

Elucidating the Effect of Silver on Ethylene Signaling
in *Arabidopsis thaliana*

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Brittany Kathleen McDaniel
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“There is no theory of evolution; just a list of creatures Chuck Norris has allowed to live”

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ABSTRACT

Ethylene, a gaseous plant hormone, is involved in numerous plant developmental processes such as seed germination, senescence, and fruit ripening. In *Arabidopsis thaliana*, ethylene is perceived by a family of five membrane-bound receptors, which upon binding ethylene trigger downstream effects. At the receptor level, it is known that the coordination of a copper ion is necessary for ethylene to bind, resulting in a conformational change of the receptor and the initiation of the ethylene signal transduction pathway. Interestingly, silver ions are also able to support binding of ethylene but ethylene responses are blocked in the presence of silver. When etiolated seedlings are exposed to ethylene, a reduction in growth occurs that we quantitate with high-resolution, time lapse imaging. In wild-type plants silver blocks this reduction in growth. Single *etr1* loss-of-function mutants have a diminished response to silver ions. In other words, they show a partial response to ethylene in the presence of silver ions. Conversely, knocking out any of the other receptor isoforms has little influence on the ability of silver ions to block ethylene responses. These results suggest that silver's effect is mediated through ETR1. By taking a genetics and biochemical approach and analyzing various receptor loss-of-function mutants in the presence and absence of silver and ethylene, a more complete understanding of which receptors are directly involved in silver's effects was studied.

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CHAPTER I

INTRODUCTION

Introduction to the Phytohormone: Ethylene

As sessile organisms, plants must employ both chemical and physical adaptations to respond to their dynamic environments. To quickly respond to abiotic and biotic stresses, plants harness the effects of phytohormones. There are five major phytohormones that plants use to regulate various developmental processes such as growth, germination, abscission and senescence. One of the first hormones identified and the only gaseous hormone was ethylene [1]. Plants tightly regulate the biosynthesis of ethylene as well as modulate the level and activity of key components in the ethylene signal transduction pathway to control such developmental programs.

Depending on the plant species, ethylene can have very different roles in development. For example, in deep water rice ethylene stimulates growth, yet in the plant model organism *Arabidopsis thaliana*, ethylene inhibits growth [2, 3]. In *Arabidopsis*, the physiological responses to ethylene have been extensively characterized. When dark-grown, or etiolated, seedlings are exposed to ethylene there are three basic physiological responses, which together are termed the “triple response” [4]. The triple response is denoted by a shorter root and hypocotyl, radial expansion of the hypocotyl and the presence of an exaggerated apical hook. The radial expansion helps with penetration of the soil and the exaggerated apical hook helps to protect the fragile cotyledons. In addition to the triple response, ethylene stimulates the nodding back and forth, or nutation, of the hypocotyl, which also aides in penetration through the soil [5].

History Behind the Discovery of Ethylene

It was first discovered that ethylene elicits a physiological effect in plants in the late 1800's by an assiduous graduate student, Dimitry Neljubow [6, 7]. Leading up to his discovery, there were multiple reports across Germany of trees adjacent to street lamps shedding their leaves prematurely [6]. Also, Neljubow observed that pea seedlings grew

horizontally in the greenhouse at his school, the Botanical Institute of St. Petersburg University [7]. After completing a series of experiments, Neljubow determined that the abnormal growth was caused by the air quality in the greenhouse. Subsequently, Neljubow discovered that the street lamps were leaking illuminating gas and, similarly, illuminating gas was accumulating in his greenhouse, which was causing the aberrations in pea seedling growth [7]. Neljubow screened multiple candidates for the active component in illuminating gas. Ultimately, he found that the hydrocarbon gas, ethylene, was the active component causing the aberrations in plant development [7].

Even though the effects of ethylene were not discovered until the late 1800's, it has been used in agriculture dating back to the ancient Egyptians and biblical times when farmers would gash figs to hasten ripening [8-10]. Such wounding causes an accumulation of ethylene in the fruit, thereby stimulating ripening [11]. Similarly, ancient Chinese farmers would accelerate ripening of pears by burning incense in ripening rooms [10]. At the time the farmers were unaware that ethylene was produced when burning incense, and this is what caused the hastening of ripening. The purpose of using smoke to ripen fruits became apparent with Neljubov's discovery.

Applications for Ethylene

In addition to ethylene having an effect on plants, it also has an effect on animals. Up until the late 1920's, ethylene was commonly used as an anesthetic for patients undergoing surgery [12]. Compared to the common anesthesia we use today, nitrous oxide, ethylene was far more potent and effective when putting a patient under before surgical procedures [12]. The drawback of ethylene, however, was that when mixed with oxygen it was highly flammable. There are multiple reports of ethylene-fueled explosions in the operating rooms, some of which resulting in the deaths of the patient and doctors [12]. However, ethylene was not the only flammable anesthetic used in the operating rooms. Doctors also employed other flammable gases for the same purpose with, unfortunately, the same explosive conclusion [12]. As a result, there was a significant incentive to develop methods to anesthetize patients using less explosive materials.

Ethylene has also had a large impact on the agriculture industry. With the emergence of large scale farming came the issue of getting produce to consumers before over-ripening. One method to address this is to harvest climacteric fruits, or fruits that ripen in response to ethylene, prematurely and then exogenously add ethylene prior to shipment. This prevents bruising and spoiling of produce [13]. The first commercialized genetically modified crop was the tomato, and it was engineered to initiate ripening once exogenous ethylene was applied. An additional example of an ethylene-insensitive crop that subsequently followed is soybeans [14].

Ethylene research has applications in the horticulture industry, as well. As beautiful as flowers are, they lose their luster with the shedding of their colorful petals. One factor positively regulating this developmental process is ethylene [15]. As a result of the 1976 study by Beyer, the horticulture industry sprays flowers with silver thiosulfate; however this practice is becoming less common. Beyer showed that silver nitrate blocks ethylene's ability to promote leaf, flower and fruit abscission [15]. Therefore, to extend the life of flowering plants and flowers for distribution and subsequently avoid money loss, gardeners and florists can employ silver's effect to delay petal abscission.

Ethylene: an Air Pollutant

While ethylene is endogenously produced in plants, which will be discussed in more detail below, it is also an air pollutant with multiple points of origin. Natural sources of ethylene are produced from plants, fungi, soil microorganisms, forest fires and volcanic activity [16]. Ethylene is a byproduct of combustion, thus when volcanoes erupt, or forests are engulfed in flames, ethylene is released. Non-natural sources make up a larger percentage of the total amount of ethylene in our atmosphere [16]. Examples of non-natural sources are automobile exhaust, shale gas and petroleum refining [16]. High levels of ethylene in the atmosphere due to both natural and non-natural sources pose vast problems for farmers. It is predicted that the atmospheric ethylene costs farmers millions of dollars each year due to crop-yield reduction. Due to ethylene's large impact on the agriculture and horticulture industry, it is important to

understand ethylene signal transduction in plants.

