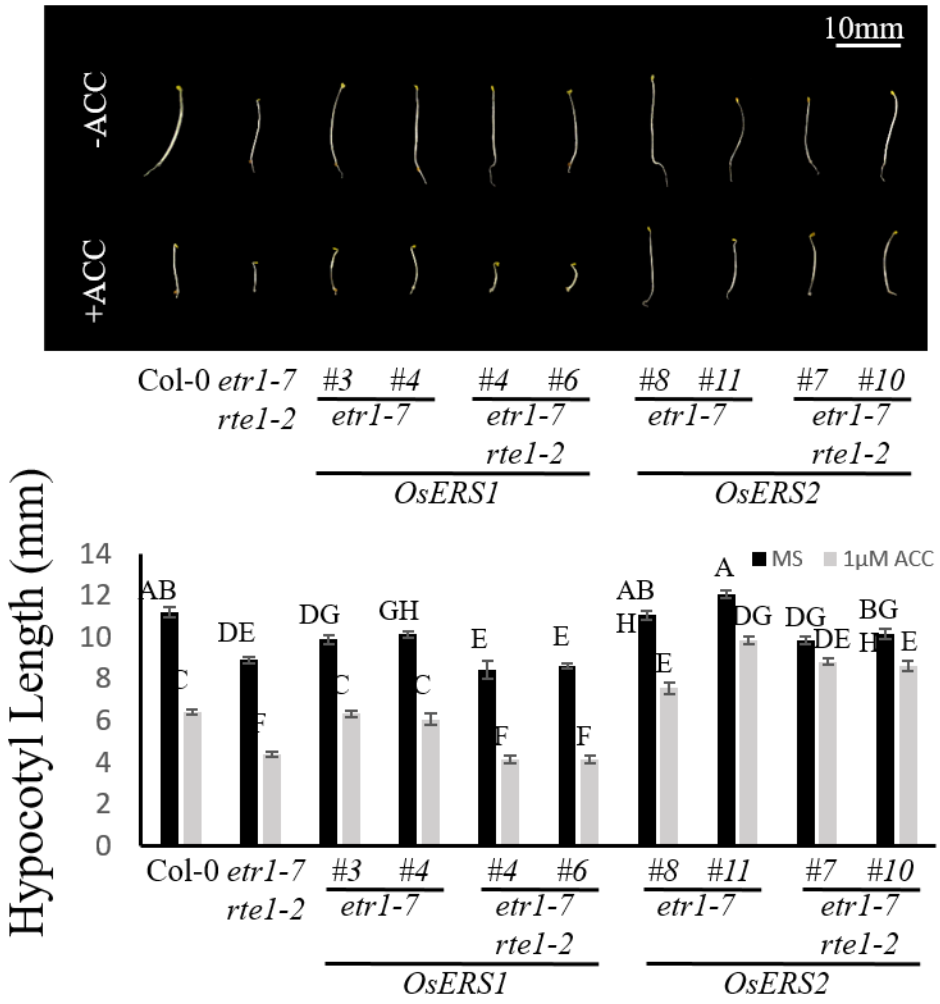


Figure 3-7: Osers1 is RTE1-dependent

Upper: Representative 4-day-old etiolated Arabidopsis seedlings grown on MS and MS + 1μM ACC. Two lines of transgenic Osers1 in etr1-7 rte1-2 displays an enhanced ethylene phenotype similar to etr1-7 rte1-2 compared to OsERS1 in the

etr1-7 background confirming OsERS1 is RTE1-dependent. OsERS2 in both etr1-7 and etr1-7 rte1-2 display an ethylene insensitive phenotype suggesting OsERS2 is RTE1-independent.

Lower: Measurement of hypocotyl lengths of 4-day-old etiolated Arabidopsis seedlings described above on MS ± 1μM ACC. Values indicate means ± standard error. Different letters above bars indicate samples that are significantly different.

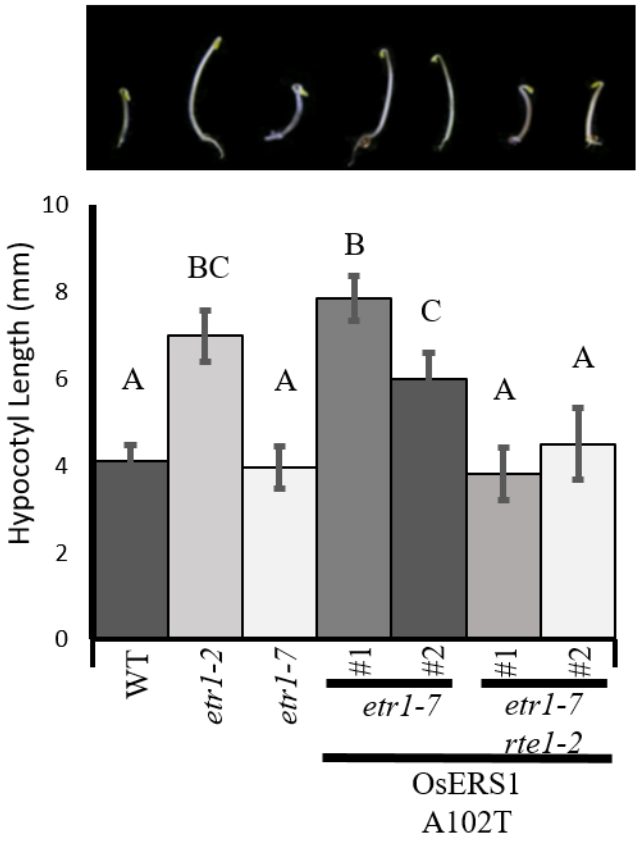


Figure 3-8: Osers1 A102T and OsERS2 A103T is RTE1-dependent

Upper: Representative 4-day-old etiolated Arabidopsis seedlings grown on MS plates containing 1μM ACC. Transgenic Osers1 A102T confers ethylene insensitivity in the etr1-7 background similar to etr1-2 (A102T). This same transgene osers1 A102T does not confer insensitivity in the etr1-7 rte1-2 background indicating this construct is RTE1-dependent.

Lower: Measurement of hypocotyl lengths of 4-day-old etiolated Arabidopsis seedlings described above grown on MS + 1μM ACC. Values indicate means ±

standard error. Different letters above bars indicate samples that are significantly different.

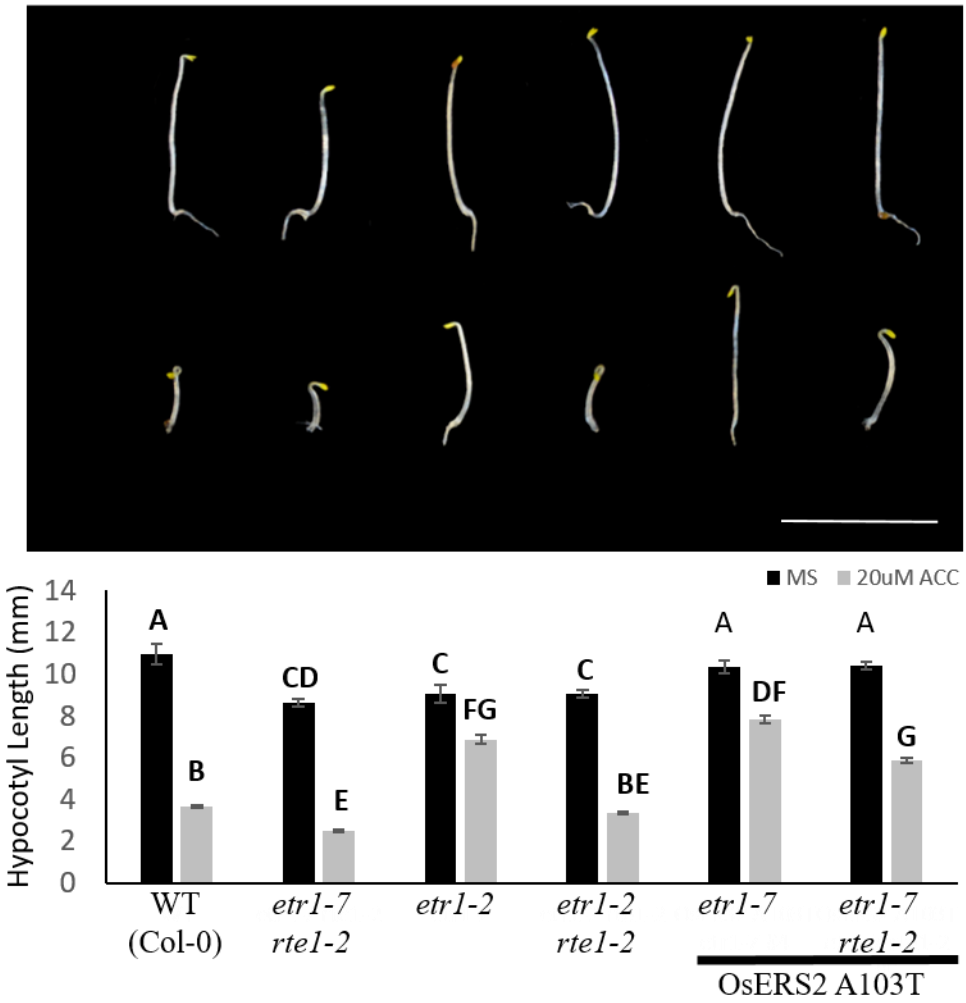


Figure 3-9: OsERS2 A103T is partially RTE1-dependent

Upper: Representative 4-day-old etiolated Arabidopsis seedlings grown on MS plates with and without 20μM ACC. Transgenically expressed Osers2 A103T stably transformed into the etr1-7 background was crossed with etr1-7 rte1-2 to generate Osers2 A103T etr1-7 rte1-2. Osers2 A103T etr1-7 seedlings display a strong ethylene

insensitive phenotype while Osers2 A103T etr1-7 rte1-2 seedlings display a weak ethylene insensitive phenotype.

Lower: Measurement of hypocotyl lengths of 4-day-old etiolated Arabidopsis seedlings described above grown on plates with MS media ± 20μM ACC. Values indicate means ± standard error. Different letters above bars indicate samples that are significantly different.

why it was difficult in determining RTE1-dependence of the wild-type OsERS2 receptor.

Discussion

In this chapter, we sought to understand why some receptors, like AtETR1, were dependent on RTE1 and other receptors with very similar amino acid sequences, such as AtERS1, were RTE1-dependent. We were able to identify that proline 9, a proline conserved in ETR1 receptors as well as monocot ERS1 receptors but not found in dicot ERS1 receptors, was important for determining whether a receptor was RTE1 dependent. The *A. thaliana* ERS1 receptor mutated to have this proline, ers1 (T9P), and the two ERS receptors from *O. sativa* that naturally have this proline are RTE1-dependent.

Although proline 9 appears to be an important amino acid in determining whether a receptor is RTE1-dependent, it is not the sole determining factor. The etr1(P9T A102T) receptor, which does not have this proline, is still RTE1-dependent. To accurately determine the impact of adding and removing proline 9 from these receptors and to predict what other amino acids may be involved we should consider 3D structure of these receptors, however, the crystal structure of the N-terminal transmembrane domain of the ethylene receptors has not yet been solved.

OsERS1 and OsERS2 are the first known naturally occurring RTE1-dependent ERS1 receptors. Although these receptors share a more recent common ancestor with the dicot ERS1 receptors, they have the conserved proline 9 like ETR1

receptors. Monocots, like *O. sativa,* may have ERS1 receptors that are functionally more similar to ETR1 receptors to compensate for monocots lacking ETR1 receptors.

Proline 9 was originally an amino acid of interest due to its proximity to cysteines 4 and 6. Given that prolines can disrupt protein structure which could disrupt disulfide bond formation and that cytochrome b5, a known redox molecule, interacts with RTE1, we felt that RTE1 may help serve as a chaperone using electrons provided by cytochrome b5 to help assist in the redox folding of the ETR1 receptor. However, we disproved this hypothesis when we found that the ETR1 receptor without these cysteines (etr1 4a6a) is still dependent on RTE1. This does not rule out the possibility that RTE1 functions as a chaperone. Given the ability for prolines to disrupt secondary structures and cause protein rigidity, RTE1 may still be required to help the ETR1 receptor for an active conformation.

We continue to narrow down the portion of the ETR1 receptor that RTE1 functions on. Previously it was reported that RTE1 acts on an ETR1 receptor that contains only transmembrane and GAF domains (Zhou et al., 2007). The RTE1-dependent OsERS1 and OsERS2 receptors lack receiver domains further confirming that this portion of the receptor is required for RTE1-dependence. Proline 9 is located in the ER lumen. It is unclear if this proline is interfering with the short N-terminal lumen tail or disrupting the transmembrane domain, but this N-terminal/transmembrane portion of ETR1 appears to be affected by RTE1.

The OsERS2 receptor is different from the other receptors examined in this chapter. First, OsERS2 is only partially dependent on RTE1. Without the A102T

equivalent mutation, there were no statistically significant difference between seedlings expressing T-*OsERS2* in the etr1-7 background versus the etr1-7 rte1-2 background. When the equivalent A102T mutation was mutated into OsERS2 receptors and expressed in these backgrounds, the differences were much more distinct. However, *T-Osers2(A103T) etr1-7 rte1-2* seedlings still displayed a significant level of ethylene insensitivity compared to *etr1-7 rte1-2* seedlings without the transgene. Second, The OsERS2 receptor both with and without the A103T mutation displays a greater than expected repression of ethylene signaling. The hypocotyl of T-*OsERS2 etr1-7* seedlings were larger than wildtype Col-0. *T-Osers2(A103T) etr1-7* seedlings displayed a very strong ethylene insensitivity phenotype much more insensitive than *etr1-2*. This may be an artifact of transgenically expressing this rice gene under the control of the AtETR1 promoter in *Arabidopsis* instead of its native plant. It is also possible that this OsERS2 is less responsive to ethylene. This may be advantageous when the plant is in an environment of increased ethylene concentrations such as when a plant is submerged and ethylene is not efficiently diffusing into air.

Recently, a new insensitive allele of AtERS1, ers1-4 was identified and concluded to be partially RTE1-dependent (Deslauriers et al., 2015). This conclusion was made after observing a partial suppression of insensitivity of *ers1-4* in an *rte1* loss-of-function mutant compared to a wild-type background. However, these observations were made in plants that have a functioning ETR1 receptors. In fact, similar suppression of ethylene insensitivity conferred by *ers1-4* was also observed in

the double *ers1-4 etr1* loss-of-function background. Only by comparing *ers1-4 etr1* loss-of-function background to the triple *ers1-4 etr1 rte1* loss-of-function background could confirm if ERS1 is partially RTE1-dependent.

The AtETR1, OsERS1 and OsERS2 receptors are RTE1-dependent suggesting that their common ancestor, an early angiosperm from approximately 100 million years ago, was also likely RTE1-dependent. The early ERS1 receptors, like OsERS1 and OsERS2, were likely RTE1-dependent and after the divergence of monocots and dicots, the ERS1 receptors became no longer dependent on RTE1. Interestingly, several offshoots of early angiosperms have either an ERS1 or ETR1 receptor, but not both (Gallie, 2015). It is possible that functionally speaking, these early angiosperms and monocots have ERS1 receptors that are similar to AtETR1 and that these early angiosperms had no need for functionally redundant receptors and lost either their ETR1 or ERS1 receptor.

Materials and Methods

Ethylene Receptor Alignments

Ethylene receptor amino acid sequences were obtained using the *Arabidopsis* ETR1 amino acid sequence as a query in a protein BLAST search of the National Center for Biotechnology Information's (NCBI) Non-redundant protein sequences (nr) database. Select representative sequences were then aligned with Biomatters Limited's Geneious software using the MUSCLE Alignment plugin (Edgar, 2004).

Plant growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild type. All mutants and transgenes used were generated in this ecotype. Plants were grown in soil in environmental growth chambers at 22°C under white light with a 16-hour light/8-hour dark period. Triple response assays were performed on Murashige and Skoog (MS) agar plates with 0.8% agar with and without 1-aminocyclopropane-1-carboxylic acid (ACC). Hypocotyl lengths were measured from digital photographs using ImageJ (https://imagej.nih.gov/ij/).

Generation of mutant receptor constructs

FvERS1 was amplified from *F.vesca* cDNA using phusion polymerase (www.neb.com) and primers listed in Table3-1. . FvERS1 PCR product was then cloned into the entry vector pDONR221 using BP Clonase II (Invitrogen) and subsequently switched into the pEarleygate100 expression plasmid. pCambia1301-AtETR1p::OsERS1 and pCambia1301-AtETR1p::OsERS2 were gifts from Dr.Chi-Kuang Wen.

AtERS1, AtETR1, FvERS1 A102T OsERS1 and *OsERS2* were mutagenized using site-directed mutagenesis using Quikchange II XL site-directed mutagenesis kit (Stratagene) and the primers listed in Table S-1.

Generation of Stably Transformed *Arabidopsis* Lines

Binary vectors were transformed into the Agrobacterium GV3101, which was then used to transform into *Arabidopsis thaliana* using the floral dip method (Clough and Bent, 1998). Transformants were then selected by either BASTA spraying or selection on MS plates containing either Hygromycin, Gentamycin or Kanamycin.

Generation of *etr1 4A 6A etr1-7 ers1-2 rte1-2*

We received seeds expressing etr1 4A 6A under control of the *ETR1* promoter in the etr1-7 ers1-2 background from Chi-Kuang Wen. These plants were crossed with etr1-7 rte1-2 and then selected for plants that were homozygous for etr1 4A6A, ers1-2 and rte1-2.