Ethylene Biosynthesis

Plants are able to produce ethylene as a result of reactions branching from the Yang cycle [17]. The two key steps in ethylene biosynthesis are the conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylate (ACC) and the oxidative cleavage of ACC to form ethylene [18]. These two steps occur through the activity of ACC synthase and ACC oxidase (ACO), respectively [18]. Regulation of ethylene production is accomplished through modulating the levels and activity of both ACC synthase and ACO. The conversion of SAM to ACC by ACC synthase is regarded as the rate-limiting step of the ethylene biosynthesis pathway [19]. Both enzymes are affected by different stimuli such as auxin and wounding for ACC synthase and fruit ripening for ACO.

Almost all tissues in the plant are able to produce ethylene, but the amount of ethylene produced, depends on the tissue-type as well as the abiotic and biotic cues. For instance, ethylene levels increase during germination and positively regulate seed germination [3]. Data support that other phytohormones also affect ethylene biosynthesis. For example, ethylene can positively regulate synthesis of auxin, cytokinins and abscisic acid [20, 21]. Conversely, these phytohormones can positively regulate ethylene biosynthesis.

General Overview of Ethylene Signal Transduction

Over the past 30 years, a model has developed from the many studies that aimed to identify components of the ethylene signaling pathway (Fig. 1). In Arabidopsis, ethylene responses are mediated by a family of five disulfide-linked homodimer receptors located in the endoplasmic reticulum membrane: ETHYLENE RESPONSE-1 and 2 (ETR1 and ETR2), ETHYLENE RESPONSE SENSOR-1 and 2 (ERS1 and ERS2) and ETHYLENE INSENSITIVE-4 (EIN4). The five ethylene receptors share significant similarity in their amino acid sequence and modular structure (Fig. 2) [22]. All receptor isoforms contain an N-terminal ethylene-binding domain with three

transmembrane α -helices, a GAF domain suggested to mediate receptor-receptor interactions, and a kinase domain [23]. Also, a subset of the receptors (ETR1, ETR2, and EIN4) contain a C-terminal receiver domain, which as a unit resembles bacterial two-component receptors [24]. A survey of the receptors exogenously expressed in yeast, as well as genetic data, identified that all family members bind ethylene with equally high-affinity and negatively regulate ethylene responses [25-27]. However, the exact output signal of the receptors is not well understood.

The coordination of a copper ion in the binding domain formed at the interface of the two monomers is a requirement for ethylene to bind to the receptor is [28]. Studies have identified RAN1 (RESPONSE TO ANTAGONIST1) as the metal transporter responsible for delivering of copper to the receptors [29, 30]. As further evidence for the importance of copper delivery, in *ran1* null mutants the biogenesis of functional receptors is altered [31]. Gold and silver ions can also support ethylene binding to ETR1 [32]. This is intriguing since silver is used as an inhibitor of ethylene signaling [15]. The focus of my thesis is on the effects of silver at the receptor-level. This will be discussed in more detail below

The ability of the receptors to negatively regulate ethylene responses is due to the physical interaction with CTR1 (CONSITUTIVE IRIPLE RESPONSE1) [33, 34]. CTR1 is most similar to the Raf family of serine/threonine protein kinases, suggesting it might function in a mitogen-activated protein (MAP) kinase cascade [33-35]. According to the current model, when ethylene binds to the receptors, a conformational change occurs in the receptor leading to a reduction in CTR1 kinase activity, which in turn relieves the inhibition on ethylene signaling [36]. Notably, a mutation that disrupts the stable interaction between CTR1 and the kinase domain of ETR1 renders CTR1 inactive [34]. Therefore, the interactions between CTR1 and the receptors are required for ethylene responses but, again, the exact output signal is not clear.

Using a genetic approach, researchers have identified EIN2 as a central, positive regulator of ethylene signaling downstream of CTR1 [37]. Loss-of-function (LOF) *ein2* mutants are the only LOF mutants identified so far that lead to complete ethylene insensitivity [37]. EIN2 is an integral membrane protein located in the endoplasmic

reticulum with two well-defined domains: (1) an N-terminus resembling the NRAMP family of metal-ion transporters and (2) a unique C-terminus soluble region which is required to elicit an ethylene response [37]. Despite the homology to metal-ion transporters, EIN2 does not possess any metal-transporting activity [37]. When ethylene is present, EIN2 accumulates and signals to down-stream elements in the pathway.

A downstream effect of EIN2 activity is the accumulation of at least two transcription factors, EIN3 and EIL1 (EIN3-LIKE-1) [38]. Both transcription factors are essential, positive components in the ethylene signaling pathway [38]. Mutants overexpressing EIN3 exhibit ethylene hypersensitivity or a constitutive ethylene response [39]. The levels of EIN3 and EIL1 are regulated by ubiquitination and subsequent degradation through the action of two F-box proteins, EIN3 BINDING F-BOX1 and 2 [39-41]. EIN3 and EIL1 work by promoting transcription of *ETHYLENE RESPONSE FACTOR* genes in response to ethylene [42]. The expression of *ETHYLENE RESPONSE FACTOR* genes ultimately leads to the physiological responses regulated by ethylene exemplified by the triple response.

A Closer Look: Ethylene Receptors

As previously stated, the focus of this thesis is on the ethylene receptors. The major advancement in understanding the role of the receptors stems from the study examining the dominant ethylene-insensitive *etr1-1* mutant in *Arabidopsis* [3]. This mutation resulted in reduced levels of ethylene binding in the plant, which suggested the mutation resided in a receptor for ethylene. Cloning and sequencing of *ETR1*, and subsequent expression in yeast, led to the observation that ETR1 exhibited ethylene-binding activity [43]. Also, specific missense mutations within the transmembrane domain of ETR1 led to ethylene-insensitivity [3, 44, 45]. Together, these data illustrated that ETR1 is a *bona fide* ethylene receptor with the ethylene binding pocket in the transmembrane domain. Subsequent studies used a reverse-genetics approach to identify that there were four other receptor isoforms (*ERS1*, *ETR2*, *ERS2*, *EIN4*) in *Arabidopsis* that bind ethylene with high-affinity [3, 26, 43, 46-48].

The five members of the receptor family share three conserved domains: (Fig.2) a transmembrane domain, a GAF domain, [49] and a kinase domain [50]. Based on genetic analysis, the receptors are further classified into two subfamilies. Subfamily I receptors (ETR1 and ERS1) possess His-kinase activity and contain three transmembrane domains. Subfamily II receptors (ETR2, ERS2 and EIN4) are noted for their Ser/Thr-kinase activity and have four transmembrane domains [50]. It is hypothesized that the fourth transmembrane domain is a signal sequence which aids in proper targeting within the cell. Furthermore, the ETR1, ETR2 and EIN4 receptors contain a receiver domain. The receiver domain contains a conserved aspartate residue, also present in canonical bacterial two-component receptors [51]. With the presence of a His-kinase domain and a conserved aspartate residue, it was predicted that the ethylene receptors signaled via a phospho-relay dependent manner similar to bacterial two-component receptors [51, 52]. The autophosphorylation on a conserved histidine residue followed by the transfer of the phosphate group to the conserved aspartate residue allows for a signal to propagate in the bacterial component system. However, studies on ETR1 kinase-deficient mutants indicated that kinase activity is not required for ethylene responses [53-55].

Higher-Order Receptor Complexes

In bacterial two-component systems, a signal is perceived by His-kinase receptors and then propagated through a phospho-relay mechanism to a response regulator [24]. Two-component receptors involved in chemotaxis exist as homodimers which associate in trimers and higher-order signaling complexes [56]. By functioning as receptor clusters, chemoreceptors are able to activate adjacent receptors, resulting in signal amplification [24]. Current data supports a similar model that ethylene receptors also function as higher-order signaling complexes [49, 55, 57-60]. It is thought that receptor clustering and trans-activation of receptors fosters signal amplification at the receptor level leading to the ability of plants to respond to ethylene at concentrations 300-fold below the K_d for ethylene binding [43, 53].

Ethylene receptors are able to non-covalently interact via the GAF domain and

form higher order complexes. One hypothesis is that higher-order clustering leads to the ability of one receptor to affect the signaling state of an adjacent receptor. Evidence for this is that ethylene-insensitive mutations in the binding domains of the receptors exhibit greater dominance than loss of the receptor isoform [60]. Also, a truncated *etr1-1* mutant lacking the cytosolic domain still confers ethylene insensitivity [49, 55, 60]. Altogether, this indicates that ethylene-insensitive mutants are able to affect the signaling states of adjacent wildtype receptor isoforms.

Non-Overlapping Receptor Functions

All five receptor isoforms are similar in sequence and modular structure and have functional overlap in mediating ethylene signaling. Single LOF ethylene receptor mutants exhibit little to no effect on seedling growth, which suggests functional overlap [53, 61, 62]. However, genetic evidence indicates that there are non-overlapping functions of individual receptors, as well. For example, ETR1 has a unique function in mediating ethylene-stimulated nutations and ETR2 is the only ethylene receptor required for trichome branching [5, 63]. Additional evidence for non-overlapping receptor functions is illustrated in the subfamily I LOF double mutant. In Arabidopsis, *etr1;ers1* LOF mutant exhibits a more severe constitutive ethylene response in comparison to any other receptor mutant combination, including a quadruple LOF mutant [64]. The current model attributes the greater role of subfamily I receptors in ethylene signaling to a stronger interaction with CTR1 [64].

Ethylene Receptor Domain Structure

The initial step in ethylene signaling is the binding of ethylene to the ethylene receptor binding domain located in the membrane of the endoplasmic reticulum [65]. The ethylene binding domain is contained within the N-terminal transmembrane domains linked via a disulfide-linkage at conserved cysteine residues. Within the binding domain resides a copper ion, which is necessary for ethylene binding [28]. Analyses of the ethylene binding domain in ETR1 containing point mutations have led to a model that proposes three conformational signaling states during ethylene response

[22]. Conformational state I: in the absence of ethylene, the receptor is in the low-energy, transmitter-on state which is actively suppressing ethylene responses. Conformational state II: ethylene is bound, but the receptor is in the unstable, transmitter-on state. Conformational state III: ethylene is bound to the receptor and the receptor undergoes a conformational change in the binding domain leading to a transmitter-off state, which is then propagated to the C-terminal domain [22]. The amino acid residues in ETR1 identified to foster the conformational states are strongly conserved between the ethylene receptor isoforms and between receptors from various plant species [22]. This conservation suggests that the same residues that are important for signaling in ETR1 are also important in the remaining four receptor isoforms.

The least characterized domain of ethylene receptors is the cytosolic GAF domain. The specific function of the GAF domain has not been identified in plants, but homologues in bacteria exhibit cGMP binding and light regulation [66]. A possible function supported by previous data is mediation of receptor-receptor interactions [49]. Yeast two-hybrid assays showed that the GAF domain alone was sufficient to support heteromeric interactions between ETR1 and ETR2 receptor isoforms [49].

Adjacent to the GAF domain is the kinase domain, which is shared among all ethylene receptor isoforms. Analysis of *in vitro* kinase assays showed that all receptor isoforms possess functional kinase domains [50]. Unlike ETR1, which contains a complete histidine kinase domain, all other receptor isoforms predominantly autophosphorylate on serine and threonine residues, with the exception of ERS1 [33, 50, 67]. ERS1 contains a degenerate histidine kinase domain which is capable of autophosphorylating on both histidine and serine residues [50]. A hallmark characteristic of bacterial two-component receptors is the role the canonical histidine kinase domain plays in propagating a signal [24]. Therefore, it was hypothesized that ethylene signaling required kinase activity from the ethylene receptors. Interestingly, a kinase-deficient mutant of ETR1 could rescue the *etr1;ers1* mutant, indicating that a functional kinase domain in the ethylene receptor was not necessary for ethylene signaling [54]. While it is unclear what the downstream targets are for the kinase domain, it is known that the kinase domain is involved in mediating interactions with the negative regulator,

CTR1 [64].

As previously mentioned, a subset of the receptors (ETR1, ETR2, and EIN4) contains a C-terminal conserved receiver domain that resembles the response regulator of bacterial two-component receptors. Similar to other response regulators, the receiver domain possesses a conserved aspartate residue that receives the phosphate group transferred from the kinase domain. The receiver domain is the only crystalized domain of the receptor, and based on the crystal structure, the receiver domain is structurally similar to well-characterized response regulators in bacteria [68]. Since kinase activity is not required for ethylene signaling, the role of the receiver domain is unclear.

Metal Cofactor Requirement for Ethylene Receptor Function

Using graphite furnace atomic absorption spectroscopy, it was shown that the metal cofactor required for ethylene binding to ETR1 was a copper [28]. Subsequently, it was established that the remaining four receptors required the same copper cofactor for ethylene to bind [25].