Cloning Primers
FVERS1 ATTB1 F: GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGATGGAGTCATGTGAT TGTGTTG
FVERS1 ATTB2 R: GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAAAGACTTCTTTGATAA CGTGC

Mutagenesis Primers
ERS1 P9 FOR: CATGCGAT TGTTTTGAGCCGCATGTGAATCAAGATGATG
ERS1 P9 REV: CATCATCTTGATTCACATGCGGCTCAAAACAATCGCATG
ETR1 T9 FOR: GTCTGCAATTGTATTGAAACGCAATGGCCAGCGG
ETR1 T9 REV:CCGCTGGCCATTGCGTTTCAATACAATTGCAGAC
OSERS1 G304A: GTTGTGTCCTGTGCGACAACTTTGATGCTTGTACATA
OSERS1 G304A_ANTISENSE: TATGTACAAGCATCAAAGTTGTCGCACAG GACACAA
OSERS2 G307A: GGTTGTTTCGTGCATCACAACTTTGATGCTTGTGCATAT
OSERS2 G307A_ANTISENSE: ATATGCACAAGCATCAAAGTTGTGATGCACGAAACAACC
FVERS1G304AF: TTGTGTCGTGTGCAACGACGTTGATGCTTGTTCAC

FVERS1G304AR: GTGAACAAGCATCAACGTCGTTGCACACGACACAA

Table 3-1: Primers used to generate transgenic ethylene receptors described in this chapter

Chapter 4: Ethylene biosynthesis in the charophycean green alga Spirogyra pratensis

Introduction

Ethylene is a gaseous plant hormone involved in many aspects of growth and development as well as responses to abiotic and biotic stresses (Abeles et al., 1992). Studies in *Arabidopsis thaliana* and other angiosperms have uncovered the molecular pathways for ethylene biosynthesis (Vanderstraeten and Van der Straeten 2017) and ethylene signalling (Ju and Chang 2015). With increasing availability of nucleotide sequence data for a range of species in the plant lineage (Figure 4-1A), the evolutionary history of ethylene as a plant hormone is starting to be addressed as well. Based on sequence homologies and in some cases functional studies, the ethylene signalling pathway is thought to be conserved in land plants other than the seed plants, such as *Selaginella moellendorffii*, *Physcomitrella patens* (moss) and *Marchantia polymorpha* (liverwort) (Banks et al., 2011; Bowman et al., 2017; Rensing et al., 2008; Yasumura et al., 2012). Interestingly, the conservation of ethylene signalling extends to the freshwater green algae known as charophytes (Ju *et al*. 2015), indicating that ethylene may have been a plant hormone in the common ancestor of charophytes and land plants at least 450 million years ago, prior to the evolutionary transition to land. However, there is no evidence of ethylene receptor homologs in other algae (chlorophytes, rhodophytes, or glaucophytes) that are more distantly related to land plants (Ju *et al.* 2015) (Figure 4- 1A). In contrast to ethylene signalling, the evolutionary history of ethylene biosynthesis is unclear. By definition,

a hormone not only induces a response in an organism but must be produced by that organism as well. However, it is uncertain to what extent the ethylene biosynthesis pathway is conserved in the non-seed land plants and in charophycean algae.

In angiosperms, ethylene biosynthesis begins with S-adenosyl-L-methionine (SAM), which is made within the methionine salvage cycle also known as the Yang cycle (Figure 4-1B) (Adams and Yang, 1977, 1979). Ethylene is synthesized from SAM via a two-step conversion. In the first committed step, SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS) (Yu et al., 1979). This step is highly regulated and is generally rate limiting in ethylene biosynthesis (Argueso et al., 2007; Vogel et al., 1998). In the second step, ACC is oxidized to ethylene via ACC oxidase (ACO) (Hamilton et al., 1991). In seed plants (gymnosperms and angiosperms), ACS and ACO enzymes are encoded by multigene families. For example, *A. thaliana* has 12 *ACS* genes and 5 *ACO* genes. ACSs are within the pyridoxal-dependent aminotransferase family (Vanderstraeten and Van der Straeten 2017). ACOs belong to the large family of 2-oxoglutarate dioxygenase (2OGD) proteins (Kawai et al., 2014). 2OGD proteins catalyze a wide range of reactions in bacteria, plants, fungi and animals (Kawai et al., 2014). Plant 2OGDs are involved in DNA methylation, proline hydroxylation and synthesis of plant hormones and metabolite intermediates (Meza et al., 2012; Thomas et al., 1999; Vlad et al., 2007).

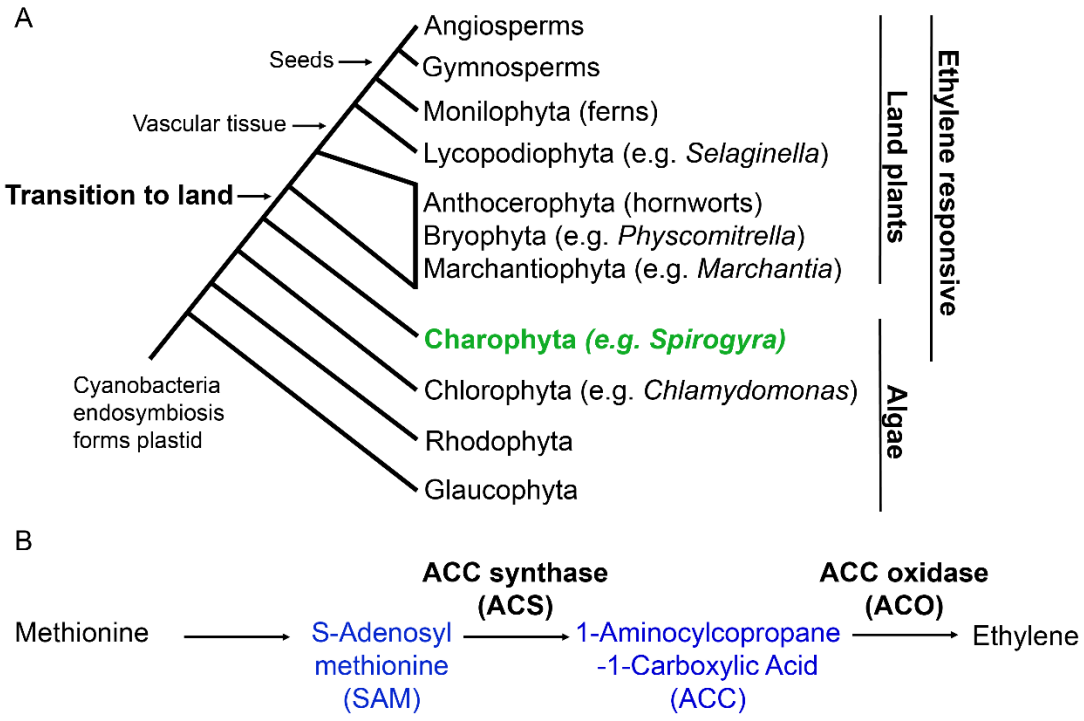


Figure 4-1: Ethylene biosynthesis pathway in plants.

A. Plant phylogeny from algae to land plants. **B.** The two-step ethylene biosynthesis pathway as known in the seed plants (angiosperms and gymnosperms). Methionine is converted to S-adenosyl methionine (SAM) in the methionine recycling pathway (not shown). In the first committed step of ethylene biosynthesis, the ACS enzyme converts SAM to ACC. In the second step, the ACO enzyme converts ACC to ethylene gas.

Current evidence indicates that the ethylene biosynthesis pathway described for angiosperms is specific to seed plants. Sequence analyses suggest that *ACO* genes are present only in seed plants (Kawai *et al.* 2014), consistent with experimental data on ACC-induced ethylene production in a wide range of plant species (Osborne 1989; Osborne *et al.* 1996). Other studies, however, suggest that ACC can be produced by non-seed land plants (Rohwer and Bopp 1985), as well as by rhodophytes (Garcia-Jimenez and Robaina 2012; Garcia-Jimenez *et al.* 2013) and chlorophytes (Vanden Driessche *et al.* 1988; Maillard *et al.* 1993; Plettner *et al.* 2005), which are algal lineages that shared a common ancestor with land plants nearly 1 billion years ago. Moreover, two rhodophyte species (Garcia-Jimenez and Robaina, 2012; Garcia-Jimenez et al., 2013) and two chlorophyte species (Vanden Driessche et al., 1988; Maillard et al., 1993) have been reported to produce ethylene when treated with ACC.

Land plants evolved from ancestral charophycean green algae more than 450 million years ago (Sanderson et al., 2004). Because extant charophytes are the closest living relatives of land plants, the study of these algae is valuable for insight into the common aquatic ancestor prior to the evolutionary transition from the aquatic to terrestrial lifestyle (Becker and Marin, 2009; Delwiche and Cooper, 2015; Timme and Delwiche, 2010; Wodniok et al., 2011). *Spirogyra pratensis* is a filamentous freshwater alga in the charophycean lineage of *Zygnematophyceae*, one of the lineages most closely related to land plants (Karol, 2001; Leliaert et al., 2012; Turmel et al., 2002) (Figure 4-1A). It was recently discovered that *S. pratensis* has an ethylene signalling pathway similar to that in angiosperms; all of the major

components of ethylene signalling are conserved in *S. pratensis*, and several of these components possess conserved function (Ju *et al.* 2015). Ethylene treatment induces cell elongation in *S. pratensis* (Ju et al., 2015). In addition, several abiotic stresses, such as osmotic and/or nutrient stress, were found to induce cell elongation that is ethylene-dependent (Van de Poel et al., 2016).

Although *S. pratensis* is clearly capable of ethylene signalling, there are remaining questions concerning ethylene biosynthesis in *S. pratensis* (and charophytes, in general). Ju *et al.* (2015) reported the presence of *ACS* and *ACO* homologs in *S. pratensis*, yet ethylene levels are considerably lower in *S. pratensis* compared to those in flowering plants (Ju et al., 2015; Van de Poel et al., 2016). Even when treated with a high dose of ACC (500 μM), *S. pratensis* produced low levels of ethylene (Ju *et al.* 2015), the basis for which was unknown. In this chapter, we examined the ethylene biosynthesis pathway in *S. pratensis* more closely. We evaluated the identification *ACS* and *ACO* homologs in *S. pratensis* and infer their functionality by determining, for example, whether *S. pratensis* produces ACC. Additionally, we tested whether ethylene is produced via the SAM-ACC pathway in *S. pratensis*. Our findings provide insight into the difference in ethylene biosynthesis efficiency between *S. pratensis* and angiosperms and contribute to our understanding of the evolution of ethylene biosynthesis in plants.

Results

S. pratensis expresses ACS-like homologs

In higher plants, the conversion of SAM to ACC by ACS is the first dedicated step in the ethylene biosynthesis pathway. Using the *A. thaliana* ACS1 sequence to query an existing assembly of *S. pratensis* RNAseq reads (DDBJ/EMBL/GenBank TSA accession number GFWN00000000) (Van de Poel et al., 2016), we identified three putative *S. pratensis* ACS homologs (comp6893_c0, 1E-88; comp13204_c0, 2E-57; comp6865_c0, 1E-14) with BLAST *E*-values <1E-10. This is consistent with the previously published suggestion that there are three ACS homologs in *S. pratensis* (Ju et al., 2015). Reciprocal BLAST analyses for the three homologs indicated that comp6893_c0 and comp13204_c0 are likely ACS orthologs, while comp6865_c0 shows more sequence similarity to *A. thaliana* amino acid transferases. Comp6893_c0 was previously named Sp-ACS1 (Ju et al., 2015), and here we refer to comp13204_c0 as Sp-ACS2.

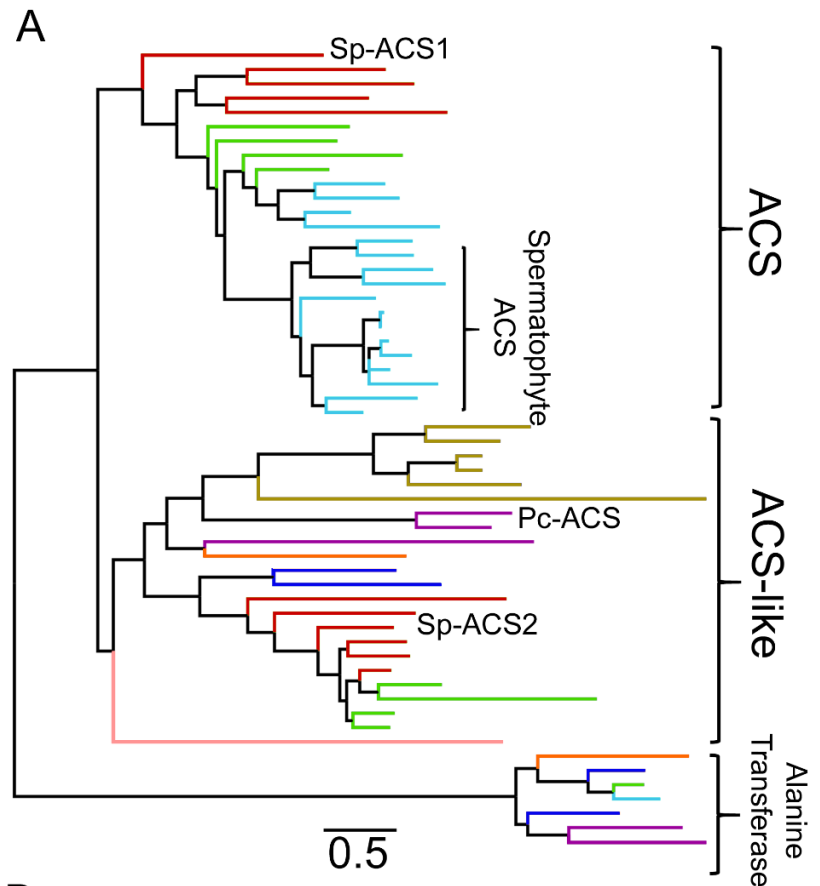
Phylogenetic analyses place Sp-ACS1 within a clade containing ACS proteins from other charophytes, non-seed land plants (such as *Physcomitrella patens* and *Marchantia polymorpha),* gymnosperms and angiosperms (Figure 4-2A), indicating strong conservation of *ACS* genes in plant evolution. The other *S. pratensis* ACS homolog, Sp-ACS2, falls into a separate clade that contains ACS-like homologs found in lower land plants, charophycean green algae, chlorophycean green algae, rhodophytes, glaucophytes, fungi and metazoans (Figure 4- 2A).

S. pratensis produces ACC using pathway intermediates known in land plants

We next measured the ACC content of *S. pratensis* by grinding *S. pratensis* filaments and chemically liberating ethylene from ACC with NaOCl in the presence of Hg^{2+} and then measuring evolved ethylene by gas chromatography (Lizada and Yang 1979; Bulens *et al.* 2011) (Figure 4-2B). We found that the ACC concentration in *S. pratensis* (cultured in Bolds 1NV media) was 0.068 nmol g FW^{-1}, which is approximately half of the ACC concentration in tomato leaves (Hall et al., 1993). When we added methionine to the *S. pratensis* growth medium, we detected higher levels of ACC (Figure 4-2B), suggesting that methionine can be converted to ACC, presumably via SAM and ACS activity.

No evidence of the plant-like ACO enzyme in *S. pratensis*

The second step in ethylene biosynthesis is the oxidation of ACC to ethylene via the ACO enzyme, which belongs to the large family of 2OGDs (Kawai et al., 2014). The phylogenetic analysis by Kawai *et al.* (2014) indicated that ACOs are only present in gymnosperms and angiosperms, indicating that ACOs are a relatively recent derivation in plant evolution. Using the *A. thaliana* ACO1 sequence to query the same *S. pratensis* transcriptome assembly as above (*E*-value <1E-5), we identified four 2OGD-like sequences (comp6684_c0, 4E-28, 22% identity + 11% similarity; comp13325_c0, 9E-14, 25% identity + 12% similarity; comp11895_c0, 1E-9, 19% identity + 15% similarity; comp7597_c0, 1E-7, 22% identity + 14%


A
Sp-ACS1
Spermatophyte ACS
ACS
Pc-ACS
Sp-ACS2
ACS-like
Alanine Transferase
0.5

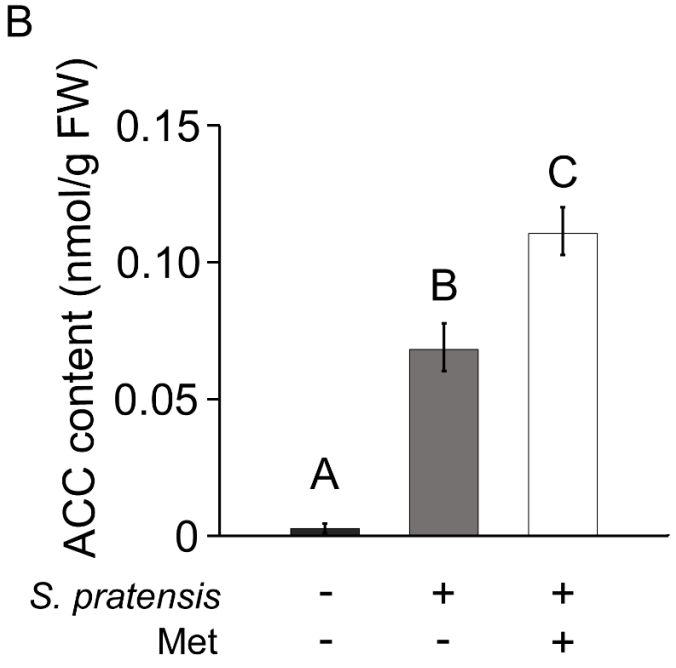

B
ACC content (nmol/g FW)
0.15
0.10
0.05
0
A
B
C
S. pratensis - + +
Met - - +

Figure 4-2: S. pratensis produces ACC.

A. Phylogenetic tree of ACS homologs in plants, metazoans and fungi. ACS homologs fall into two clades: ACS and ACS-like. *S. pratensis* ACS1 (Sp-ACS1) is in the ACS clade, which includes ACS proteins known to synthesize ACC in angiosperms. *S. pratensis* ACS2 (Sp-ACS2) belongs to the ACS-like clade, which has no members present in seed plants but includes an ACS-like enzyme (Pc-ACS) from the fungus *Penicillium citrinum* that is known to make ACC (Kakuta et al., 2001). Pink=Glaucophyte, Orange=Rhodophyte, Dark Blue=Chlorophytes, Red=Charophytes, Green= Non-Seed Land Plants, Light Blue= Seed plants, Gold= Metazoans, Purple = Fungi. Branch length scale bar is located below tree. **B.** ACC content in *S. pratensis*. The addition of methionine (1 mM) to the growth medium increases the ACC content in *S. pratensis*. The different letters above the bars indicate significant difference as determined by Tukey-Kramers HSD.

similarity). A previous, more stringent analysis (*E*-value <1E-10) of the *S. pratensis* transcriptome had identified two ACO1-like homologs (Ju et al., 2015). When we conducted reciprocal BLAST to the *A. thaliana* protein database with the four identified Sp-2OGD-like homologs, the results suggested that none of these homologs are ACOs. One homolog, *Sp-7597* (comp7597_c0), appeared to be most closely related to fungal 2OGDs, and was found in only two other species, *Spirogyra Sp. and Mougeotia scalaris*, both in the Zygnemataphyceae (Cooper and Delwiche, 2016). Another homolog, Sp-*11895* (comp11895_c0), was substantially shorter than the others, possibly representing a pseudogene or the result of incomplete sequence data. We incorporated the Sp-2OGD-like amino acid sequences in a phylogenetic analysis of ACO and other 2OGD subfamily C enzymes as described by Kawai *et al.* (2014) (Figure 4-3A). Sp-7597, which likely has a fungal origin, was not included in this plant 2OGD tree. The results indicated that the three Sp-2OGD-like sequences, Sp-6684 (comp6684_c0), Sp-11895 and Sp-13325 (comp13325_c0) all appeared to have origins in the plant 2OGD family, falling into an ancient clade of 2OGDs conserved in higher land plants, lower land plants and the chlorophyte *Chlamydomonas*. The activities of the proteins within this clade are unknown, but these 2OGDs are distantly related (Figure 4-3A) to the ACOs of seed plants. Additionally, we performed reciprocal best hit analysis of predicted proteins from the *Klebsormidium nitens* genome (Hori et al., 2014) and could not find any ACO orthologs. The lack of ACO enzymes in these charophytes is consistent with ACOs appearing much later in land plant evolution.