Copper is essential to life on earth because of its prevalence as a cofactor in numerous proteins, mainly proteins involved in electron transport as well as enzymes involved in redox reactions [69]. The utility of copper in biochemical reactions stems from its ability to cycle between an oxidized Cu(II) and reduced Cu(I) state. Plants are able to use copper as a redox cofactor in a wide range of processes such as photosynthesis, reactive oxygen metabolism, superoxide scavenging, mitochondrial respiration and cell wall remodeling [69]. However, copper is a toxic agent when in excess and if copper homeostasis is altered, many controlled processes can be adversely affected [70]. As a result, there are multiple proteins responsible for maintaining copper homeostasis. In Arabidopsis, four homologues of COPT-transporters located on the surface of root cells mediate copper uptake [69]. The copper transporter responsible for transporting copper across the endoplasmic reticulum membrane into the lumen is the P-type ATPase RAN1 [29-31]. Partial LOF *ran1* mutants (*ran1-1* and *ran1-2*) exhibit an increased sensitivity to copper chelators [31]. In addition, *ran1-1* and *ran1-2* mutants exhibit reduced sensitivity to silver nitrate, which

suggests RAN1 is able to transport silver ions, as well [31].

Silver ions block ethylene perception in plants [15] but support ethylene binding to ETR1 [28]. This engenders the question of how silver can support ethylene binding, yet block signaling. One possible explanation for this is that silver ions are 70% larger than copper ions and the bulkiness of the silver ion blocks the necessary conformational change in response to ethylene [32]. An alternative possibility is that the silver-receptor complex is not as stable as the copper-receptor complex, which leads to a quicker dissociation of ethylene. Experimental and computational evidence showed that the silver-olefin bonds have approximately 72% the bond energy when compared to copper-olefin bonds [71-76].

The focus of the thesis project was to: (1) elucidate the role of each receptor isoform in mediating silver's effect and (2) exogenously express in yeast the ethylene binding domain of each receptor isoform and measure ethylene binding in the presence of copper and silver. By taking both a genetics and biochemical approach, the goal is to obtain a clear description of which receptors are required for silver's effect and determine the mechanism for how they mediate the responses to silver.

CHAPTER II

MATERIALS AND METHODS

Seed Preparation

The *etr1-6*, *etr1-7*, *etr2-3*, *ers2-3* and *ein4-4* mutants were from Elliot Meyerowitz [27], the *ers1-3* and *etr1-9* mutants were from Eric Schaller [64], the *ers1-3;etr2-3;ein4-4;ers2-3* quadruple mutants were from Chi-Kuang Wen [58, 77] and the *rte1-2* (reversion to ethylene sensitivity 1-2) mutants were from Caren Chang [78]. Other combinatorial mutants used in this study have been described [54, 61, 64]. All mutants are in the Columbia (Col) background except for *etr1-9*, *ers1-3* and *ers1-2* that are in the Wassileweskija (Ws) background. All transgene constructs and transgenic plant lines have been described previously [5, 53, 54, 77]. *Arabidopsis* (*Arabidopsis thaliana*) seeds were imbibed with distilled water at 4°C for 2 days, and then light-treated under continuous fluorescent lights for 3 to 5 h. Following light-treatment, the seeds were transferred to agar plates containing one-half-strength Murashige and Skoog basal salt mixture [79], 0.8% agar, and B5 vitamins consisting of inositol (100 mg mL⁻¹), nicotinic acid (1 mg mL⁻¹), pyridoxin HCl (1 mg mL⁻¹), and thiamine HCl (10 mg mL⁻¹) with no added sugar. For silver treatment, 100 µM AgNO₃ was added to the agar. These seeds were then used for either end-point or time-lapse imaging.

End-Point Assay

For end-point experiments, plates were wrapped with aluminum foil and transferred to gas-tight chambers with a continuous flow of 100 ppm ethylene or hydrocarbon-free air at a flow rate of 100 mL min⁻¹. The seedlings were grown vertically for 4 days in the darkness at 22°C. The plates were then scanned on a flat-bed scanner and hypocotyls were measured with the computer program, ImageJ (ver. 1.34; <http://rsb.info.nih.gov/ij>).

High Resolution Time-Lapse Imaging

Seedlings were allowed to grow in the dark to a hypocotyl length of 2 to 5 mm

before measurements were taken. The agar plates were fitted with a lid which allowed for continuous gas flow and placed vertically in a stand. At this time growth measurements were taken every 5 minutes for 6 hours using a computer-driven digital camera system as described previously [80, 81] under infra-red radiation. Seedlings were grown in air for 1 hour before the addition of 1 ppm ethylene for 5 hours. In the chamber, which was 25 mL the gas flow rate was maintained at 100 mL min⁻¹. Experiments under all conditions were repeated at least 3 separate times. To determine the growth rate, the height in mm of each seedling in each frame was analyzed using custom software written by Edgar Spalding [81, 82] as previously described [53].

DNA Constructs, Cell Strains, Growth Conditions and Membrane Isolation

Pichia pastoris (Invitrogen, Carlsbad, CA) was used to express the binding domain of each receptor fused to glutathione-S-transferase (GST). We used the following nomenclature for these constructs: ETR1[1-128]-GST, ETR2[1-157]-GST, ERS1[1-128]-GST, ERS2[1-160]-GST, EIN4[1-151]-GST for the binding domains of ETR1, ETR2, ERS1, ERS2 and EIN4 fused to GST respectively [25]. Similar constructs were described and characterized previously using *Saccharomyces cerevisiae*, strain LRB 520 [25]. We switched to *P. pastoris* as an expression system for ethylene binding experiments because this expression system gave over 10-fold more protein expression than *S. cerevisiae* (data not shown).

The sequence encoding the ethylene binding domain of each receptor was amplified by PCR using cDNA generated from Columbia seedlings. The receptor specific primers introduced the EcoRI restriction site at the N terminus and KpnI at the C terminus and the GST specific primers introduced KpnI at the N terminus and ApaI at the C terminus. The ETR1[1-128] construct was generated by PCR amplification using the forward primer 5'AATTCATAGCCACCATGGAAGTCTGCAAT3' and the reverse primer 5'ATATAGGTACCCTCAGCAGCTTTATTTTCA3', ERS1[1-128] using the forward primer 5'AATTCATAGCCACCATGGAGTCATGCGAT3' and the reverse primer 5'CTAATGGTACCCTCATCAGCTTTCTTC3', ETR2[1-157] using the forward primer 5'AATTCATAGCCACCATGGTTAAAGAAATAGCT3' and the reverse primer