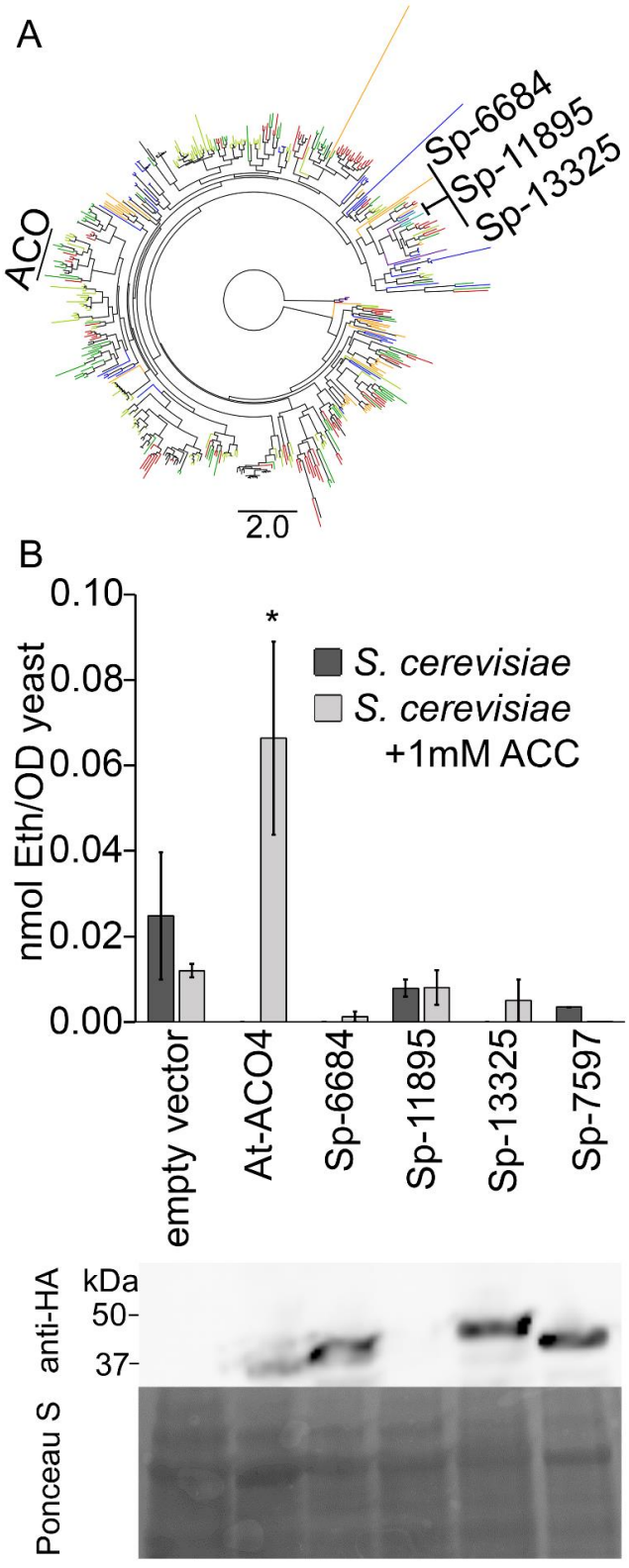

A
ACO
Sp-6684
Sp-11895
Sp-13325
2.0
B
nmol Eth/OD yeast
0.10
0.08
0.06
0.04
0.02
0.00
*
S. cerevisiae
S. cerevisiae
+1mM ACC
empty vector
At-ACO4
Sp-6684
Sp-11895
Sp-13325
Sp-7597
kDa
anti-HA
50-
37-
Ponceau S

Figure 4-3: Sp-2OGDs are not ACOs.

A. Phylogenetic tree of several plant/algae 2OGD (subfamily C) reproduced from Kawai *et al* (2014) including three Sp-2OGD homologs (Sp-6684, Sp-11895, Sp-13325). The clade of ACOs is indicated. Red=*A. thaliana*, Dark Green= *Oryza sativa*, Light Green= *Picea abies*, Orange= *P. patens*, Blue= *S. moellendorffii*, Teal= *S. pratensis*, Purple= *C. reinhardtii*. A fourth Sp-2OGD homolog (Sp-7597) (not shown) has fungal origins. Branch length scale bar located below tree. **B. Upper-** Ethylene production assay in yeast expressing Sp-2OGD homologs indicates that these 2OGDs do not produce ethylene from ACC. An anti-HA antibody was used to detect the HA-tagged 2OGD proteins of the expected size. Asterisk indicates sample that is significantly different from others as determined by Tukey-Kramer HSD. **Lower-** Western blot to confirm 2OGD protein expression in yeast. Labels same as above. The blot was stained with Ponceau S to indicate protein loading. Sp-11895 appears to be a truncated gene and was not expressed in yeast.

Since ethylene-producing enzymes have evolved from the 2OGD gene family at least twice (once in higher plants to form ACO and once in bacteria to form what has been termed as Ethylene Forming Enzyme (EFE) (Jacobsen and Wang, 1968; Kawai et al., 2014; Weingart and Völksch, 1997)), we tested whether the Sp-2OGD homologs could potentially represent an additional derivation of an ethylene forming enzyme that converts ACC to ethylene. We individually expressed the Sp-2OGD proteins in the yeast *Saccharomyces cerevisiae*, treated the yeast with ACC, and then measured ethylene production by gas chromatography. We confirmed yeast expression of Sp-6684, Sp-7597 and Sp-13325 by western blotting, but could not detect expression of the shorter protein Sp-11895 (Figure 4-3B). After treating yeast with a high concentration of ACC, no ethylene was detected from yeast expressing any of these Sp-2OGD homologs, whereas ethylene was easily detected in yeast expressing the *A. thaliana ACO4* gene as a positive control (Figure 4-3B). These results indicate that at least three of the Sp-2OGDs do not efficiently convert ACC to ethylene, and suggest that these proteins do not possess ACO functionality.

S. pratensis can produce ethylene via an ACC-intermediate pathway

The likely absence of ACO in *S. pratensis* raised the question of whether ACC serves as a precursor for ethylene production in *S. pratensis*. Some fungi and bacteria, such as *Pseudomonas syringae*, can synthesize ethylene from 2-oxoglutarate (Jacobsen and Wang, 1968), while other bacteria, such as *Escherichia coli*, and some fungi, such as *Cryptococcus albidus*, are able to synthesize ethylene from 2-keto-4-

methylthiobutyric acid (Mansouri and Bunch, 1989). Only the higher land plant ethylene biosynthesis pathway is known to have SAM and ACC as intermediate substrates. To test whether *S. pratensis* can synthesize ethylene from the precursors known in angiosperms, we measured ethylene production after treating *S. pratensis* separately with methionine, SAM and ACC. *S. pratensis* produced ethylene when treated with any of these three substrates (Figure 4-4A), supporting the presence of a conserved ACS activity capable of converting SAM to ACC. Moreover, this indicates that *S. pratensis* can produce ethylene from ACC despite lacking an identifiable *ACO*-like gene using our search protocol.

Recently, it was reported that cell elongation is induced in *S. pratensis* by several abiotic stresses, such as stress from growth in deionized H_2O in place of nutrient growth media and that the stress-induced cell elongation was ethylene dependent (Van de Poel et al., 2016). To obtain further evidence of the presence of an ACS-like enzyme and absence of an ACO enzyme in *S. pratensis*, we used ethylene biosynthesis inhibitors (as established in higher plants) to test whether they could block the ethylene-mediated cell elongation observed during growth in deionized H_2O. We found that cell elongation was blocked when we treated the culture with aminoethoxyvinylglycine (AVG), an inhibitor of the ACS enzyme in higher plants (Amrhein and Wenker, 1979; Even-Chen et al., 1982)(Figure 4-4B,C). This provides further evidence that *S. pratensis* produces ethylene via a pathway that involves an ACS-like enzyme and ACC. In contrast, aminoisobutyric acid (AIB), which inhibits

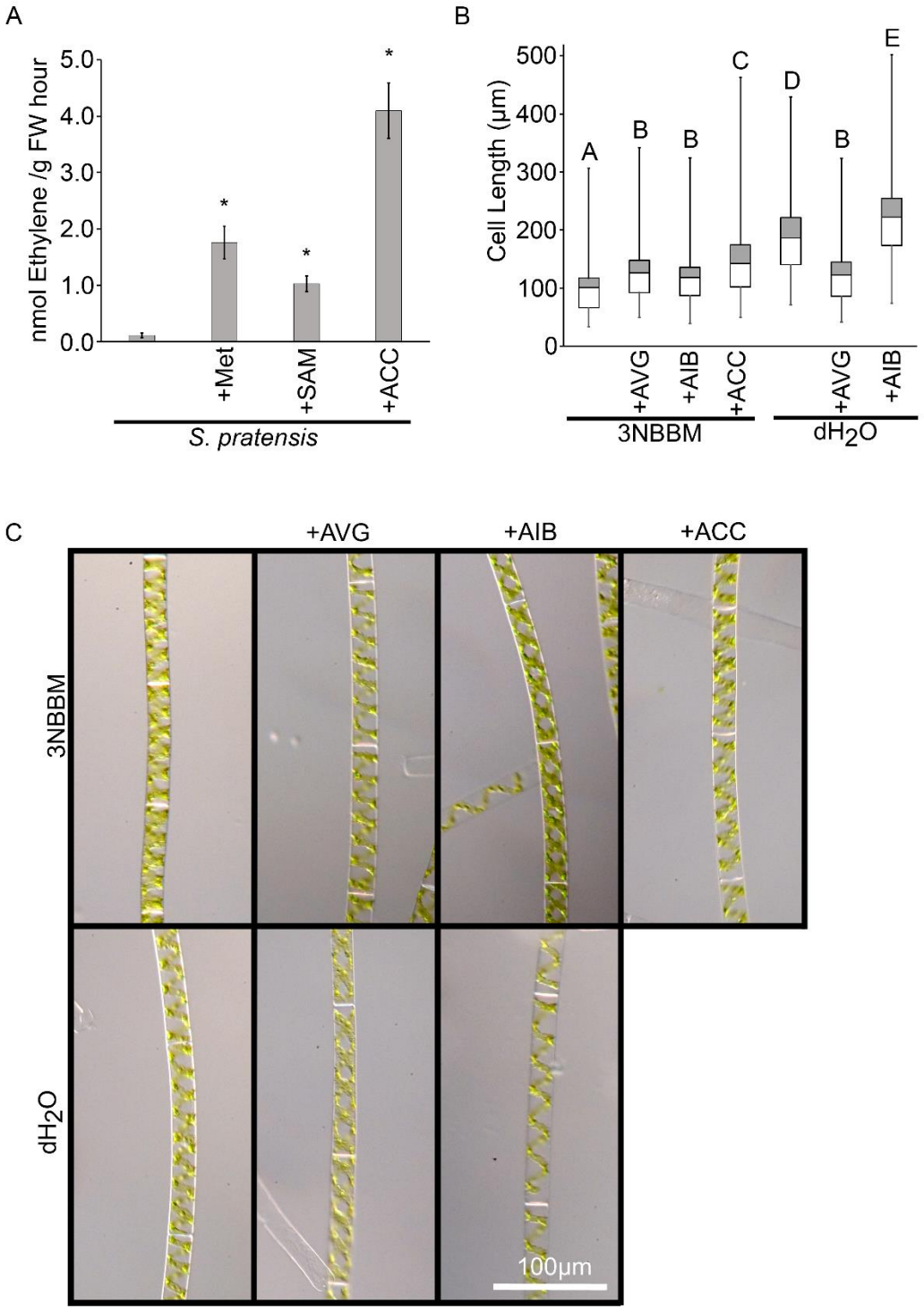

A
nmol Ethylene /g FW hour
5.0
4.0
3.0
2.0
1.0
0.0
*
*
*
+Met
+SAM
+ACC
S. pratensis
B
Cell Length (µm)
500
400
300
200
100
0
A
B
B
C
D
B
E
+AVG
+AIB
+ACC
+AVG
+AIB
3NBBM
dH2O
C
+AVG
+AIB
+ACC
3NBBM
dH2O
100µm

Figure 4-4: S. pratensis can synthesize ethylene via an ACC-intermediate pathway.

A. *S. pratensis* produces ethylene when treated with the three ethylene precursors, known in the angiosperm ethylene biosynthesis pathway, methionine (1 mM), SAM (1 mM), and ACC (0.5 mM). * indicates a P-value less than 0.05 when compared to the no treatment sample using Student's t-test. **B.** Stress-induced cell elongation mediated by ethylene in *S. pratensis* is blocked by ACS inhibitor AVG (10 μM) but not ACO inhibitor AIB (500 μM). *S. pratensis* filaments were stressed by treatment with a dH_2O-only medium. Cell elongation is also observed in filaments treated with ACC (500 □M). Box and whisker plot indicates individual cell size within a filament. Center line of each box indicates average cell length. Edges of each box indicate 25th and 75th percentiles. Whiskers indicate minimum and maximum cell lengths. **C.** Representative images of *S. pratensis* filaments treated as in 4B. The cell wall between cells appears as a white line. Scale bar = 100 μm.

ACO enzyme activity in flowering plants (Amrhein and Wenker, 1979; Even-Chen et al., 1982), did not block ethylene-mediated cell elongation in H_2O-treated *S. pratensis*. This is consistent with the hypothesis that *S. pratensis* can produce ethylene through an ACC-intermediate pathway independent of a functional ACO.

Discussion

Ju *et al.* (2015) previously found that the charophycean green alga *S. pratensis* possesses a functionally conserved ethylene signaling pathway and suggested that *S. pratensis* also has *ACS* and *ACO* homologs that could play a role in ethylene biosynthesis. Here, we have examined ACC and ethylene biosynthesis in *S. pratensis* in greater detail. We demonstrated that *S. pratensis* contains measurable levels of ACC and confirmed the presence of ACS-like activity in *S. pratensis* by demonstrating that *S. pratensis* produced more ACC when provided methionine. Furthermore, *S. pratensis* produced more ethylene when treated with either methionine or SAM. Finally, AVG, an ACS-specific inhibitor, was able to block the ethylene-mediated cell elongation phenotype of stress-treated *S. pratensis*.

S. pratensis and other charophytes have two ACS homologs based on transcriptomic data, suggesting our results for *S. pratensis* may extend to other charophytes. Sp-ACS1 falls into a clade of enzymes known to produce ethylene in angiosperms (Liang et al., 1992, 1995; Sato and Theologis, 1989; Van der Straeten et al., 1990, 1992; Yamagami et al., 2003). Sp-ACS2 lies within an ACS-like clade that may also be involved in ACC synthesis. An ACS-like protein in this clade from the

fungus *Penicillium citrinum* has been shown to synthesize ACC when expressed in yeast treated with 0.1% methionine (Jia et al., 1999; Kakuta et al., 2001). Moreover, three chlorophyte species and two rhodophyte species have been suggested to be capable of synthesizing ACC (Vanden Driessche et al., 1988; Garcia-Jimenez et al., 2013; Maillard et al., 1993) although rhodophytes and chlorophytes lack a homolog in the ACS clade. Therefore, it is possible that enzymes in the ACS-like clade could be responsible for ACC synthesis in chlorophytes and rhodophytes. Thus, both the ACS and ACS-like clade could produce enzymes capable of synthesizing ACC.

Having confirmed ACC production in *S. pratensis* I investigated whether *S. pratensis* possesses *ACO* genes as described in angiosperms. I found several ACO homologs in *S. pratensis*, but none are closely related to the ACO clade of 2OGDs, and do not confer any measurable ACO activity in our yeast-based assay. Given that the *S. pratensis* genome has yet to be sequenced, there is a slim possibility that *Sp-ACO* genes have yet to be uncovered. However, my finding that the ACO inhibitor AIB did not block stress-induced cell elongation in *S. pratensis* is consistent with the absence of a seed plant-like ACO enzyme, as suggested by Kawai *et al.* 2014, and in contrast to the reported Sp-ACOs in Ju *et al.* (2015). We also did not identify any *ACO* homologs in the genome of *Klebsormidium nitens*, which is in the Klebsormidiophycae lineage of charophytes.

If *S. pratensis* lacks a true *ACO* ortholog, how is *S. pratensis* capable of producing low levels of ethylene when treated with ACC? A possible explanation is that ACC can be converted to ethylene non-specifically by 2OGDs or by other

enzymes. For example, Osswald *et al.* (1989) demonstrated that horseradish peroxidase was capable of converting ACC to ethylene in an *in vitro* system. Additionally, Bousquet and Thimann (1984) were able to show that an *in vitro* lipoxygenase-linoleate system converted ACC to ethylene, and they suggested this system was responsible for ethylene in senescent oak leaves. An alternate possibility is that *S. pratensis* oxidizes ACC to ethylene non-enzymatically. Beaulieu *et al.* (1998) found that both aldehydes and basic pHs could increase ethylene production from ACC *in vitro* in the absence of enzymes. Any of these explanations could be consistent with the low levels of ethylene observed in *S. pratensis*. A similar scenario might apply to non-seed land plants and other algae that lack ACOs.

Not having an ACO enzyme to efficiently convert ACC to ethylene makes sense for organisms in aqueous habitats. Ethylene diffuses 10,000-fold faster in air than in water (Jackson, 1985). Therefore, any ethylene produced in submerged organisms would persist longer than in an organism on dry land, and high levels of ethylene would seem to be counterproductive if ethylene is to act as a hormone. Instead, an inefficient ethylene biosynthesis pathway that produces very low levels of ethylene would be more effective. Thus, an aquatic organism, such as *S. pratensis*, would only want to produce small quantities of ethylene when needed and then, when ethylene is no longer needed, these low levels of ethylene would slowly diffuse out into the water to reset the ethylene response.

We speculate that charophyte green algae and the non-seed land plants could potentially take advantage of the dramatic difference in the rate of diffusion of

ethylene in air versus water to serve as a signal for submergence. When submerged, low levels of ethylene, as a byproduct of ACC production, would be an indicator that the plant is under water. However, when exposed to air, ethylene would be able to quickly diffuse out of the plant, thus signalling exposure to dryer conditions. Submerged algae could take advantage of this system to prepare for times of possible desiccation, while early land plants may have used this system to indicate submergence. Interestingly, several species of rice utilize ethylene diffusion to determine whether the plant is submerged (Fukao and Bailey-Serres, 2008; Hattori et al., 2009; Métraux and Kende, 1983). This could be an important feature of any plant that is transitioning from water to terrestrial land.

Our findings have demonstrated the likely conservation of ACC synthesis in *S. pratensis*. Interestingly, some species of fungi, rhodophytes and chlorophytes have also been found to produce ACC (Vanden Driessche *et al.* 1988; Maillard *et al.* 1993; Kakuta *et al.* 2001; Plettner *et al.* 2005, Garcia-Jimenez and Robaina 2012; Garcia-Jimenez *et al.* 2013), despite not having ethylene receptors to perceive ethylene. It is thus tempting to speculate that ACC may have had a role other than in ethylene production, potentially serving as a signalling molecule on its own (Van de Poel and Van Der Straeten, 2014; Yoon and Kieber, 2013). Indeed, examples exist of ACC acting separately from ethylene in *A. thaliana* roots (Tsang et al., 2011; Tsuchisaka et al., 2009; Xu et al., 2008). Rhodophytes, chlorophytes and fungi may be synthesizing ACC for non-ethylene functions with some of the ACC being non-enzymatically oxidized to ethylene. Ancestral charophyte algae perhaps took advantage of this

ethylene production, thus helping to establish ethylene as a plant hormone, e.g., as a sensor for submergence. As land plants evolved to become more resistant to desiccation, ancestral gymnosperms, no longer constrained to wet areas for reproduction, most likely derived ACO activity from 2OGDs to efficiently synthesize higher levels of ethylene in terrestrial environments.

Materials and Methods

S. pratensis growth conditions

Spirogyra pratensis (UTEX 928) was cultivated in either Bold's Basal Medium with 3X Nitrogen (3NBBM; UTEX) or Bold 1NV medium (UTEX) in stationary flasks in controlled temperature growth chambers at 18°C under a 16-hour light/8-hour dark cycle at approximately 100 µmol m^{-2} s^{-1}.

Measurement of cell length

Ethylene-mediated cell elongation was observed in *S. pratensis* filaments (approximately 0.15 g wet weight) grown in 800 µL of either deionized water (osmotic/nutrient stress treatment) or 3NBBM (control) with specified ethylene biosynthesis inhibitors in a Petri dish sealed with parafilm for 10 days. Digital images of *S. pratensis* cells were captured with a Zeiss Axioskop through a 20X objective. For each treatment, at least 500 individual cells were measured with ImageJ (https://imagej.nih.gov/ij/).

Identification of *Sp-ACS* and *Sp-ACO* homologs

An existing assembly (DDBJ/EMBL/GenBank TSA accession number GFWN00000000) of *S. pratensis* RNAseq reads (DDBJ/EMBL/GenBank SRA accession number SRP081241) (Van de Poel et al., 2016) was converted to a BLAST database using the BLAST+ (v2.3.0) suite of programs (Camacho et al., 2009). Putative homologs of *ACS* and *ACO* genes were identified using *A. thaliana* proteins to search the database using TBLASTN. Hits were then used to reciprocally search for best hits in an *A. thaliana* database (Swarbreck et al., 2008). Because no Sp-2OGD had a reciprocal best hit for ACO in *A. thaliana*, we examined the activity of all the Sp-2OGD-like sequences with an *E*-value <1.0E-5.

Generation of phylogenetic trees for the ACS and 2OGD homologs

ACS homologs were identified in many species using the *A. thaliana* ACS1 sequence to query NCBI's Model Organisms/Landmark protein database using NCBI's BLASTp search tool (NCBI Resource Coordinators, 2017). Additionally, the *A. thaliana* ACS1 sequence was used to search the protein databases of *Marchantia polymorpha* (Bowman et al., 2017), *Physcomitrella patens* (Rensing et al., 2008), *Selaginella moellendorffii* (Banks et al., 2011), *Picea abies* (Nystedt et al., 2013), *Cyanophora paradoxa* (Price et al., 2012) and *Galdieria sulphuraria* (Schonknecht et al., 2013). We identified ACS homologs in the chlorophyte *Elakothrix viridis*, and the charophytes *Coleochaete orbicularis, Caetophaeridium globosum, Entransia fibriata. Nitella mirabilis* and *Penium margaritaceum* using *A. thaliana* ACS1 and TBLASTN to query existing transcriptome assemblies of these charophytes (Cooper and Delwiche, 2016). The amino acid sequences of Sp-ACS1, Sp-ACS2, *Penicllium*

citrinum ACS (Kakuta et al., 2001) and other putative ACS homologs were aligned using the Muscle (Edgar, 2004) plugin in Geneious (version 5.0.3, www.geneious.com) with default parameters.

2OGD (subfamily C) proteins from *Chlamydomonas reinhardtii* and several plants were previously identified (Banks et al., 2011; Kawai et al., 2014; Merchant et al., 2007; Nystedt et al., 2013; Ouyang et al., 2007; Rensing et al., 2008; Swarbreck et al., 2008). Sp-2OGDs identified as described above were aligned with the previously identified 2OGDs in Kawai *et al.* (2014) using the Muscle plugin in Geneious (version 5.0.3) with default parameters. A maximum-likelihood analysis was used to generate ACS and 2OGD phylogenetic trees in RAxML 8.2.7 (Stamatakis, 2014) with an LG substitution model (Le and Gascuel, 2008).

Plasmid constructs for yeast expression

To generate plasmid constructs for yeast expression, *S. pratensis* and *A. thaliana* RNA was isolated using the RNeasy kit (Qiagen), followed by cDNA synthesis using an iScript cDNA Synthesis Kit (Bio-Rad). The protein coding sequences of the Sp-2OGD-like (identified as described above) and At-ACO4 cDNAs were PCR-amplified using Phusion DNA Polymerase (New England Biolabs) and gene-specific primers Table 4-1]. The PCR products were cloned into pDONR221 (Life Technologies) using the Gateway cloning system (Life Technologies). The pDONR221 constructs were confirmed by DNA sequencing and transferred into a yeast expression vector, pAG426GPD-ccdB-HA, carrying the constitutive GPD promoter. pAG426GPD-ccdB-HA was a gift from Susan Lindquist (Addgene

plasmid #14252). The expression plasmids were transformed into the yeast *Saccharomyces cereviseae* strain BY4741 (gift from Sébastien Thomine).

Confirmation of expression in yeast by western blotting

To confirm protein expression in yeast, total protein extract was isloated from yeast using Clontech's Urea/SDS extraction method (Clontech, 2009). Protein extracts were run on SDS-PAGE and transferred to PVDF membrane (Bio-Rad). HA-tagged proteins were detected with 1:4000 anti-HA antibody (Roche) visualized by chemiluminescence with the ECL Plus Western Blotting Substrate kit (Pierce Chemical). Protein loading levels were confirmed by visualization of proteins on the membrane using Ponceau S (Sigma).

Measurement of ethylene production in yeast

S. cereviseae constitutively expressing Sp-2OGD-like or At-ACO4 proteins in log-phase growth were used to start 1 mL cultures in 6 mL glass vials. The specified ethylene precursor was added to the cultures, and then the vials were sealed with septa fitted caps (Fisher Scientific). Cultures were grown at 28°C with agitation. After 20 hours, 1 mL of headspace was removed from each vial using a syringe fitted with a 22-gauge needle (Hamilton), and injected into an HP 6890 gas chromatograph (Agilent) equipped with an activated alumina packed column and a Flame Ionization Detector (FID). The chromatographic retention time was compared with standards of pure ethylene diluted in carbon-free air.

AtACO4F- GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAGCTCATGGAGAGTTTCCCGATCATC
AtACO4R2- GGGGACCACTTTGTACAAGAAAGCTGGGTTCGCAGTGGCCAATGGTCC
comp6684F- GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAGCTCATGAATCATTTCAGAAATAAGT TACC
comp6684R2- GGGGACCACTTTGTACAAGAAAGCTGGGTTTAATGAAAAGTTATTATAAACTTTTTTAAC C
comp7597F- GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAGCTCATGGCTCCACGATACATTCC
comp7597R2- GGGGACCACTTTGTACAAGAAAGCTGGGTTAACTCGAATGGCAGCAGG
comp11895F- GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAGCTCATGCATGTGCATGTGCGTG
comp11895R2- GGGGACCACTTTGTACAAGAAAGCTGGGTTAAGTTTCTTTAAGTTTATATTATCAAAGTT G
comp13325F-

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAGCTCATGCCTCCTCACACGAGCTAC
comp13325R2- GGGGACCACTTTGTACAAGAAAGCTGGGTTTTTTTCTAGTTTAATATTATCGAATGTTTG

Table 4-1: Primers used to clone AtACO4 and Sp2OGDs

Measurement of ethylene production in *S. pratensis*

S. pratensis filaments cultured in 3NBBM media were transferred to 6 mL glass vials with 800 µL 3NBBM with the indicated ethylene precursors, and then the vials were sealed and cultured as described above. After 48 hours of treatment, 1 mL of headspace was removed from the vial and injected into a gas chromatograph as described above.

Detection of ACC in *S. pratensis*

ACC was extracted and quantified according to Bulens et al (2011). ACC was extracted from 0.5 g of ground *S. pratensis* filaments treated with 1mL of 5% sulfosalicylic acid for 30 min at 4°C. Next, the sample was centrifuged for 10 min at 4°C at 3,000 x g, and the supernatant was collected. ACC was converted to ethylene in a 10 mL airtight vial by mixing 400 µL extract with 600 µL water, 200 µL of 10 mM $HgCl_2$ and 100 µL mixture of saturated NaOH:NaClO (1:2) for 5 min on ice. Evolved ethylene was measured by injecting 1 mL of the headspace in a gas chromatograph (Shimadzu GC-2014) equipped with a packed column (porapak R 50/80 mesh, 3 m, 1/8 inch) and a FID.

Statistical analyses

Significant differences were determined using JMP Pro v13.1 software (SAS Institute) to carry out either the Student t-test paired comparison of means with an alpha of 0.05 or the Tukey-Kramer HDS comparison of means with an alpha of 0.05.

Chapter 5: Identification and functional conservation of ETHYLENE INSENSITIVE2 (EIN2), a central regulator of ethylene signaling, in the charophycean green alga Spirogyra pratensis

Introduction

Ethylene is a gaseous plant hormone involved in growth, development and stress response in land plants and charophyte green algae (Abeles et al., 1992). Ethylene is perceived at the ER-membrane by a family of ethylene receptors. The ethylene receptors function together with CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), a serine/threonine kinase, to negatively regulate ethylene response (Clark et al., 1998; Kieber et al., 1993). In the model plant *Arabidopsis thaliana*, dark-grown *ctr1* loss-of-function mutants exhibit a constitutive ethylene "triple response" consisting of a shortening and thickening of the hypocotyl, an inhibition of root elongation and the formation of an apical hook (Bleecker et al., 1988; Guzman and Ecker, 1990; Kieber et al., 1993). In the absence of ethylene, CTR1 phosphorylates ETHYLENE INSENSITIVE2 (EIN2) to block ethylene response (Chen et al., 2011; Ju et al., 2012). When ethylene is present, CTR1 no longer phosphorylates EIN2, allowing a carboxyl terminal portion (C-TAIL) of EIN2 to be cleaved and translocated into the nucleus (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). EIN2 C-TAIL activates ethylene response by binding to the 3'UTR of the F-box proteins EBF1 and EBF2, which are involved in degrading the ethylene transcription factors EIN3/EIL1, and targeting these transcripts to processing bodies for

degradation (Li et al., 2015; Merchante et al., 2015). Additionally, the carboxyl terminal end of EIN2 interacts with EIN2 NUCLEAR-ASSOCIATED PROTEIN1 (ENAP1) and together they help regulate histone acetylation allowing for EIN3 binding to promote transcription and activate ethylene responses (Zhang et al., 2017).

EIN2 is one of the most important components of the ethylene signaling pathway. Loss-of-function mutations in EIN2 cause complete ethylene insensitivity (Alonso et al., 1999). EIN2's importance is highlighted in its function to not only transmit ethylene signal from the ER to the nucleus but also its direct involvement in stabilizing ethylene transcription factors and helping the transcription factors bind to ethylene responsive genes (Li et al., 2015; Merchante et al., 2015; Zhang et al., 2017). EIN2 has two domains: an amino-terminal domain that has sequence similarity to the Nramp family of metal ion transporters found in prokaryotes and eukaryotes(first ~480 amino acids of *A. thaliana* EIN2), and a carboxyl-terminal signaling portion (everything excluding the Nramp domain, termed C-END) (Alonso et al., 1999). Although the Nramp domain of EIN2 has been predicted to transport a metal due to its similarity with known metal ion transporters, no transport activity has yet been published. The only known function of the twelve predicted transmembrane domains of the Nramp domain are to help anchor EIN2 to the ER membrane. EIN2 C-END contains the putative cleavage sight (Qiao et al., 2012), a nuclear localization signal (Bisson and Groth, 2011) and is sufficient for activating ethylene responses (Alonso et al., 1999).

Recently, several papers examined the evolution of the ethylene signal transduction pathway by identifying ethylene responses and conserved ethylene signaling proteins in basal land plants such as *Physcomitrella patens* (Rensing et al., 2008; Yasumura et al., 2012) and the charophyte green alga *Spirogyra pratensis* (Ju et al., 2015). Green algae are divided into chlorophytes and charophytes. Chlorophytes, which include the model system *Chlamydomonas reinhardtii*, are a large diverse group of green algae thought to appear somewhere between 0.7 and 1.5 billion years ago (Berney and Pawlowski, 2006; Douzery et al., 2004; Hedges et al., 2004; Herron et al., 2009; Leliaert et al., 2012; Sanderson et al., 2004). Charophytes are freshwater green algae that diverged from chlorophytes. Charophytes are the closest living algal relatives of land plants and therefore are important to study to gain insights into the transition of life in the water to life on land (Becker and Marin, 2009; Delwiche and Cooper, 2015; Timme and Delwiche, 2010; Wodniok et al., 2011). The most recent common ancestor of charophytes and land plants existed 450 million years ago (Sanderson et al., 2004). The filamentous charophyte, *S. pratensis*, belongs to the order of zygnematales and is thought to be one of the charophytes that is most closest related to land plants (Timme and Delwiche, 2010; Zhong et al., 2013, 2014). Sequence data from *S. pratensis*, together with functional analyses, have indicated that a plant-like ethylene signaling pathway was present prior to the evolutionary transition to land (Ju et al., 2015).

While much of the land plant ethylene signal transduction pathway was determined to be functionally conserved in *S. pratensis*, a full-length functioning

EIN2 was absent (Ju et al., 2015). Without a full-length EIN2 to relay the ethylene signal from the ER to the nucleus, then ethylene signaling in *S. pratensis* must function differently than in land plants. Furthermore, the *Klebsormidium flaccidum* genome, the only published charophyte genome, does not contain any EIN2 carboxyl-terminal domain homologs (Hori et al., 2014). Moreover, a best BLAST hits analysis of several charophyte transcriptomes suggest that only a small portion of the EIN2 C-END was conserved in *S. pratensis* and *Coleochaete orbicularis* (Ju et al., 2015), which is thought to belong to a sister clade of the zygnematales. Charophytes that are thought to have diverged earlier, *Nitella mirabilis*, *Klebsormidium nitens* and *Mesostigma viride*, did not have EIN2 C-EIN2 containing best BLAST hits (Ju et al., 2015). This led to the hypothesis that the EIN2 carboxyl-terminal signaling domain did not appear evolutionarily until the more recent charophyte lineages and that a full-length EIN2 may not have appeared until after early land plants.

Here, we investigated *EIN2* transcripts in *S. pratensis* and uncovered the presence of a full-length *EIN2* cDNA homolog. Moreover, we have identified several other full-length *EIN2* cDNA homologs in other charophytes as well as chlorophyte green algae. Furthermore, we have identified putative EIN2 Nramp and C-END domains in several additional species of chlorophytes and charophytes. In order to assess whether the function of the land plant EIN2 is conserved in *S. pratensis*, we attempt to complement an *A. thaliana* loss-of-function *ein2* mutant with the full-length and C-END portion of *SpEIN2*.

Results

Identification of a full-length *EIN2* homolog in *S. pratensis*

To search for potential EIN2 Nramp and carboxyl-terminal sequences in *S. pratensis*, we performed tBLASTn using the *A. thaliana* and *Marchantia polymorpha* (liverwort) EIN2 sequences to query two published *S. pratensis* transcriptomes (Ju et al., 2015; Van de Poel et al., 2016). These searches yielded the previously identified homolog of a portion of the EIN2 C-END (Ju *et al.* 2015) as well as two Nramp homologs (*E-value* <1e-10), neither of which overlapped with the EIN2 C-END homolog To see if we could uncover overlapping sequences, we generated a new assembly of existing RNAseq reads using an updated version of Trinity (v2.3) (Clay et al., 2017; Van de Poel et al., 2016). Using the *M. polymorpha* EIN2 sequence to query the new assembly, we identified the same partial EIN2 C-END hit (DN5242_c1, *E-value* 3e-56) and the two Nramp encoding sequences (DN6454_c0, *E-value* 2e-72; DN5698_c0, *E-value* 5e-20). Now, however, we detected a small hit that has sequence similarity to a middle portion of EIN2 (DN5242_c2, *E-value* 8e-4). Upon visual inspection, this portion that we call "middle EIN2" (DN5242_c2) appeared to bridge the gap between the Nramp homolog, DN5698_c0 and the partial EIN2 C-END homolog DN5242_c1 (Figure 5-1); 63 of the last 65 nucleotides of DN5698_c0 were identical to the first 65 nucleotides of DN5242_c2. In addition, 28 of 34 nucleotides were identical between middle EIN2 and the partial EIN2-CEND sequence, thus potentially linking together all three contigs.

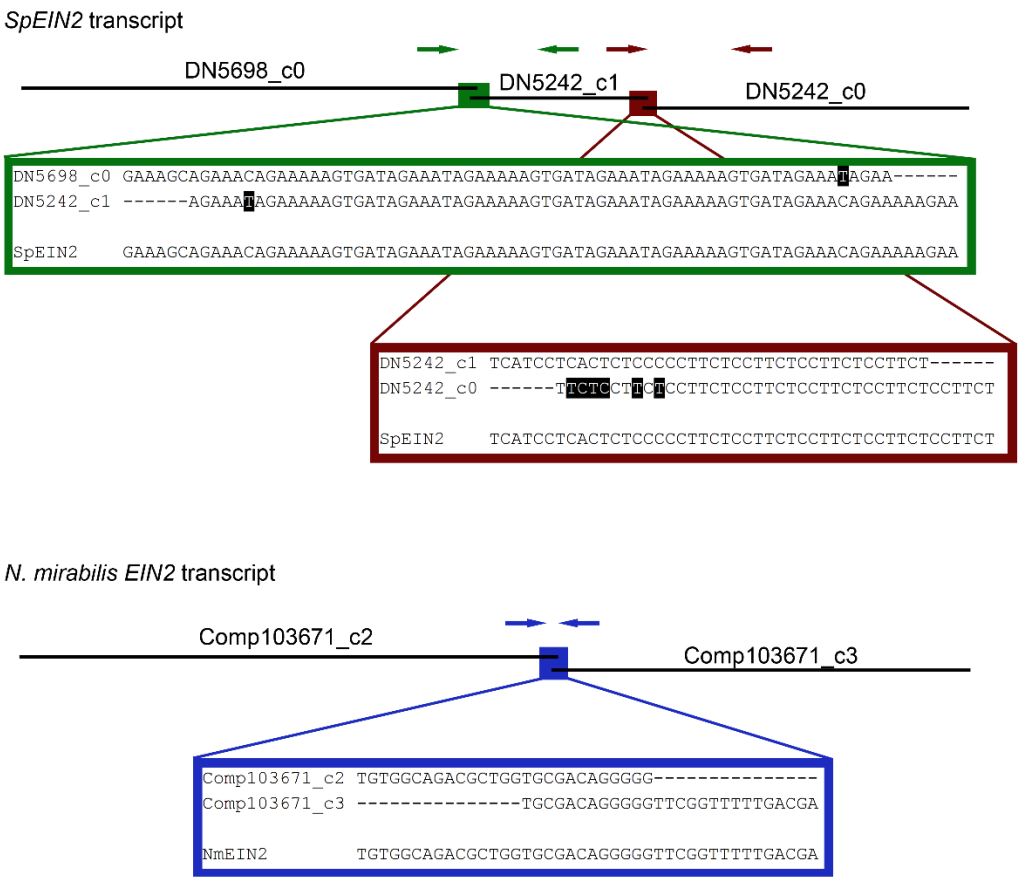


Figure 5-1: Identification of full-length EIN2 transcripts in *S. pratensis* and *N. mirabilis*

Boxed alignment sequences indicate overlapping nucleotide sequences from different assembled contigs that encode for full-length EIN2 homologs. Arrows indicate relative position of primers used to amplify these overlapping regions to confirm that these contigs are part of a single transcript. Actual sequence is listed below contig sequences. Nucleotides with black background are mismatched from the confirmed

sequence. Upper- Green box indicates overlapping DN5698_c0 and DN5242_c1 *S. pratensis EIN2* sequences. Red box indicates overlapping DN5242_c1 and DN5242_c0 *S. pratensis EIN2* sequences. Lower- Blue box indicates overlapping Comp103671_c2 and Comp103671_c3 *N. mirabilis EIN2* sequences.