5'ACGATAGGTACCCTCATGAGCTTTCTT3', ERS2[1-160] using the forward primer 5'AATTCATAGCCACCATGTAAAGACATTG3' and the reverse primer 5'CTAATGGTACCCTCTCTGGTCTTCTTAC3', and EIN4[1-151] using the forward primer 5'AATTCATAGCCACCATGTAAAGATCTTTA3', and the reverse primer 5'ATATAGGTACCCTCCAACACATTCTG3'. The GST sequence was amplified using the forward primer 5'ATAGGTACCATGTCCCCTATACTAGGT3', and the reverse primer 5'ATAATTGGGCCCTTATCAGTCACGATGCG3'. Following PCR amplification, each fragment was gel purified, digested using EcoRI and KpnI, ligated into the pPICZ A vector, and subsequently transformed into *Escherichia coli*. Plasmids were isolated from positive colonies and receptor and GST gene fragments were digested with KpnI and ApaI and ligated together. Plasmids isolated containing the complete receptor-GST construct were linearized and transformed into *P. pastoris* using electroporation transformation. Yeast cultures expressing each construct were grown under conditions described in the Invitrogen Pichia manual for membrane-bound proteins. Following a 48h induction, the yeast cultures were centrifuged at 11,500 x g for 10 min. The cells were resuspended, disrupted and the membranes isolated using previously described methods [43, 83]. Membranes were rapidly frozen in liquid nitrogen and stored at -80° C until used.

Radio-Labeled Ethylene Binding Assay

For ethylene binding assays, membranes from yeast expressing the binding domain constructs described above were incubated in either 300 μ M CuSO₄, 300 μ M AgNO₃, or no metal in darkness for 30 minutes prior to starting the binding assays. For each condition, 1 g of membranes was transferred to a piece of filter paper inserted into a microcentrifuge tube, and incubated in an air-tight chamber for 4 hours containing either 1 ppm ¹⁴C₂H₄ or 1 ppm ¹⁴C₂H₄ + ¹²C₂H₄ [43]. The jars were allowed to air out for 10 min, thus releasing unbound ethylene. The samples were then transferred to individual jars containing vials with 0.3 mL of mercuric perchlorate, which trapped the specifically-bound ethylene released from the receptors. Following, the samples were incubated in the jars at 65°C for 90 min. After allowing the jars to cool for 17-24 hours,

scintillation fluid was added to the vial of mercuric perchlorate and radioactivity was measured using a scintillation counter. Assays were done in triplicate.

Ethylene dissociation measurements were made on samples labeled with $^{14}\text{C}_2\text{H}_4$ as described above. Labeled samples were aired for 10 minutes and then incubated in the dark in a humidified chamber with a continuous flow of humidified-air at (30 mL min^{-1}) for 2, 4, 8, 16 and 24 hours. Radioactivity of these samples was then determined as described above.

To measure the affinity of ethylene to ETR1 in the presence of 300uM CuSO_4 and 300uM AgNO_3 , increasing concentration of $^{12}\text{C}_2\text{H}_4$ was added. The binding assay was conducted as mentioned above with two differences. Samples for all conditions were incubated in 0.1 ppm $^{14}\text{C}_2\text{H}_4$ and for each condition 0, 0.1, 0.5, 1, 1000 ppm of $^{12}\text{C}_2\text{H}_4$ was added, respectively.

CHAPTER III

RESULTS

Non-Overlapping Function of Ethylene Receptors in the Presence of Silver

Silver's effect on ethylene responses differs between mutants lacking subfamily I or subfamily II receptors [55, 62]. Loss of subfamily II receptors has a minimal effect on silver's ability to inhibit ethylene responses, but mutants lacking subfamily I receptors lack sensitivity to silver, and thus exhibit ethylene responses [55]. To characterize the role that all five ethylene receptors play in mediating the response to silver nitrate, various single and combinatorial LOF receptor mutants were analyzed. Hypocotyls were measured of etiolated seedlings grown in the presence or absence of 100 μM AgNO_3 for 4 days in chambers with air or 100 ppm ethylene continuously flowing. In the wildtype backgrounds of the various receptor mutants (Col, WS), AgNO_3 blocked growth inhibition caused by addition of ethylene (Fig. 3). All single LOF receptor mutants exhibited insensitivity to ethylene in the presence of AgNO_3 with the exception of *etr1-7* and *etr1-9* (Fig. 3a,b). To assess this in more detail, combinatorial mutants were analyzed. Consistent with previous studies, the triple LOF mutant *etr1-6;etr2-3;ein4-4* exhibited a constitutive ethylene response in air and growth was inhibited further by application of ethylene [55] (Fig. 3). Surprisingly, this mutant had a normal response to ethylene in the presence of 100 μM AgNO_3 . In contrast, the *etr2-3;ein4-4;ers2-3* mutant lacking the subfamily II receptors was insensitive to ethylene in the presence of 100 μM AgNO_3 . Also, in air, the hypocotyls of *etr2-3;ein4-4;ers2-3* were longer when grown on 100 μM AgNO_3 when compared to seedlings grown without AgNO_3 and ethylene.

Due to the visible importance of ETR1 in mediating silver's effect, we wanted to further characterize the role of ETR1. One unique aspect about ETR1 is that it is positively regulated by RTE1 [78, 84-88]. The *rte1-2* LOF mutants exhibited ethylene insensitivity in the presence of 100 μM AgNO_3 indicating that silver is affecting ETR1 in an RTE1-independent manner (Fig. 3a). Another unique aspect about ETR1 is that it is the only receptor isoform that has both a His kinase domain and a receiver domain,

which are required for a phosphotransfer signaling mechanism. Therefore, we examined the ability of a phosphotransfer-deficient mutant (*getr1[D]*), which has the conserved aspartate residue mutated, to rescue responses to silver in the *etr1-6;etr2-3;ein4-4* mutant. The *getr1[D]* transgene was able to fully rescue the silver response, indicating that silver's effect is mediated via a phosphor-transfer-independent manner (Fig. 3c). Together, these data suggest that ETR1 is important for silver's effect. However it remains to be shown what aspect of ETR1 is important for this function.

Growth Inhibition Kinetics of Single and Combinatorial Receptor Mutants in the Presence of Silver

In end-point experiments, transient and rapid responses to ethylene are not visible. To further characterize the effect of silver on the ethylene growth response, we used high resolution time-lapse imaging to analyze rapid growth kinetics of single and combinatorial mutants in the presence of 1 ppm ethylene.