We confirmed the assembly of these three contigs by PCR-amplification of the overlapping sequences using *S. pratensis* cDNA as the template and PCR primers flanking the overlaps as shown in Figure 5-1. We cloned the PCR fragments into pGEM-T using TA cloning and confirmed the sequence of the overlapping region. Notably, the overlapping regions between the three contigs contain repeated sequences (Figure 5-1) with either single mismatches or potential sequencing errors that may have made it difficult for the assembler to accurately piece together.

Expression of the SpEIN2 carboxyl terminal domain confers ethylene hypersensitivity in an Arabidopsis ein2 mutant

To determine if SpEIN2 is functionally conserved with EIN2 in land plants, we tested whether the full-length *SpEIN2* sequence is able to rescue the *Arabidopsis ein2-5* loss-of-function mutant. We cloned the full-length *SpEIN2* coding sequence (from *S. pratensis* cDNA) into a binary transformation vector (pEarleyGate101) carrying the constitutive CaMV 35S promoter and a C-terminal YFP-HA tag, and stably transformed the construct into the *Arabidopsis ein2-5* null mutant. All seven stably transformed lines of full-length *SpEIN2* in the *ein2-5* background remained ethylene insensitive similar to the untransformed *ein2-5* mutant with or without the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Figure 5-2 A,B). Consistent with these results, no YFP signal could be detected in the nucleus of cells in the hypocotyl of seedlings from these transformed lines.

Expression of the AtEIN2 C-END in *A. thaliana* confers a constitutive ethylene response. To determine if the SpEIN2 C-END also confers a constitutive

response, we cloned the *SpEIN2* C-END (from nucleotide 1461, resulting in a protein that begins three amino acids after the last predicted transmembrane domain of the Nramp domain) into pEarleyGate101 and stably transformed the construct into *Arabidopsis ein2-5*. On MS medium containing no ACC, dark-grown *SpEIN2c-tail ein2-5* seedlings were smaller than those of the untransformed *ein2-5* background as well as the wild-type Col-0 control, consistent with a weak constitutive ethylene response (Figure 5-3 A,B). On MS plates with 20μM ACC, *SpEIN2c-tail ein2-5* seedlings displayed a strong hypersensitive ethylene phenotype (5 of 5 lines observed). Lines 2 and 5 displayed the strongest hypersensitive phenotypes. YFP signal was easily detected in the nuclei of hypocotyl cells in lines 2 and 5 with the addition of ACC (Figure 5-3C, Table5-1). Lines 1, 3 and 4, which showed weaker hypersensitivity to ACC correspondingly displayed a weaker YFP signal in the nucleus after the addition of ACC (data not shown). Without ACC treatment, lines 2 and 5 displayed much weaker YFP signal in the nucleus consistent with a weaker ethylene response without ACC (Table 5-1).

Identification of full-length EIN2 homologs in chlorophyte and charophyte species

Once we identified a full length EIN2 homolog in *S. pratensis* and found that *SpEIN2 C-END* was capable of conferring ethylene hypersensitivity, we were interested in determining the extent to which EIN2 is conserved in green algae, especially since *K. nitens*, the only charophyte with a sequenced genome, appears to

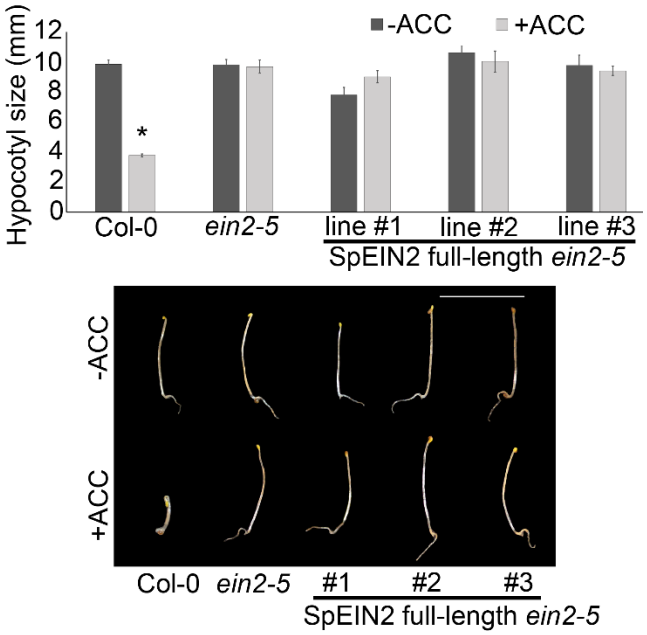


Figure 5-2: Full-length *SpEIN2* does not rescue *A. thaliana ein2-5* mutant

Upper- The hypocotyl lengths of 4-day-old etiolated seedlings treated with or without 20µM ACC indicate *A. thaliana* seedlings expressing full-length *SpEIN2* are insensitive to ethylene. Bar lengths indicate mean hypocotyl lengths ± standard error. Measurement significantly different ($P < 0.05$) from other measurements is indicated by an asterisk.

Lower- Representative 4-day-old etiolated wild-type (Col-0), *ein2-5* and *SpEIN2 ein2-5* (3 independent lines) seedlings treated with or without 20µM ACC.

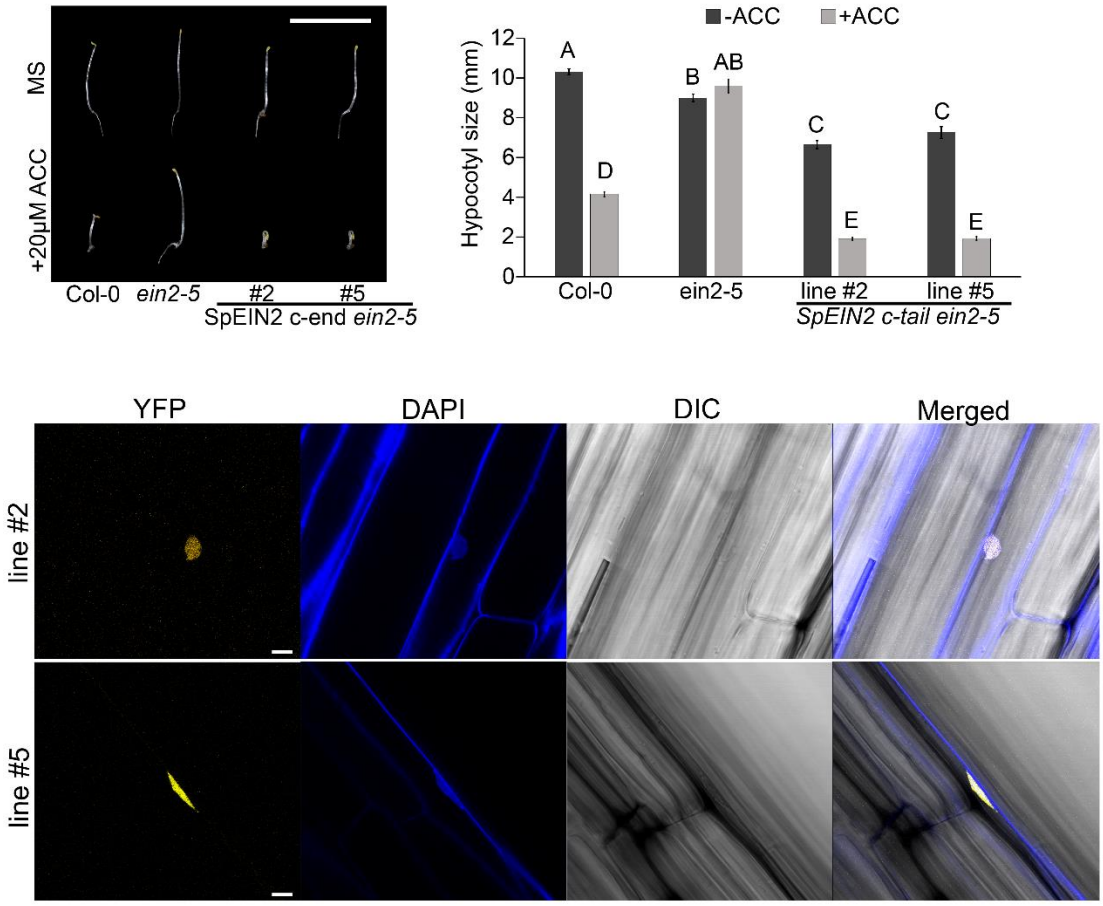


Figure 5-3: *SpEIN2 C-END* confers ethylene hypersensitivity in *A. thaliana*

Top left- Representative 4-day-old etiolated wild-type (Col-0), *ein2-5* and *SpEIN2 C-END ein2-5* (2 independent lines) seedlings indicate that *SpEIN2 C-END ein2-5* seedlings are hypersensitive to 20µM ACC.

Top right- The hypocotyl lengths of 4-day-old etiolated seedlings treated with or without 20µM ACC. Bar lengths indicate mean hypocotyl lengths ± standard error.

Significant differences ($P < 0.05$) in measurements are indicated by different letters above the bar.

Bottom- Localization of *SpEIN2 C-end- YFP* in *A. thaliana* hypocotyl cells. Seedlings were pretreated with DAPI to help identify nuclei. Scale bar = 10µm

	treatment	easily detectable signal	detectable signal	no signal
SpEIN C-END #2-5	+ACC	20/20	0/20	0/20
	-ACC	8/20	8/20	4/20
SpEIN C-END #5-6	+ACC	20/20	0/20	0/20
	-ACC	0/20	7/20	13/20

Table 5-1: SpEIN2 C-END YFP fluorescence is stronger with ethylene treatment.

Quantification of YFP fluorescence intensity of the hypocotyls of two lines of *A. thaliana* seedlings expressing *SpEIN2 C-END-YFP*. Twenty seedlings pretreated with water (control) or 100 µM ACC were visualized. YFP-signal was considered easily detectable it multiple fluorescence were detected after focusing the confocal microscope. YFP-signal was classified as detectable if any fluorescence was detected.

lack *EIN2* (Hori et al., 2014). We carried out BLAST searches of the published transcriptome assemblies of twenty chlorophytes and nine additional charophytes (Cooper and Delwiche, 2016) for full-length EIN2 sequences using *M. polymorpha* EIN2 to query the assemblies. In the assembly of another *Spirogyra* species, *Spirogyra sp.*, we identified a previously unpublished full-length EIN2 sequence (comp9361_c0). An assembly for *Nitella mirabilis* contained a contig containing both the NRAMP sequence as well as the sequence similar to the middle region of EIN2 (comp103671_c2). A second contig contained the rest of an EIN2 C-END sequence (comp103671_c3). Again, we visually examined the two sequences, found overlap in the sequences, PCR-amplified the overlapping region of these two comps and confirmed the sequence (Figure 5-1). In addition to these two additional charophyte species, we were able to identify full-length EIN2 sequences in five of the twenty chlorophytes (*Hormotilopsis gelatinosa*, *Elakatothrix viridis* (SAG 9.94), *Phaeophila dendroides*, *Tetraselmis striata* and *Watanabea reniformis*) (Table 5-2). In all, we were able to find at least partial EIN2 C-END sequences in 12/20 chlorophyte species and 9/10 charophyte species (*K. nitens* does not have EIN2).

Identification of EIN2 regions conserved in green algae and land plants

Having identified several full-length algal EIN2 homologs, we were interested in identifying regions of EIN2 that are conserved among green algae and land plants. To identify conserved regions, we aligned the protein sequences of the full-length charophyte and chlorophyte EIN2s as well as EIN2 protein sequences from *C.*

Species	Region of EIN2 contig aligns to:				
Chlorophyte	**Nramp**	**middle**	**c-end**	**Top CTR1 hit**	**Other Nramps**
Ankistrodesmus falcatus	comp10893_c2	comp10567_c2	comp10567_c3	comp6417_c0	comp12283_c0, comp11255_c12
Atractomorpha echinata	comp7744_c0	comp24040_c0	comp7758	comp10121_c3	comp2686_c0
Bracteacoccus aerius	comp5224_c0 + comp28340_c0	comp5224_c2		comp8383_c8	comp3225_c0, comp8005_c2, comp223992_c0, comp1665_c0
Cephaleuros parasiticus				comp19140_c0	comp13182_c0, comp17535_c0
Elakatothrix viridis	comp6695_c1	comp6695_c0		comp7240_c0	comp6001_c0, comp32399_c0, comp13994_c0
Eremochloris sp.				comp14748_c0	comp9018_c0, comp6813_c0
Hormotilopsis gelatinosa	comp24698_c0			comp24856_c0	comp19992_c0, comp21099_c0
Leptosira terrestris				comp8893_c0	comp5489_c0, comp4432_c0, comp5062_c0
Nephroselmis pyriformis				comp22327_c0	comp6345_c0
Oedogonium cardiacum					comp49463_c0, comp41533_c1
Oltmannsiellopsis unicellularis	comp138324_c0 + comp25661_c0		comp157677_c0	comp11538_c0	comp3978_c0
Oocystis solitaria	comp38015_c1	comp37668_c0		comp39325_c0	comp29565_c1, comp35773_c1
Phaeophila dendroides	comp9087_c0			comp15449_c1	comp24080_c0
Prasiolopsis sp.				comp27588_c0	comp25661_c0, comp25650_c0
Pyramimonas parkeae				comp16142_c0	comp22033_c0, comp15870_c0, comp16915_c0
Tetraselmis striata	comp27626_c2			comp26594_c0	comp27417_c0, comp19718_c0, comp23881_c0
Tetraselmis suecica	comp25758_c1	comp18680_c0		comp22987_c0	comp19744_c1, comp24930_c0, comp25476_c0
Trebouxia aggregata				comp11576_c0	comp12128_c0, comp11905_c0, comp9520_c0, comp116730
Trentepohlia annulata					comp15937_c0(has stops), comp17335_c0, comp10770_c0
Watanabea reniformis	comp7024_c0			comp6213_c0	comp6557_c0, comp7295_c0, comp5990_c0

Charophyte					
Chaetosphaeridium globosum	comp14505_c0	comp21197_c0	comp30276_c0	comp40690_c0	comp8180_c0, comp19465_c0, comp36971_c0, comp23903_c0
Coleochaete orbicularis	?	comp28893_c0		comp38388_c0	comp27322_c0, comp33604_c0, comp756495_c0
Entransia	comp24899_c0	comp10479_c0		comp27909_c1	comp27054_c0, comp29146_c1, comp29692_c1
Klebsormidium nitens				comp10124_c0	comp3995_c0, comp6331_c0, comp4741_c0
Mesostigma viride	comp42519_c0	comp24445_c0	comp19651_c0	comp36186_c0	comp36043_c1, comp40966_c0
Mougeotia scalaris	comp11874_c0	comp14101_c0		comp14465_c1	comp9357_c0, comp12121_c0, comp4488_c0, comp5349_c0
Nitella mirabilis	comp103671_c2		comp103671_c3	comp103372_c21	comp102493_c0, comp104726_c5, comp31121_c0
Penium margaritaceum	comp319066_c0	comp29458_c2	comp32850_c2	comp35410_c1	comp30240_c0, comp33995_c1, comp36450_c2, comp34042_c0
Spirogyra pratensis	DN5698_c0	DN5242_c2	DN5242_c1	DN6131_c0	DN6354_c0
Spirogyra sp.	comp9361_c0			comp10492_c1	comp5216_c0, comp7086_c0, comp8613_c0, comp291_c0

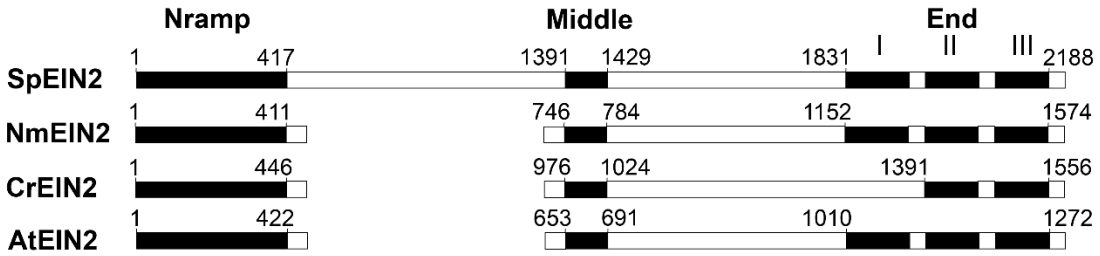
Table 5-2: Identification of EIN2, Nramp and CTR1 homologs in green algae

Listed in this table are the contig identification number for at least portion of EIN2, Nramp, and CTR1 homologs from several charophyte and chlorophyte green algal transcriptomes. For EIN2 homologs, we identify the portion of EIN2 contained in each contig. EIN2 Nramp homologs are distinguished from other Nramp containing sequences in figure 5-5.

reinhardtii and several land plants. We found three primary regions that are highly conserved in all of these sequences (Figure 5-4). As known previously, the N-terminus of EIN2 always features an Nramp domain (amino acids 1-422 in AtEIN2) (Alonso et al., 1999). While most of the EIN2 C-END in conserved among the land plants, among land plants and green algae most of the EIN2 C-END is not conserved with the exception of two domains. One conserved domain is a span of amino acids (amino acids 653-691 in AtEIN2) that we call the middle domain. The middle domain is encoded by the "middle" *SpEIN2* contig as well as the *NmEIN2* Nramp/middle region contig that were used to bridge the Nramp and C-END domains to form full-length EIN2. The second conserved C-END domain has a series of 2 or 3 closely spaced conserved regions (within amino acids 1010-1272 in AtEIN2) that we refer to as the C-terminal domain. The EIN2 amino acid sequence is not conserved outside of the Nramp domain, middle domain and C-terminal domain among the highly diverged species that we examined.