When exposed to saturating amounts of ethylene, wildtype seedlings exhibit a rapid, well-described two-phase growth inhibition response [89] (Fig. 4a,e). Approximately 10 minutes after ethylene addition, there is a sharp decrease in growth rate that plateaus 20 minutes later. The first plateau in growth rate lasts for 30 minutes and is followed by a second decrease in growth rate that lasts for 15 minutes. Following the second growth decrease is a second plateau in growth rate that is maintained for as long as saturating ethylene levels are maintained. While the first phase of growth inhibition is sensitive to levels of ethylene as low as 0.2 ppb [90], the second phase is much less sensitive (≥ 1 ppm) [90]. EIN2 is required for both first and second phases of rapid growth inhibition [89]. For the second phase of growth inhibition to occur the activity of the transcription factors EIN3 and EIL1 are required.

In the absence of AgNO₃, wildtype seedlings exhibited the two defined phases of growth inhibition; however, with the exception of a short, transient response, the presence of 100 μ M AgNO₃ abolished both phases (Fig. 4). In the *etr1-7* mutant without addition of silver nitrate, a wildtype two-phase response to ethylene was observed and growth inhibition was maintained for as long as ethylene was applied (Fig. 4b).

Interestingly, silver treatment resulted in a two-phase response but 2 hours after the addition of ethylene a reversal of growth inhibition occurred. A similar gradual reversal of ethylene-driven growth inhibition was observed in Columbia seedlings exposed to sub-saturating levels of the ethylene antagonist, 1-MCP [90]. A two-phase growth inhibition response occurred in *etr1-6;etr2-3;ein4-4*, but there was no reversal of growth inhibition observed in the presence of 100 μ M AgNO₃. This suggests that ETR1 does not solely mediate the effect of silver on ethylene responses. Interestingly, *etr2-3;ein4-4* double mutants exhibit ethylene insensitivity in the presence of AgNO₃, suggesting that they are having indirect effects to modulate the effects of AgNO₃ (Fig. 4c). In the presence of silver nitrate, *ers1-3;ers2-3* exhibited a transient response to ethylene which completely reverted 2 hours after the addition of ethylene (Fig. 4f). This suggests ERS1 and ERS2 are not required for a full response to silver. Additional evidence that ETR1 is important for silver's effect is that the *ers1-3;ers2-3;etr2-3;ein4-4* mutant, that only expresses ETR1, exhibits ethylene insensitivity when silver is added (Fig. 4d). Together, this suggests silver's inhibitory effect on ethylene growth responses acts predominantly through ETR1 with minor contributions to this phenotype from other receptor isoforms.

Rescue of the Silver Response in etr1-6 etr2-3 ein4-4 Triple Mutants

Analysis of end-point and time-lapse imaging data suggests that *ETR1* is playing the predominant role in mediating silver's response and that other receptors contribute to silver's effect in an ETR1-dependent manner. There is a slight difference in ethylene growth inhibition between *etr1-7* and *etr1-7;etr2-3;ein4-4*. To identify the individual roles of each receptor isoform in mediating silver's effect, each receptor isoform was transformed into the *etr1-7;etr2-3;ein4-4* under the control of the *ETR1* promoter. Transformants were previously analyzed and shown to be functional and express all transgenes at similar levels [77].

Comparison of hypocotyl growth of each transformant line showed that the *ETR1* transgene could fully rescue the effects of silver (Fig. 5). Surprisingly, the *ERS1*, *ERS2*, and *EIN4* transgenes also rescued the sensitivity to silver (Fig. 5). The only transgene

unable to complement silver's inhibitory effect was *ETR2*. Use of high resolution time-lapse imaging allowed us to further elucidate the roles of each receptor isoform in responding to silver nitrate and ethylene. Analysis of the rapid kinetics revealed 3 distinct ethylene responses in the presence of AgNO_3 among the 5 receptor isoforms. First, the *ETR1* transgene was the only receptor isoform that completely restored silver's effect (Fig. 6a). Second, the *ETR2* transgene could not rescue the silver phenotype to any degree (Fig. 6c). Third, the remaining 3 transgenes, *ERS1*, *ERS2* and *EIN4* elicited a partial response to silver (Fig. 6b,d,e) where the addition of 1 ppm ethylene caused an initial growth inhibition that then partially reversed with time.

Comparison of Ethylene Binding to Receptors in the Presence and Absence of Silver Nitrate

There are two hypotheses that explain why *ETR1* plays a larger role than the other 4 receptor isoforms in mediating silver's effect. Either (1) silver is only incorporating in the binding pocket of *ETR1*, and subsequently locking *ETR1* into the active position or (2) silver is incorporating into all, or a subset, of the ethylene receptors, yet only affecting the conformational change in *ETR1*. To parse the two possibilities, the binding domain of each receptor isoform was expressed in yeast, isolated, and used in radiolabeled ethylene binding assays.

It was previously shown that *ETR1* exogenously expressed in yeast can bind approximately 33% the amount of ethylene in the presence of silver nitrate when compared to copper sulfate [32]. However, silver's ability to support ethylene binding to the remaining 4 receptor isoforms is not known. We generated constructs with the gene encoding the ethylene binding domain of each receptor fused to a C-terminal GST tag in the *P. pastoris* expression vector, pPICZ-A. The membrane proteins were isolated and radiolabeled ethylene binding assays were performed. Consistent with previous results, we observed ethylene binding to *ETR1*[1-128]-GST incubated with 300 μM AgNO_3 was roughly 30% that of binding to *ETR1*-[1-128]-GST incubated with 300 μM CuSO_4 [28, 32] (Fig. 7). Also, silver ions supported ethylene binding to *ERS1*-[1-128]-GST at levels roughly 30% that of binding in the presence of 300 μM CuSO_4 (Fig. 7). Conversely,

there was no measurable ethylene binding to ETR2-[1-157]-GST, ERS2-[1-160]-GST or EIN4-[1-151]-GST pre-incubated in 300 μM AgNO_3 even though they bound ethylene in the presence of CuSO_4 (Fig. 7).

Since silver ions support ethylene binding to the ETR1 receptor yet does not confer an ethylene response *in vivo*, one hypothesis is that the affinity for ethylene is lower with ETR1 bound to Ag(I) compared to ETR1 bound to Cu(I) . Previously, it was shown that the theoretical nature of bonding is different between copper and silver with ethylene, which could have implications on physiological responses [72-74, 76, 91]. The K_d for ethylene binding to ETR1 was determined in the presence of either 300 μM CuSO_4 or 300 μM AgNO_3 using the methods of Blankenship and Sisler [92] (Fig. 8). To do this, $^{14}\text{C}_2\text{H}_4$ binding was evaluated in the absence of added $^{12}\text{C}_2\text{H}_4$ and in the presence of increasing concentrations of $^{12}\text{C}_2\text{H}_4$. The data was evaluated with Scatchard analysis to determine the K_d . We found that the K_d for ethylene was 1.51 ppm with CuSO_4 and 0.98 ppm with AgNO_3 . Additionally, the time-course of $^{14}\text{C}_2\text{H}_4$ dissociation from ETR1-[1-128]-GST was determined with these metals (Fig. 9). Consistent with prior studies [43], the half-time of ethylene release in the presence of copper sulfate was 12h, and in the presence of silver nitrate, this time shortened slightly to 10h.