Identification of putative EIN2 Nramp domains

While identifying full-length EIN2 sequences in chlorophytes and charophytes, we discovered many species that contained partial EIN2 sequences. Hits encoding Nramp domains were present in all species examined but we lacked the information required to determine whether these Nramp domains represent EIN2 or a non-EIN2 Nramp protein. The middle domain of EIN2, due to its relatively small size, is not a good indicator of the presence of EIN2. The EIN2 C-terminal domain, however, is



Middle

```
SpEIN2  GGSGSGSLSRFSG----------LGRGARRQFAAILDEFWGMLFDLHGQ
NmEIN2  KAGAMASKAAPCS----------LGRSSRRQLANVLDDFWASVFDLHGQ
CrEIN2  SGGGSGRAGTSASAPSSSRPPHPVTRGGRRQFALLLDEFWSCLYDAHGL
AtEIN2  GGSGTGSLSRLQG----------LGRAARRHLSAILDEFWGHLYDFHGQ
```

End I

```
SpEIN2  SLWSDSAYSALFGKNKMSETQKWLWEANAKSEVIQNQKFSQSQPFSQSQQQQTSSQSPPQ
NmEIN2  SYWHKSPQEVLFPSSSIPRHPTQFETSEMAGNAAAAAAAAGAVGGSRVVGRDGQQGLGSTS
AtEIN2  SLWSRQPFE--------------------------------QFGVAERNGAVGEELRN

SpEIN2  SLSSLRLQESLSQQMSQHGIRKEGSRKADSSMAVNSLFGNLTEKSVMDRMRVYFHRLMKL
NmEIN2  SVGGRGSDQTMAAEDSNDCARSSSSFRSDTEQPSSSFVSDGPLGLLLDRLSLCVQRLRQL
AtEIN2  RSNPINIDNNASSNVD----------------------AEAKLLQSFRHCILKLIKL

SpEIN2  EGSDWLFRFDTGYDEDLI----------SCIAIREKSLHD
NmEIN2  DGAECLFHVRLSPDEIVIGWLGFGWVAYGPGQDRACAGNSVRMSRE
AtEIN2  EGSEWLFGQSDGVDEELI----------DRVAAREKFIYE
```

End II

```
SpEIN2  CEGGRCVWNTNLIISFGVWCIHRVLELSQMESRPELWGKYTYVLNRLQGIL-DKAFLQPR
NmEIN2  CGDARCVWSRELVISFAVWSSVRLLELCQMETRPELWGKYTYVLNRLQGVL-ESSFRVPR
CrEIN2  TLSSWCLFGPAVLVSFGVWCAYTLLQWCAAESRPELWGRYAAMLNRLQGVVWDSRLDVAA
AtEIN2  CGDG-CVWRADLIVSFGVWCIHRVLDLSLMESRPELWGKYTYVLNRLQGVI-DPAFSKLR

SpEIN2  SIPNLCDCF
NmEIN2  PGPPTCPCI
CrEIN2  AAAAAADAA
AtEIN2  TPMTPCFCL
```

End III

```
SpEIN2  KGALASFFLEMIKDLENAVSG----RKGRTGTAAGDVAFPKGKENLASVLKRYKRKL---
NmEIN2  NASLITSMLDLVRSVEIAVGS----RKGRAGTAAGDVAFPKGKENLASALKRYKRRLGNC
CrEIN2  PAHLALLRREELAAQLAGLEGGGTLERRGPADTAAGEVAFPRGKEILLSVMRRYRRRLG--
AtEIN2  KCTTAVTLLDLIKDVEMAISC----RKGRTGTAAGDVAFPKGKENLASVLKRYKRRL---

SpEIN2  -----VNR
NmEIN2  RANFSSNK
CrEIN2  -----IG-
AtEIN2  -----SNK
```

Figure 5-4: Identification of conserved EIN2 domains

Upper- Relative positions of conserved domains of EIN2 in representative species (Sp= *S. pratensis,* Nm= *N. mirabilis,* Cr= *C. reinhardtii,* At=*A. thaliana*). Conserved domains are indicated by black boxes. Amino acid positions of conserved domains are indicated.

Lower- Alignment of the middle and C-terminal domains of the representative EIN2. Conserved amino acids are highlighted with black backgrounds. Similar amino acids are highlighted with grey backgrounds.

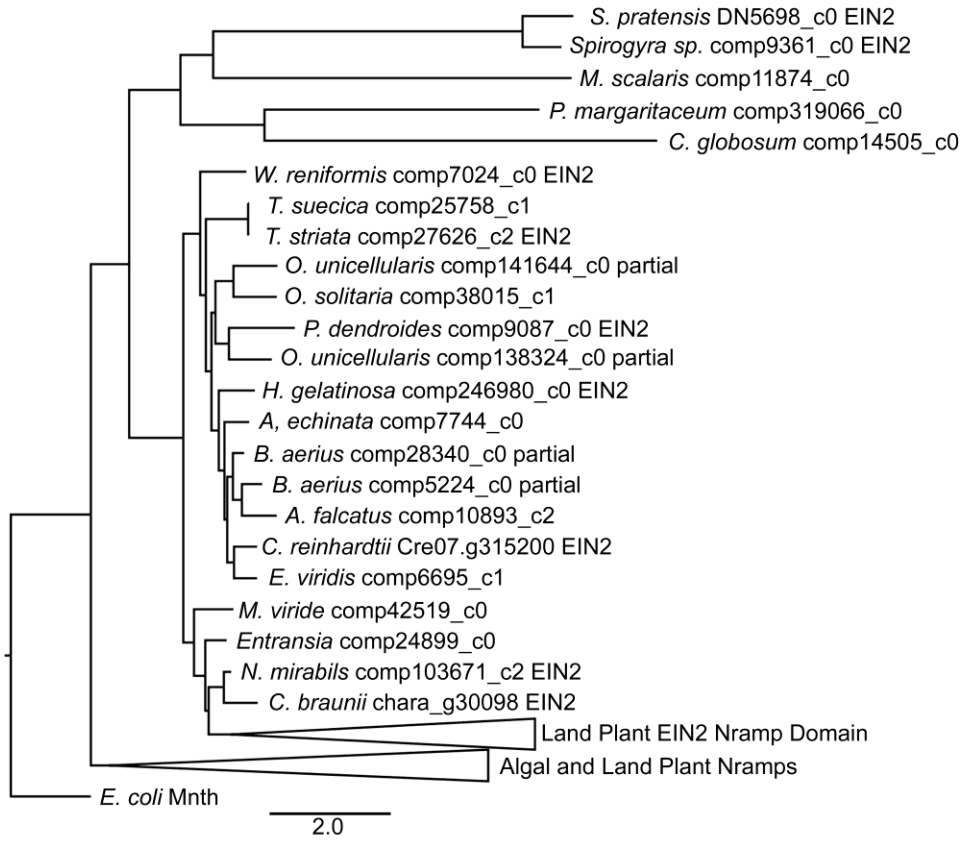


Figure 5-5: Phylogenetic analysis of land plant and algal Nramps reveals EIN2 Nramp domains

Phylogenetic tree including all Nramp-domain containing contigs from the analyzed green algal transcriptomes as well as the genomes of Chara braunii, C. reinhardtii, and several land plants . Clade containing predicted Nramp proteins and clade containing land plant EIN2 Nramps are collapsed. Contig or locus number of predicted algal EIN2 Nramps are given. Algal Nramp domains from known full-length EIN2 proteins are designated by EIN2. Incomplete algal EIN2 Nramp domains are designated as partial.

specific to plant EIN2 proteins and therefore signifies the presence of an EIN2 transcript.Having found several instances of full-length EIN2 in both chlorophytes and charophytes, we reasoned that any species that has the conserved EIN2 carboxyl terminal domain is likely to have an N-terminal Nramp domain. Determining whether Nramp-containing hits represent EIN2, however, requires the identification of distinct differences between the EIN2 Nramp and other Nramp proteins. To identify these differences, we performed a phylogenetic analysis of all the Nramp-containing hits from the available chlorophyte and charophyte transcriptomes, as well as from *C. reinhardtii* and several land plants (Figure 5-5). We included the *E. coli* Nramp MntH as an outgroup. The phylogenetic analysis revealed a single clade containing all of the EIN2 Nramp domains from known full-length EIN2 sequences. In addition, this clade contained Nramp domain hits from chlorophytes and charophytes that also have EIN2 c-terminal domain hits. Importantly, species that lack an EIN2 c-terminal domain also lack Nramp-hits in this clade. Interestingly, later charophytes, including several zygnematales and *Chaetoshpaeridium globosom*, have divergent EIN2 Nramp sequences suggesting that these EIN2 Nramp domains may have a function different from the EIN2 Nramp domains from other algae and land plants. We conclude that the Nramp domain-containing proteins found in this clade likely part of a full-length EIN2 sequence.

Discussion

Although the ethylene signal transduction pathway found in higher plants was shown to be largely conserved in *S. pratensis* (Ju et al., 2015), the absence of a full-length sequence for EIN2, the central regulator of ethylene response, had been puzzling. Here we report the identification of transcripts that encode full-length EIN2 not only in *S. pratensis*, but in several charophyte and chlorophyte algae. Moreover, we have demonstrated that the *SpEIN2 C-END* can rescue the ethylene insensitive phenotype of the *Arabidopsis ein2-5* mutant, indicating that *Sp*EIN2 is functionally conserved. We also identified putative EIN2 Nramp and EIN2 C-end sequences from additional green algae indicating that full-length EIN2 sequences are present in both charophytes and chlorophytes, despite the absence of EIN2 in the genomic sequence of *K. nitens (*Hori *et al.* 2014).

Surprisingly, the C-END portion of EIN2 exhibits functional conservation in land plants and *S. pratensis* despite the divergence of charophytes and land plants approximately 450 million years ago (Sanderson et al., 2004). The functionality of *Sp*EIN2 in *Arabidopsis* suggests that *Sp*EIN2 is capable of stabilizing and activating the EIN3 transcription factor. Moreover, the observation that *Sp*EIN2 C-END is activated upon ethylene treatment indicates that another key feature of EIN2 is conserved, regulation by the CTR1 protein kinase. In *Arabidopsis*, the ethylene receptors activate CTR1 to control EIN2 by phosphorylation in the absence of ethylene. When ethylene binds to the receptors, CTR1 no longer phosphorylates EIN2, thus triggering ethylene signaling by EIN2. Since *SpEIN2 C-END-YFP*

confers hypersensitivity to ethylene and the SpEIN2-C-END-GFP signal is detected in the nucleus, we propose that ethylene signaling from the receptors to AtCTR1 results in nuclear translocation of *Sp*EIN2. Therefore we deduce that without ethylene perception, AtCTR1 is inactivating *Sp*EIN2 by phosphorylation. Because the three important features of EIN2 (regulation by phosphorylation of CTR1, translocation to the nucleus and activation of EIN3) are conserved in the *S. pratensis* we can conclude that the main functions of EIN2 are conserved in both charophytes and land plants.

Overexpression of the *Arabidopsis EIN2 C-END* in *ein2-5* confers a strong constitutive ethylene response (Alonso et al., 1999), as the C-END is thought to constitutively translocate to the nucleus and activate ethylene responses. The *SpEIN2 C-END* confers a weak constitutive ethylene response in the absence of ethylene and requires ethylene treatment to confer a strong response. Notably, the *Sp*EIN2 C-END is nearly twice the size of the *Arabidopsis* C-END (nearly 600 amino acids larger). It is therefore possible that the *Arabidopsis* EIN2 C-END is constitutively transported to the nucleus whereas the *Sp*EIN2 C-END might be excluded due to its large size. Alternatively, the large *Sp*EIN2 C-END could be unstable in *Arabidopsis* without ethylene. The presence of ethylene could alter phosphorylation of SpEIN2 C-END leading to its stabilization. These hypotheses are supported by the observation that ACC treatment caused an increase in the nuclear-localized *Sp*EIN2-YFP signal.

We found that only three domains are highly conserved in all EIN2s. An Nramp domain is conserved near the N-terminus of the EIN2 protein (amino acids 1-422 in *At*EIN2). While this domain shows homology to the Nramp metal ion transporters, whether the EIN2 Nramp domain transports a metal is unknown (Alonso et al., 1999). This may be because a couple of amino acids necessary for metal ion transport in Nramp proteins are not conserved in the EIN2 Nramp domain. The highly conserved sequence found in this domain would suggest that this domain does serve an important function beyond anchoring the C-terminal domain to the ER membrane. The middle domain (amino acids 653-691 in *At*EIN2) of the EIN2 is considered part of the carboxyl terminal tail (C-TAIL) as it is within the portion of EIN2 that is cleaved and moved to the nucleus during ethylene exposure. This middle domain is only several amino acids from the proposed *At*EIN2 cleavage site (between amino acids 645 and 646) (Li et al., 2015). Therefore it is possible that this site is important for cleavage of EIN2. However, *At*EIN2 C-TAIL that lacked part of this domain was not able to activate ethylene response indicating that this region is important for activation of signal as well and not just cleavage (Li et al., 2015). The third conserved region is near the carboxyl terminus of the EIN2 protein. This region is also likely important for activation of downstream responses. Charophyte and land plant EIN2s have three conserved motifs in this region. Chlorophyte EIN2s lack the first of these but have the other two conserved motifs.

Previous data have indicated that EIN2 may have originated in later charophytes, and that full-length EIN2s, consisting of an Nramp domain and a C-

END portion, may not have been derived until early land plants. Here, we report several charophyte full-length EIN2 sequences. Additionally, we were able to identify EIN2 Nramp domains for all algae species with EIN2 C-END domains with one exception. Like Ju *et al.* (2015), we were unable to find a hit for an EIN2 Nramp domain in *C. orbicularis*. Given the prevalence of EIN2 in charophytes and several confirmed full-length EIN2 sequences we think it is likely that *C. orbicularis* has a full-length EIN2 with an EIN2 Nramp domain that was not assembled in this *C. orbicularis* transcriptomes. We conclude that full-length EIN2 sequences are highly conserved in most charophytes.

The presence of EIN2 in *S. pratensis* is not unexpected given that most charophytes have homologs of other components that are both upstream and downstream of EIN2 in the ethylene signaling pathway. Chlorophytes, however, lack ethylene receptors. Therefore we were quite surprised that we detected EIN2 homologs in more than half of the chlorophyte transcriptomes we examined. Without ethylene receptors, EIN2, at least in chlorophyte algae, is likely involved in other signaling pathways. Interestingly, homologs of CTR1, the protein kinase that regulates EIN2 in ethylene signaling, are also found in chlorophytes. In the current model of the evolution of ethylene signaling, the ethylene receptor originated in cyanobacteria and was incorporated into the plant lineage genome following plastid endosymbiosis (Mount and Chang, 2002). Based on our findings, the major ethylene signaling components –the ethylene receptor, CTR1 and EIN2 – were all present in the common ancestor of chlorophytes and charophytes, placing the origins of these

components around ~1 billion years ago. In this case, chlorophytes subsequently lost the ethylene receptor but maintained CTR1 and EIN2. Alternatively, ethylene receptors may not have been acquired until early charophytes. In this model, CTR1 and EIN2 may have originally functioned together in a signaling pathway and then began to interact with the ethylene receptor in early charophytes forming the core ethylene signaling complex. These models place the origin of EIN2 in the chlorophytes, and the complete ethylene signal transduction pathway to as far back as early charophytes.

Materials and Methods

Identification of EIN2 homologs

EIN2 homologs were identified from assemblies of several charophyte and chlorophyte transcriptomes [17,33] and *S. pratensis* RNA seq reads [31,32]. Assemblies were converted to a BLAST database using the BLAST+ (v2.3.0) suite of programs [35]. Putative domains of EIN2 homologs were identified using either the *At*EIN2 or *Mp*EIN2 protein sequence to query each database using tBLASTn. Hits were then analyzed for regions of sequence similarity. Hits with an E-value <1.0E5 for either the EIN2 Nramp domain or EIN2 c-terminal domain, and hits with an E-value < 10 for the EIN2 middle domain were kept for further analysis. Hits that contained both the Nramp domain and c-terminal domain were considered full-length. We further analyzed by hand the nucleotide sequences of Nramp, middle and c-terminal hits to find overlapping sequences. Potentially overlapping sequences were

confirmed by PCR amplification using primers (Table 5-3) that flanked potential overlapping regions using Phusion polymerase (New England Biolabs). PCR products were cloned by TA cloning into pGEM-T (Promega) and then sequenced to identify correct sequence.

Identification of CTR1 homologs.

CTR1 homologs were identified from assemblies of charophyte and chlorophyte transcriptomes [32,33]. Assemblies were queried with the EDR1 domain of *A. thaliana* CTR1 using tBLASTn. Best hits with an *E-value* < 1.0E5 are reported.

Alignment and Phylogenetics

To identify important EIN2 domains, the protein sequences of previously identified full-length EIN2 from charophyte and chlorophyte transcriptomes [33] and *S. pratensis* RNAseq reads as well as from the genome of *C. reinhardtii* [36], *A. thaliana* [37] and *Chara braunii* (IN MANUSCRIPT) were aligned using the Muscle [38] plugin in Geneious (version 5.0.3, www.geneious.com) with default parameters.

To identify EIN2 Nramp domain-containing hits, the Nramp domain was isolated from all Nramp containing hits from the assemblies of charophyte and chlorophyte transciptomes [33], *S. pratensis* RNAseq reads [31,32], and the genomes of *C. braunii (IN MANUSCRIPT), Marchantia polymorpha* [39], *Selaginella moellendorffii* [40], *Physcomitrella patens* [15], *Amborella trichopoda* [41], *Oryza*

Primers used to amplify contig overlapping regions	
SpEIN2NRAMPF4	TTCAAATCAAATGGTTTGTTGAATAAAG
SpEIN2MIDR2	CCCTTGCGAGTGAAATGAC
SpEIN2MidF2	GCATATGCCAACTCATCACTG
SpEIN2CendR1	ATGGAAATAAACTCTCATCCTATCC
NitellaMidR	AGCCGATGGAAAAATGAAAC
Nitella NEnd F	CCGGCTTCTACATGTGGTG

Primers used to amplify full-length EIN2	
SpEIN2N_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTATTCTGTGGCAAGGCC
SpEIN2_NR2	GCGAATCGGAGGATGAGATT
SpEIN2_NF2	AATCTCATCCTCCGATTCGC
SpEIN2C_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATTTCATCATTGAGTTTGAGTAAGC

Primers used to amplify EIN2 C-END	
SpEIN2 Cterm F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCATATCAGAAGGAATGAAACAGAAAAG
SpEIN2CendR2	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTTACTCAAACTCAATGATGAAATGA

Table 5-3 Primers used in this chapter to amplify *S. pratensis* and *N. mirabilis* EIN2

sativa [42], *Populus trichocarpa* [43], *Glycine max* [44] and *A. thaliana* [37]. The protein sequences were then aligned as described above. A maximum-likelihood analysis was used to generate a phylogenetic tree in RAxML 8.2.7 [45] with an LG substitution model [46]. Maximum-likelihood trees were evaluated by boot-strap analysis using 1000 iterations.