CHAPTER IV

DISCUSSION

Silver is known to block ethylene responses, but the mechanism by which silver acts remains obscure. Silver-induced ethylene insensitivity is intriguing because silver ions support ethylene binding to ETR1 [32]. This has led to the model where silver occupies the ethylene binding site and interacts with ethylene but fails to allow for changes in the receptor necessary to elicit downstream signaling. Previous work suggested that subfamily I receptors are more important than subfamily II receptors for silver's effect on ethylene responses [55, 62]. This begged the questions of which individual receptors played a role in silver's effect and how silver supported ethylene binding to ETR1 yet did not confer an ethylene response.

This study identified ETR1 as the predominant ethylene receptor mediating silver's response. To characterize silver's effect on ethylene responses, we analyzed both long term growth responses and rapid growth kinetics of ethylene receptor mutants. With end-point analysis, most receptor null mutants had normal responses to silver. In other words, silver blocked the effects of ethylene. The only mutants that had altered silver responses were those with null mutations for *etr1*. Time-lapse imaging revealed that these mutants initially responded normally to ethylene even in the presence of silver nitrate. However, the *etr1-7* mutants showed an increase in growth rate after about 2.5 hours. A similar reversal in growth inhibition has been observed in seedlings treated with a sub-saturating dosage of the ethylene antagonist, 1-MCP [90]. This suggests that ETR1 has a major role in mediating the effects of silver. However, other isoforms also have a role in this phenotype. Silver had no effect on ethylene response kinetics of *etr1-6;etr2-3;ein4-4* seedlings. Thus, unlike the *etr1-7* mutants that had a growth reversal, these triple mutants showed full growth inhibition throughout ethylene treatment. Interestingly, *etr2-3;ein4-4* double mutants had a normal response to silver. This suggests that one or both of these isoforms has a secondary role that only is apparent when ETR1 is removed. It is likely that EIN4 is having this modulatory role rather than ETR2 since the *cEIN4* transgene partially rescued the silver response while

the *cETR2* transgene did not. Our results also suggest that ERS2 has a secondary role in mediating the effects of silver. While ETR1 has the major role in this trait, ETR2 appears to have no role in mediating the effects of silver.

It is unclear why ETR1 has the major role in mediating the effects of silver ions. One possibility is that ETR1 is expressed at higher levels or in a specific tissue specific pattern [93]. In the current study, all five transgenes were under the ETR1 native promoter. Different patterns of rescue might be observed if they were under control of their native promoter. However, our results indicate that differential expression is not likely to be the entire explanation since the receptors also have biochemical differences. Silver nitrate only supported ethylene binding to ETR1 and ERS1. The fact that the *cEIN4* and *cERS2* transgenes rescue the silver phenotype but that silver does not support ethylene binding to these isoforms indicates our model is not complete. One possibility is that silver is incorporating into these receptors but blocking ethylene binding. This seems unlikely since silver ions bind ethylene. We also cannot rule out the possibility that silver supports ethylene binding to ERS2, ETR2 and EIN4, but at a level too low for our detection method. An alternative explanation is that silver affects the receptors outside of the ethylene binding pocket. There is evidence for the signaling state of one receptor dimer affecting the signaling state of adjacent receptors through physical interactions [55]. Thus, silver ions may be affecting clustering behavior in part via ERS2 and EIN4.

We further characterized the role ETR1 plays in mediating silver's effect. ETR1 possesses a functional histidine kinase domain, which in bacterial two-component receptors relies on a phosphor-relay mechanism to carry out its function. We found that the phosphotransfer-deficient mutant (*getr1[D]*) exhibited insensitivity to ethylene in the presence of silver, suggesting that phosphotransfer is not required for silver's effect. We also looked at silver's effect on RTE1 function. RTE1 is a novel integral membrane protein that acts through an unknown mechanism to promote ETR1 signaling. In the *rte1* LOF mutant, ethylene insensitivity occurred in the presence of silver. Together, this indicates that ETR1 is mediating silver's effect in a phosphotransfer and RTE1-independent manner.

Our results indicate that the lower ethylene-binding activity observed in ETR1 treated with silver nitrate is not due to lower affinity to or faster release of ethylene from ETR1 receptors. Therefore, to explain the reduced ethylene binding in the presence of silver nitrate, we predict there are fewer binding sites generated in the presence of silver when compared to copper. The study that identified there is one copper ion per receptor dimer noted that not all of the receptors were active and able to bind ethylene [28]. Since silver supports roughly 30% ethylene binding compared to copper in ETR1, we propose that there are more binding sites generated with copper compared to in the presence of silver. One way this could occur is by multiple copper ions coordinating to ETR1 with each copper capable of binding an ethylene molecule while only a single silver ion coordinating to ETR1. Alternatively, copper ions incorporate into more receptor molecules resulting in more binding sites for ethylene. Further studies probing the structural differences in ETR1 with copper or silver could shed light on the reason for reduced ethylene binding in the presence of silver. Also, the model that there are fewer metal binding sites in the presence of silver can be tested by using graphite furnace atomic absorption spectroscopy.

Based on the data presented in this thesis, we propose a model to address silver's effect on ethylene responses (Fig. 10). In this model, we suggest that wildtype receptors contain two copper ions in the binding pocket and each copper ion binds ethylene leading to a conformational change that results in the propagation of ethylene's signal. When silver is added, the silver ions displace the copper ions of ETR1 and ERS1 resulting in only one silver ion per receptor dimer. This leads to fewer ethylene binding sites and lower ethylene binding activity. We believe that silver has two effects on the ethylene receptors. One is on ETR1 and ERS1 to directly block ethylene signaling because of the bulky nature of the silver ion that prevents the conformational change believed to be necessary for signaling. Second, it is possible that silver ions disrupt receptor output perhaps by altering receptor clustering. This would explain the ability of ERS2 and EIN4 to rescue the silver phenotype and the modulatory role they appear to have when mutated in combination with ETR1 or ERS1.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

In *Arabidopsis*, ethylene perception involves a family of five receptor isoforms that are highly redundant, yet possess multiple non-overlapping functions. In response to silver nitrate, an identified ethylene response inhibitor, the ETR1 receptor has a unique role in mediating silver-induced ethylene insensitivity. Data presented in this thesis supports a model where silver ions prevents the putative conformational change upon ethylene perception in the ETR1 receptor binding domain and disrupts higher order clustering.