Cloning Full-length and C-tail SpEIN2

Full-length EIN2 was amplified from *S. pratensis* cDNA in two fragments (N-terminal or C-terminal) using Phusion DNA polymerase (New England Biolabs) and fragment specific primers. PCR products were digested with either XhoI (for N-terminal) or AscI (for C-terminal) and ligated into pre-digested pER10 [47] plasmid using T4 DNA ligase (New England Biolabs). N-terminal and C-terminal ends ligated in pER10 were then assembled by Gibson cloning resulting in a pER10-*SpEIN2* full-length construct. The full-length *SpEIN2* was then reamplifed by PCR from the pER10-*SpEIN2* full-length using Phusion polymerase and cloned into pDONR221 using the Gateway cloning system (Life technologies). Full-length *Sp*EIN2 sequence was confirmed by sequencing and transferred to the plant expression vector pEarleygate101 [48].

The carboxyl-terminal domain of *SpEIN2* was amplified from pDONR221-SpEIN2 full-length using CEND specific primers. The PCR product was then cloned into the entry vector pDONR221, confirmed by sequencing and transferred to the plant expression plasmid pEarleygate101.

Plant growth conditions:

Arabidopsis thaliana plants were grown under fluorescent light in a 16 hour light, 8 hour dark photoperiod in growth chambers at 22°C. Triple response assay were performed on 4-day-old dark-grown seedlings on Murashige and Skoog (MS) agar plates with and without 20µM ACC. To generate transgenic *SpEIN2 ein2-5* lines, *ein2-5* plants were transformed with *SpEIN2 full-length* and *C-END* using Agrobacterium GV3101 and the flower-dip protocol [49]. Plants homozygous for the trans gene were selected with either BASTA spray (1:3000 dilution Liberty 200, Bayer) or on agar plates containing glufosinate (Sigma).

Fluorescence microscopy

The hypocotyls of seedlings transgenically expressing *Sp*EIN2 Full-length YFP or *Sp*EIN2 C-END YFP were observed using the scanning confocal Zeiss LSM510 microcrope (www.zeiss.com). Seedlings were grown in the dark for 4 days on MS agar plates. Seedlings were moved to water with or without 100µM ACC for 4 hours. Approximately 10 minutes before visualizing seedlings, DAPI (at a final concentration of 20µg/ml) was added to visualize nuclei. At least 10 seedlings for full-length and carboxyl terminal EIN2 were observed for YFP signal. To determine differences in YFP signal strength of seedlings expressing *Sp*EIN2 C-END with and without ACC, 20 seedlings were visually scanned for YFP signal. If multiple YFP signals were easily found when hypocotyl was in focus, then signal was classified as

easy to find. If multiple YFP signal was not immediately found, signal was considered difficult to find.

Statistics

Significant differences were determined using JMP Pro v13.1 software (SAS Institute) to carry out the Tukey-Kramer HDS comparison of means with an alpha of 0.05.

Chapter 6: Conclusions

The goals of my thesis project, like that of all scientific endeavors, have changed over time. I began my project in pursuit of better understanding the function of RTE1. I advanced the projects started by a previous graduate student Jianhong Chang, to better understand how Cb5 was affecting ethylene signaling through RTE1 and the ETR1 receptor. I then became interested in elucidating why only the ETR1 receptor requires RTE1 for function. During this phase of my project, many new genomes and transcriptomes became available and I began looking more and more at the conservation of components of ethylene signaling especially in charophycean green algae. In collaboration with the Delwiche Lab, our lab found that the ethylene signaling pathway was conserved in the charophyte *S. pratensis*. The focus of the last half of my thesis is answering questions that raised by this collaborative effort. In this chapter I will discuss important conclusions from my results, raise new questions about ethylene biosynthesis and signaling and suggest future directions to answer these questions.

Cb5 and RTE1 function in ethylene signaling:

We continue to narrow down the biochemical function of RTE1. After finding that RTE1 interacts with the hemoprotein Cb5 we were able to propose of hypotheses about the function of RTE1. We hypothesized that Cb5 and RTE1 may be involved in oxygen signaling but disproved this hypothesis by testing for ethylene

sensitivity of wild-type and ethylene signaling mutant plants in hypoxic conditions. Given that Cb5 is necessary for fatty acid modifications, we tested to see if RTE1 was also involved in these modifications. Our results suggest that RTE1 is not involved in hydroxylation or desaturation of fatty acids but does not eliminate the possibility that RTE1 is involved in modifying a different lipid. Our original hypothesis suggested the Cb5 and RTE1 may be necessary for redox folding the ETR1 receptor, helping the formation of the correct N-terminal disulfide bonds. We disproved this hypothesis when we found that the *etr1* receptor without these cysteines was still dependent on RTE1.

Current Hypothesis: Cb5 and RTE1 are molecular chaperones for the ETR1 receptor

Cb5 and RTE1 function together to regulate ethylene signaling through the ETR1 receptor. In chapter 2 of this thesis, I confirmed a direct interaction between Cb5 and RTE1 *in planta* and indicated that Cb5 functions upstream of RTE1. In chapter 3 of this thesis I report that a proline localized near the N-terminus of ETR1 and monocot ERS1 ethylene receptors is partially important for RTE1-dependence. Prolines are known to disrupt the secondary structures and introduce rigid bends into proteins. From these results we have developed a model that suggests that ethylene receptors with the N-terminal proline such as ETR1, are initially translated in an inactive conformation possibly due to the proline causing the receptor to be misfolded. Cb5s receive electrons from upstream redox molecules and transfers the

electrons to the heme molecule bound to RTE1. Once reduced, RTE1 is able to activate the ETR1 receptor potentially by serving a chaperone that binds to the ETR1 receptor and allowing the receptor to refold into an active conformation.

During times of stress, plants produce reactive oxygen species such as hydrogen peroxide. To counteract this oxidative stress, cytochrome b5 activity is upregulated (Nagano et al., 2009a). Together with our current hypothesis of the function of Cb5 and RTE1, these results suggests that during times of stress, Cb5 and RTE1 are activated leading to more active ETR1 receptors. This hypothesis suggests that Cb5 and RTE1 serve as a point of crosstalk between oxidative signaling and ethylene signaling. In support of this idea, Catalase2 is an enzyme involved in eliminating hydrogen peroxide from cells while the loss-of-function mutant *cat2-1* mutant is partially insensitive to ethylene (Bueso et al., 2007).

Two main features of this hypothesis still need to be addressed. First, RTE1 heme-binding must be tested *in vivo*. I attempted to stain heme proteins in a native gel electrophoresis of an *A. thaliana* microsomal protein extract with 3,3',5,5'-tetramethylbenzidine (TMBZ) stain but could not detect any heme proteins in my extract (not shown). Alternatively, we can determine if RTE1 binds heme by purifying RTE1 protein and measuring absorbance of 418nm wavelength light. The ring structure of heme absorbs this wavelength of light and can be used to calculate the amount of heme present. Second, the structure of the ETR1 ethylene binding domain must be compared with and without RTE1. Due to the difficulty of

crystalizing transmembrane domains, no one has been able to determine the structure of this part of the ethylene receptor.

An Alternative Hypothesis: Cb5 and RTE1 are important for crosstalk between ceramide and ethylene signaling

Lipid Transfer Protein1 (LTP1) interacts with RTE1 and like Cb5, RTE1 and the ethylene receptors is a negative regulator of ethylene signaling (Wang et al., 2016). Little is known about the *A. thaliana* LTP1 other than it is homologous to known lipid transfer proteins. As discussed in Chapter 2, Cb5 proteins are important for different fatty acid modifications. Although I found no difference in fatty acid saturation or hydroxylation in *rte1* loss-of-function mutants, the fact that RTE1 interacts with two proteins known be involved with lipids and because both RTE1-interacting proteins affect ethylene signaling, it is possible that RTE1's function could still involve a lipid.

Cb5 is necessary for the synthesis of many different fatty acids including ceramide (König et al., 2012). Recently treatment with a fatty acid sphingolipid, Ceramide (specifically ceramide 24:1) was shown to enhance EIN2 translocation to the nucleus and increase EIN3 protein levels (Xie et al., 2015). Specifically, this ceramide binds to CTR1 and can inhibit CTR1 kinase activity *in vitro* (Xie et al., 2015). CTR1 is a RAF-like protein kinase. Interestingly, the human RAF protein is

activated when ceramide binds to an autoinhibitory c1 domain (Huwiler et al., 1996; Müller et al., 1998). CTR1 appears to be inactivated with ceramide.

Currently, it is unclear how the ethylene receptors signal to CTR1. One possibility is that the ethylene receptors control CTR1 kinase activity through ceramide. When ethylene binds to the ETR1 receptor, the receptor helps target ceramide to CTR1 to initiate ethylene response. When ethylene is no longer bound to ETR1, the receptor helps reactivate the CTR1 kinase by removing the ceramide. If the ETR1 receptor uses ceramide to signal to CTR1 then Cb5, RTE1 and LTP1 may be involved in reactivating CTR1. This hypothesis is consistent with the fact that loss-of-function *cb5*, *rte1* and *ltp1* mutants display ethylene hypersensitive phenotypes consistent with an inactive CTR1.

The function of RTE1 homologs in animals is unknown. The human RTE1 homolog, TMEM222 is among the top 20 differentially expressed genes in patients with Parkinson's disease (Diao et al., 2013). Furthermore, abnormal ceramide metabolism has been implicated with Parkinson's disease (Bras et al., 2008; Mielke et al., 2013; Xing et al., 2016). Moreover, LRRK2 kinase, a RAF1 homolog, is one of the main genetic targets of Parkinson's disease research (Liu et al., 2011; West et al., 2005). Furthermore a Cb5 domain-containing protein, HERC2, interacts with LRRK2 (Imai et al., 2015). These results together with the interaction between *C. elegans* Cb5 and RTE1 suggest the animal Cb5, RTE1 and CTR1 homologs play involved with Parkinson's disease through ceramide signaling or biosynthesis.

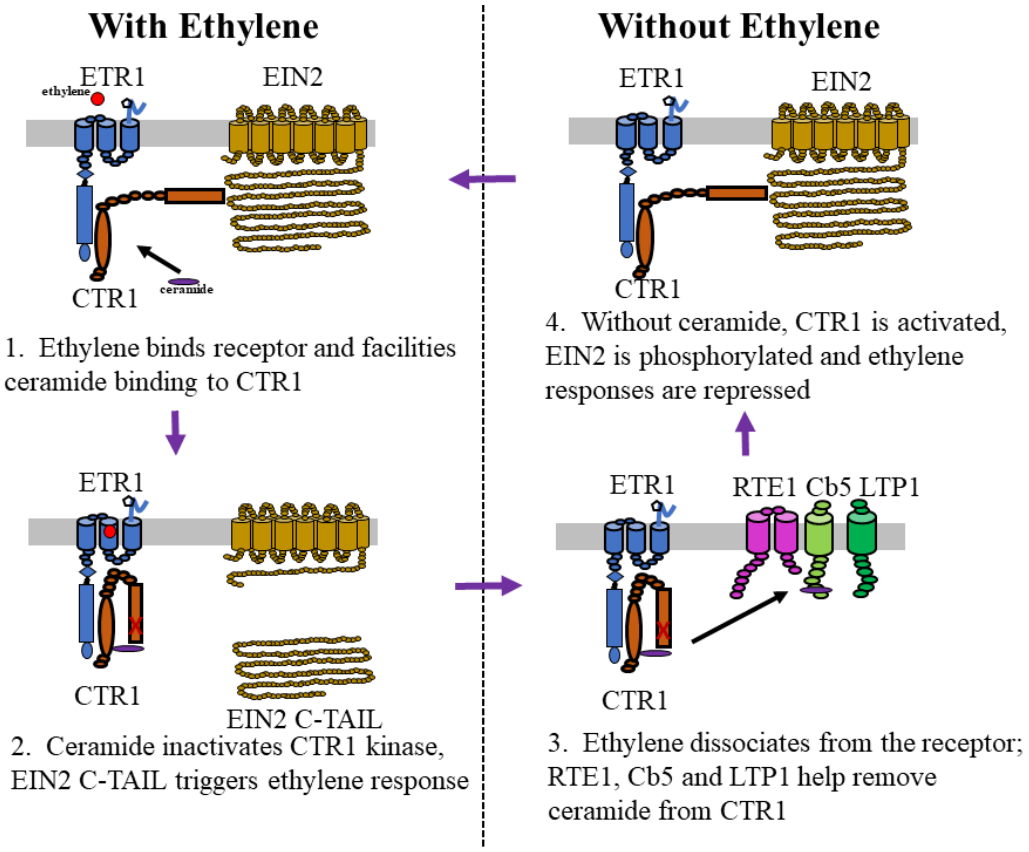


Figure 6-1: Model for ETR1 signaling to CTR1 with ceramide

To address the ceramide hypothesis, I propose testing the affect that ceramide has on gain-of-function and loss-of-function *etr1* and *rte1* mutants. For example, ceramides should cause ethylene hypersensitivity in wild-type plants. The ethylene insensitive RTE1-independent *etr1-1* mutant is normally unable to respond to ethylene and should not be affected. The RTE1-dependent *etr1-2* however is normally insensitive to ethylene but if ceramide is able to suppress the insensitivity then there may be a connection between RTE1-dependence and ceramide. Alternatively, RTE1s role in Parkinson's disease can be examined in *C. elegans* by observing the dopaminergic neurons in worms with RTE1 overexpressed or knocked down with RNA (Martinez et al., 2017).

Insights on the evolution of ethylene signaling:

RTE1-dependent ethylene receptors

In Chapter 3, we concluded that the most recent common ancestor of ETR1 and ERS1 receptors was likely a RTE1-dependent receptor suggesting that the basal angiosperms had a RTE1-dependent receptor. Furthermore, we identified an N-terminally localized proline that is partially responsible for RTE1-dependence in ethylene receptors. A quick analysis of basal land plants such as *Marchantia polymorpha* and *Physcomitrella patens* and charophyte algae finds that this proline is conserved in basal land plants but missing in charophytes. Therefore if we were to use the presence of this proline as an indicator of RTE1-dependence in a receptor, we would hypothesize that RTE1-dependent ethylene receptors appeared in basal land plants. In support of charophyte algae do not have RTE1-dependent receptors, I

expressed the *S. pratensis RTE1* homolog under the control of the 35S promoter in *etr1-2 rte1-3 A. thaliana* plants. *SpRTE1 etr1-2 rte1-3* plants were hypersensitive to ethylene indicating that *SpRTE1* could not complement the function of *AtRTE1* (unpublished). To test if basal land plants have RTE1-dependent receptors, the ETR1 receptor from *M. polymorpha* could be expressed in *A. thaliana* plants with and without a functioning RTE1.

Ethylene receptors, CTR1 and EIN2

In chapter 5, I reported that EIN2 and CTR1 homologs were not only conserved in charophytes, but chlorophyte algae as well. During the same time, I was also searched for ethylene receptor homologs in several chlorophytes, a rhodophyte and a glaucophyte but was unable to find any. The current hypothesis for the origins of the ethylene receptor in the plant lineage is that ethylene receptors came from the cyanobacteria during the plastid endosymbiosis event. If this hypothesis is correct, then that would suggest that ethylene receptors were lost several times in the algal lineage. This hypothesis is certainly still well within the realm of possibility especially given the extremely limited number of rhodophytes and glaucophytes sequences that are available. However, the number of sequences from other species of algae and bacteria is far greater now than 15 years ago when this hypothesis was first made. Ju et al. (2015) reports that ethylene receptors are widely conserved in charophytes. It may be worthwhile to reexamine whether the ethylene receptor enter

the plant lineage during plastid endosymbiosis or possibly through a different lateral transfer event in an early charophyte.

In chapter 5, we not only found that *S. pratensis* and other charophytes had a full-length EIN2 but that SpEIN2 was functional. Previously, it appeared that charophytes lacked a full-length EIN2 protein and it was unclear how ethylene signal was transduced from receptor to transcription factors. Because we were able to find a functional EIN2 in *S. pratensis* we now have a complete picture of the ethylene signaling pathway in charophytes. Moreover, because EIN2 proteins appear to be conserved in most charophytes we can conclude that early charophytes had all the components necessary to respond to ethylene.

It is interesting that EIN2 and CTR1 homologs are conserved in many of the chlorophyte transcriptomes we examined, especially given that these same chlorophytes lack ethylene receptors. We begin to ask the obvious question. What are EIN2 and CTR1 are doing in these algae? It is possible that the chlorophyte EIN2 and CTR1 homologs are involved in other signaling pathways. Furthermore, it is possible that EIN2 and CTR1 involvement in this other signaling pathway is conserved in in land plants but is masked by a stronger ethylene response. I propose taking a closer look at the *C. reinhardtii* EIN2 and CTR1. First, interaction between CrEIN2 and CrCTR1 can be tested by a yeast-2-hybrid assay and confirmed by a pull-down experiment. To test if CrCTR1 phosphorylates CrEIN2, CrEIN2 protein can be isolated from wild-type and *Crctr1*-knockout *C. reinhardtii.* The phosphorylation status of isolated CrEIN2 using mass spectrometry. To help

determine what pathways CrEIN2 and CrCTR1 may be involved with, the available *C. reinhardtii* EIN2 insertion mutant could visually compared with wild-type *C. reinhardtii* to find potential phenotypic difference caused by the insertion into the EIN2 gene. Alternatively, RNA-seq may be used to compare the two samples to identify which transcripts and corresponding pathways are most affected.

Insights on the evolution of ethylene biosynthesis:

Does ACC have a role outside of ethylene biosynthesis?

In seed plants, ethylene is synthesized from a relatively simple pathway. S-adenosylmethionine (derived from the amino acid methionine) is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the ACS enzyme which is then oxidized to ethylene via the ACO enzyme. In chapter 4, we found that *S. pratensis* is capable of producing ACC likely through one of its two ACS homologs. Furthermore, we found that *S. pratensis* can make low levels of ethylene from ACC even though it lacks an ACO enzyme. Moreover, we could block an ethylene-mediated stress response in *S. pratensis* by blocking ACC biosynthesis suggesting that *S. pratensis* does in fact use this ACC-intermediate pathway to produce ethylene. Together, these results help clarify some apparent discrepancies in the published literature. We confirm previous papers that suggest ACO enzymes do not appear until gymnosperms while at the same time we confirm that *S. pratensis*, like several other algae, makes low levels ethylene from ACC. Instead of using a specific ACC-oxidizing enzyme we propose a model that suggests these algae do not need to

produce large quantities of ethylene and therefore benefit from producing ethylene without a specific ACO enzyme.

Interestingly, we found ACS-like enzymes in fungi, metazoans and other algae such as chlorophytes and rhodophytes. The ACS-like enzyme from *Pencillium citrinum* is capable of synthesizing ACC (Jia et al., 1999; Kakuta et al., 2001) suggesting that that other ACS-like enzymes may also be able to produce ACC. Moreover, two chlorophytes and two rhodophytes have been shown to be able to synthesize ACC (Vanden Driessche et al., 1988; Garcia-Jimenez and Robaina, 2012; Garcia-Jimenez et al., 2013; Maillard et al., 1993). Why then do chlorophytes and rhodophytes, which lack ethylene receptors, make ACC? I propose that ACC's was not originally used as a precursor to ethylene but had a different function. While serving this alternative function, some of the ACC was non-specifically oxidized into ethylene. Charophytes derived the ethylene signaling pathway and began to use this ethylene byproduct as a new hormone.

Our first support for this hypothesis came when an undergraduate I mentored, Andrew Coleman, was examining ethylene responses in the liverwort *Marchantia polymorepha*. Andrew serendipitously discovered that the ethylene insensitive *ein3* knock-out in *Marchantia polymorpha* responds to ACC. Furthermore, results from studies in *Arabidopsis thaliana* suggest that ACC may still function independently of ethylene in the roots of higher plants (Tsang et al., 2011; Tsuchisaka et al., 2009; Xu et al., 2008). Our lab continues to study ethylene-independent ACC responses in *M. polymorpha* and *A. thaliana*.

Was the ACO enzyme necessary for ethylene signaling in drier environments?

Compared to angiosperms, *S. pratensis* synthesizes very low levels of ethylene from the precursor ACC (Ju et al., 2015). In this thesis we report that *S. pratensis* lacks the ACO enzyme that oxidizes ACC to ethylene consistent with previous work suggesting that ACO enzymes were first derived from the 2-oxoglutarate dioxygenase family in gymnosperms (Kawai et al., 2014; Osborne et al., 1996). Although charophytes lack the ACO enzyme, we determined that *S. pratensis* still used ACC as a precursor in an ethylene-mediated stress response. From these results, we can begin to speculate about the appearance of the ACO enzyme in gymnosperms. In chapter 5, we hypothesized inefficient ethylene production from ACC in water-bound algae may be beneficial as ethylene diffuses 10,000 times slower in water compared to air. If large quantities of ethylene were produced, it would be next to impossible to reset the ethylene signal without first allowing all the ethylene to diffuse into the aqueous surroundings. We also hypothesized that because of the difference in diffusion of ethylene in air and water, ethylene may serve as an indicator of desiccation in algae. Both of these hypotheses could also apply to basal land plants as well. Liverworts, hornworts, bryophytes, lycophytes and ferns are all restricted to moist environments for sexual reproduction (as well as a lack of vascular tissue in all plants before the ferns). These basal land plants must be able to prepare

for submergence as many of these moist environments could be prone to flooding. Submergence would allow for the accumulation of low levels of ethylene in these basal land plants. Ethylene could then trigger submergence responses allowing the plant to adapt to their submerged environment. This hypothesis is supported by recent work suggesting that the submergence response in the bryophyte *Physcomitrella patens* is controlled by ethylene (Yasumura et al., 2012). With the evolution of pollen, early gymnosperms are no longer restricted to wet environments, decreasing the likelihood of the plant being completely submerged. Without the threat of being submerged, it was no longer beneficial to synthesize low levels of ethylene as ethylene diffuses quickly into the air. Indeed, because ethylene diffuses faster in the air, it was likely now beneficial to produce large quantities of ethylene. It is in these early gymnosperms that we see the first ACO enzymes derived from the 2-oxoglutarate dioxygenase family.

Model of the evolution of the ethylene biosynthesis pathway

S-adenosylmethionine is an important molecule that serves as a co-substrate in methyl transfer reactions. We hypothesize that at some point an aminotransferase evolved the ability to convert SAM to ACC. ACC, in algae such as rhodophytes and chlorophytes, served as a signaling molecule. As a byproduct of ACC signaling, small amounts of ethylene were produced when ACC was non-specifically oxidized. Later, charophyte algae derived the ethylene signaling pathway and began to respond to not only ACC, but the oxidized ethylene by-product as well. Restricted to wet environments, basal land plants maintained the non-specific ethylene oxidation

pathway. As plants transition to dryer and dryer environments, plants then benefitted from the ability to produce larger quantities of ethylene and the ACC-specific ACO oxidase was derived from a 2-oxoglutarate di-oxygenase.

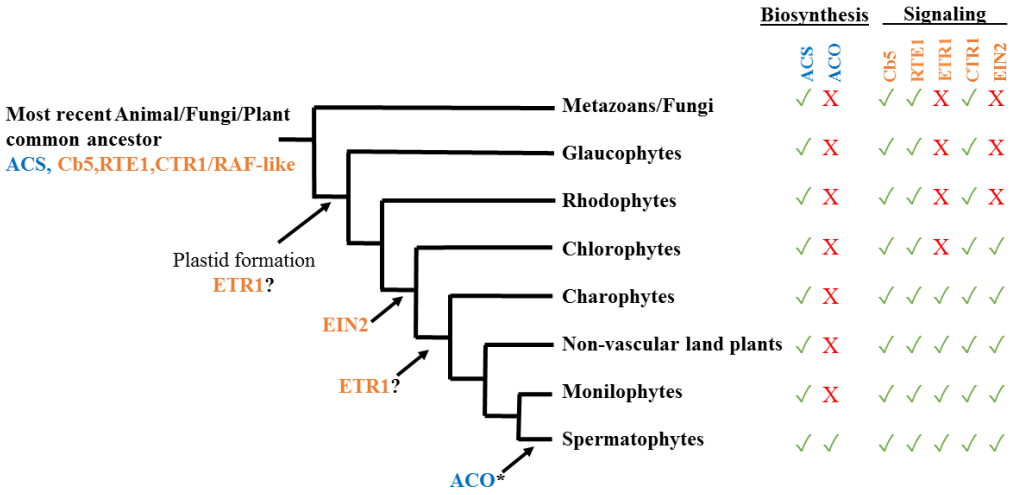


Figure 6-2: Appearance of ethylene biosynthesis and signaling homologs

Left: A simple phylogentic tree of the evolution of plants. The appearance of ethylene biosynthesis (blue) and signaling (orange) genes are noted. Asterisk indicates ACO homologs (2OGDs) are present earlier but our work indicates true ACOs do not appear until angiosperms and Spermatophyte.

Right: Checkmarks indicate whether ethylene biosynthesis or signaling homologs are present.

Appendices

Appendix A: Calcium and Glutathione affect ethylene biosynthesis/signaling

Cb5 and RTE1 affect ethylene signaling through the ETR1 receptor by an unknown biochemical function. In Chapter 2 of this thesis, I tried to determine the function of Cb5/RTE1 by determining whether one of Cb5's known pathways activities, serving as electron transport protein in fatty acid desaturation and hydroxlation, was altered in *rte1* mutants. My results indicated the RTE1 was not involved in these fatty acid modification reactions. Cb5 has also been shown to interact with the Bax-Inhibitor1 (BI-1), an ER-localized cell-death repressor (Nagano et al., 2009b). BI-1 is important for calcium homeostasis and interacts with calcium binding protein calmodulin. Increased calcium levels are thought to activate Cb5 through BI-1 to increase fatty acid hydroxylation (Nagano et al., 2009b). I hypothesized that if increased calcium concentration activate Cb5 activity, then plants that are grown on low calcium levels would have decreased Cb5 activity, resulting in decreased RTE1 activity causing these plants to be hypersensitive to ethylene. Contrary to my hypothesis, wild-type plants grown on plates containing no calcium were less sensitive to the ethylene precursor ACC (Figure). Our lab continues to explore potential roles of calcium in the ethylene signaling pathway.

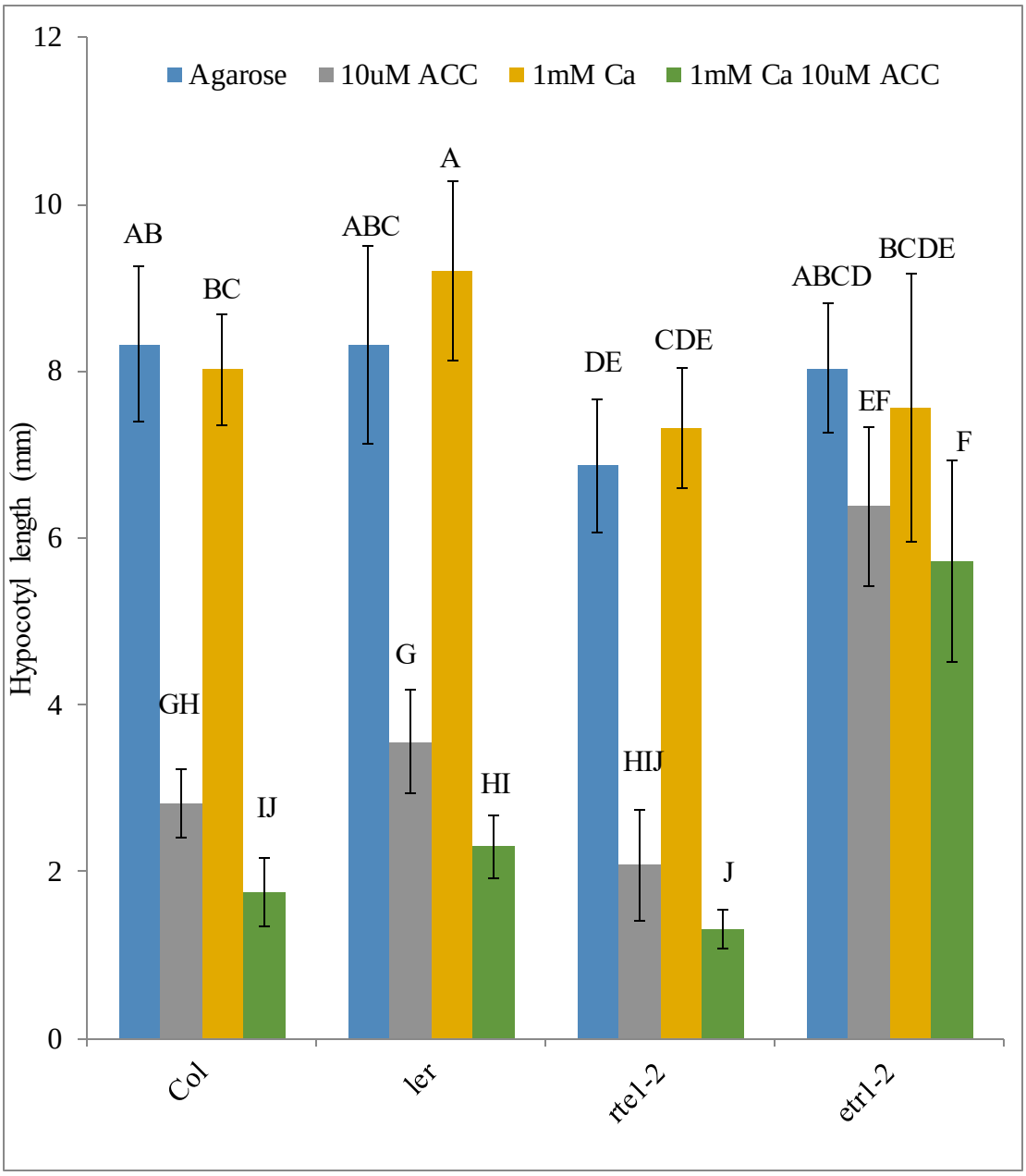


Figure A-1: Calcium is necessary for ethylene response

Measurement of hypocotyl lengths of 4-day-old etiolated Arabidopsis seedlings described above grown on plates with Agarose media ± ACC ±$CaCl_2$. Values indicate means ± standard error.

We hypothesize that Cb5 and RTE1 use upstream redox potential to activate the ETR1 receptor. According to this hypothesis, a stressed plant that produces more reactive oxygen species (ROS) such as H_2O_2, should have altered ethylene sensitivity.

To test the redox stress hypothesis I tried inducing ROS stress chemically with H_2O_2 and NaCl but found that seedling germination was greatly affected but there were no observable difference in ethylene sensitivity in ROS-induced plants. As an alternative approach I tested mutants for ethylene sensitivity in mutants that have increased ROS levels. Catalases (cat) and glutathione peroxidases (gpx) protect the plant from oxidative stress by breaking down H_2O_2. The *A. thaliana cat2* loss-of-function mutant not only has increased levels of H_2O_2 but was also reported to be partially insensitive to ethylene (Bueso et al., 2007). In my hands, two tested *cat2* mutants were just as sensitive to ethylene as a wild-type control. However, loss-of-function *gpx3* mutant plants were insensitive to ethylene.

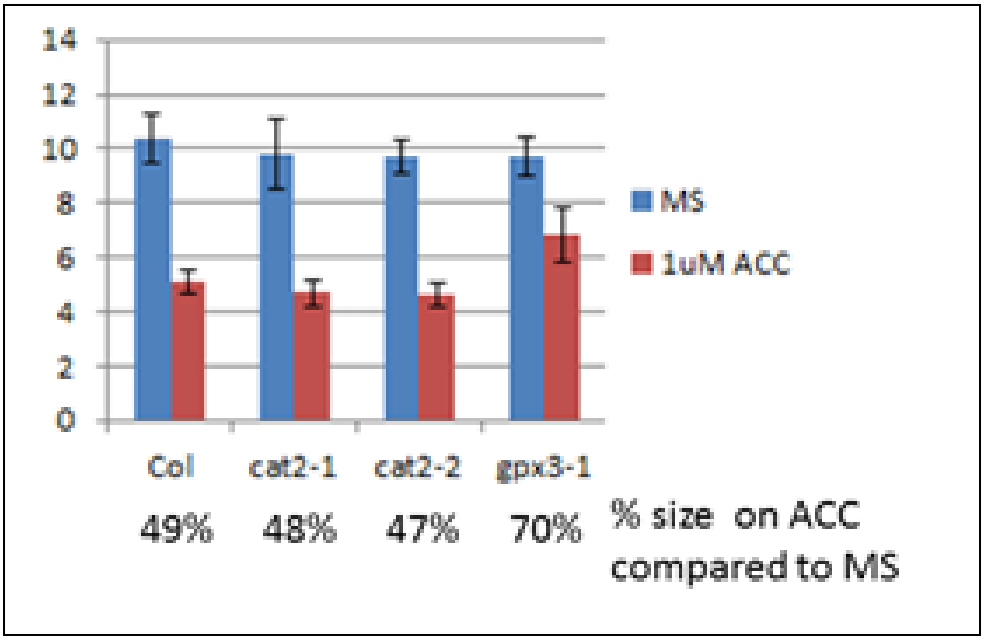


Figure A-2: Glutathione peroxidase mutants are ethylene insensitive

Hypocotyl measurements of various ROS scavenging enzymes with and without the ethylene precursor ACC. CATALASE2 null alleles cat2-1 and cat2-2 show a normal ethylene sensitive phenotype. GLUTATHIOINE PEROXIDASE3 null allele displays an obvious ethylene insensitive phenotype. Percents below graph represent the perfect size of plants grown on 1uM ACC versus no ACC.

Appendix B: Identification of a histone-deacetylase in an EIN2-interacting split ubiquitin screen

One of the major missing components of the ethylene signaling pathway is the protease that cleaves EIN2 allowing the EIN2 C-tail to translocate to the nucleus to activate ethylene response. A split-ubiquitin library screen was used to try to identify to protease as well as other potential EIN2-interacting proteins. The screen yielded many colonies many of which are believed to be false positives. Some of the most interesting genes pulled out of the screen include At2G32720 (Cb5B, which as shown in chapter 2, interacts with RTE1), At4G27500 (Proton pump interactor1, interesting because Nramps require a proton driving force to transport metal), At2G37190 (ribosomal L11 protein) and At5G22650 (histone deacetylase). The ribosomal L11 protein and histone deacetylase were originally considered potentially important for EIN2 signaling but shortly after, EIN2 was shown activate ethylene signaling by transporting breaking down F-box protein transcripts, stabilizing the EIN3 transcription factor (Li et al., 2015; Merchante et al., 2015). More recently, EIN2 has been shown to be also involved in a complex that helps acetylate histones to help activate ethylene responsive genes (Zhang et al., 2017). If EIN2 is involved in histone acetylation to activate genes, it is possible that EIN2 can interact with histone deacetylases to turn off genes that are inactivated by ethylene. At5G22650 should be pursued further to determine if it is involved in ethylene response.

Appendix C: *Marchantia polymorpha* is hypersensitive to ACC-synthesis inhibitor, AVG

In chapter 4, we hypothesized that ACC may have a function outside of ethylene biosynthesis. This hypothesis is supported by ongoing work from our lab (Andrew Coleman, unpublished; Wangshu Mou, unpublished) as well as some previous studies of *A. thaliana* roots (Tsang et al., 2011; Tsuchisaka et al., 2009; Xu et al., 2008). To pursuit the function of the ACC in *Marchantia polymorpha* we have collaborated with Eduardo Flores and Dr. John Bowman from Monash University. They have been able to produce several knock-out *M. polymorpha* mutants for us but have been unable to successfully produce any viable knockout lines of *acs* or *acs-like*. This may suggest that knocking out either of the genes causes lethality. Furthermore, in an effort to examine ACC and ethylene effects, I have treated *M. polymorpha* with the ACS inhibitor, AVG. AVG is not known to effect any other biosynthesis or signaling pathways. To inhibit ACC synthesis in the model plant, *A. thaliana*, plants are treated with 10μM AVG. In comparison, *M. polymorpha* plants grown on plates containing 1μM AVG are stressed while plants treated with 10μM AVG appear dead (Figure C-1). Together with results that inhibition of ethylene signaling is not lethal (Andrew Coleman, unpublished) suggests that ACC is necessary for *M. polymorpha* growth and viability.

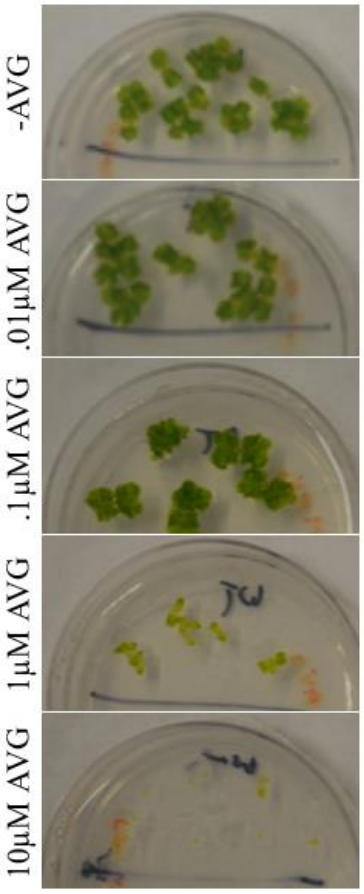


Figure C-1: M. polymorpha is hypersensitive to the ACS-inhibitor ACC.

Images of *M. polymorpha* gemmalings were grown for two weeks on agar plates with or without AVG.

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