To further ascertain why ETR1 is unique in mediating silver's effect, Nuclear magnetic resonance (NMR) could facilitate the characterization of structural differences. Since silver ions are larger than copper ions, it is possible the bulky nature of silver ions disrupts the structure of the binding domain. NMR could be employed to identify the differences.

A remaining question is the role of the ERS1, ERS2, ETR2 and EIN4 ethylene receptors in mediating silver's effect. We measured each receptor's ability to rescue silver's effect under the control of the ETR1 promoter. Future work could be done to test if each receptor isoform has an effect on silver's response under the respective, native promoter. In addition, further work needs to be done looking at the ability of silver to incorporate into the binding domains of ETR2, ERS2 and EIN4. It is possible these receptors are able to coordinate silver into the binding domain but are incapable to binding ethylene.

Understanding how silver causes ethylene insensitivity in plants has far-reaching advantages. In regards to fruit ripening, ethylene insensitivity has the potential to extend product life and quality of fruits and vegetables. Also, there are many examples of post-harvest disorders of fruit and vegetables caused by ethylene [94]. If we can develop technologies that allow us to control sensitivity to ethylene, we can reduce having to discard spoiled fruits and vegetables, extend the life of picked flowers and much more.

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FIGURES

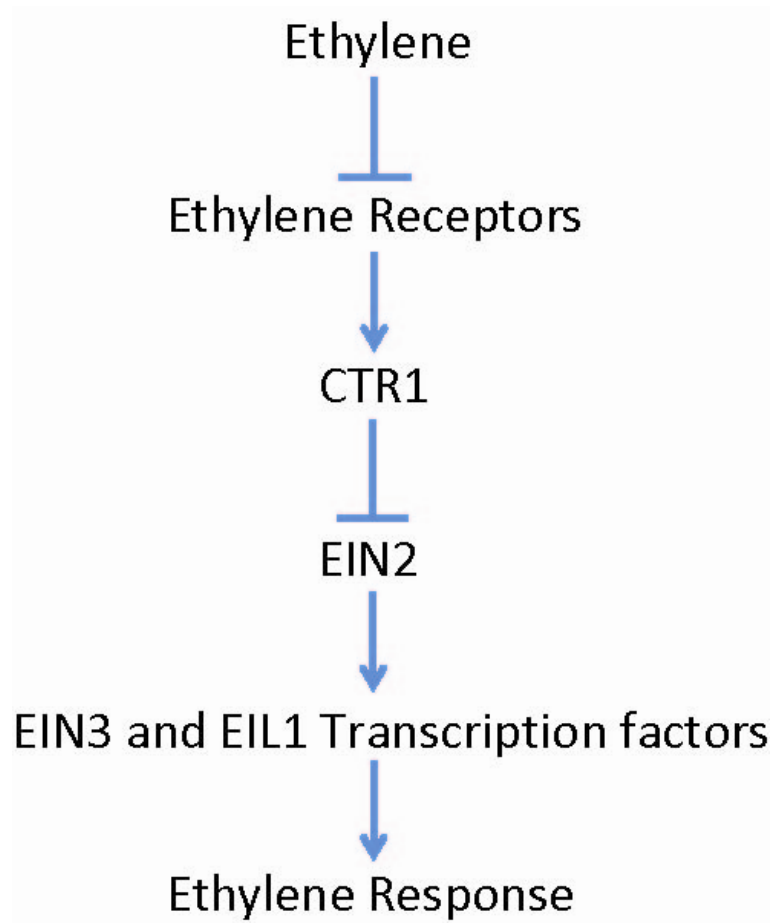


Figure1. Schematic representation of ethylene signaling in *Arabidopsis thaliana*. The ethylene signal transduction pathway is initiated when ethylene binds and inhibits the ethylene receptors. The inhibition of the receptors leads to the inhibition of CTR1 activity, which allows for EIN2 to accumulate. Subsequently, the EIN3 and EIL1 transcription factors accumulate and activate transcription of ethylene response genes, leading to a physiological response.

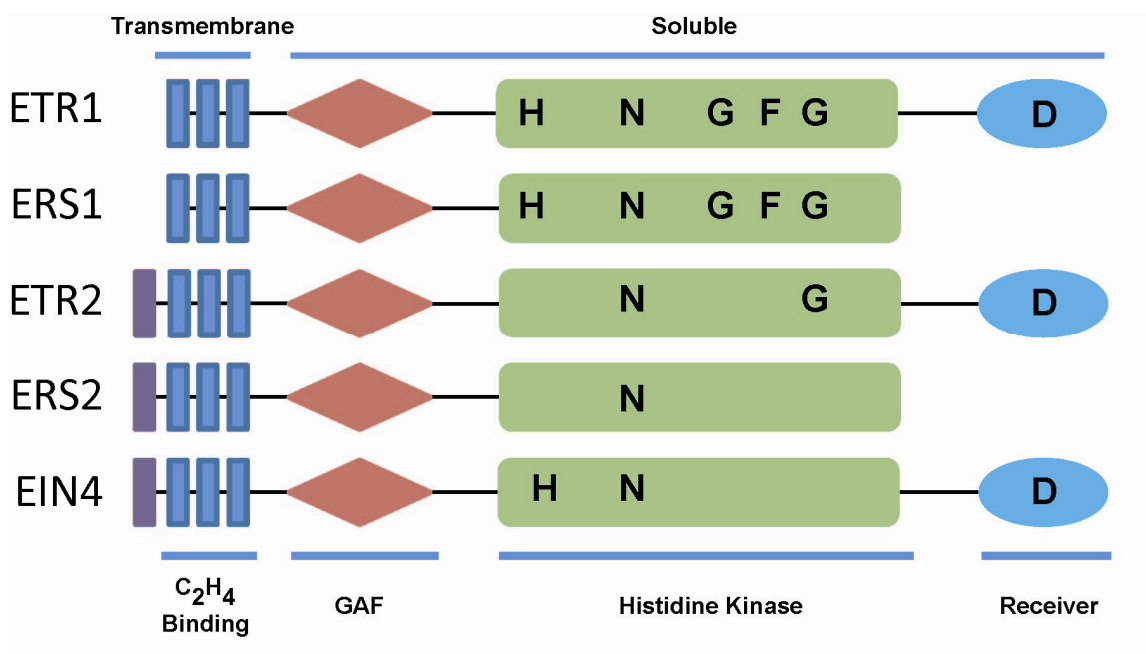


Figure 2. The domain structure for ethylene receptors in *Arabidopsis thaliana*. All five ethylene receptors in *Arabidopsis* contain a transmembrane domain consisting of 3 alpha-helices (blue rectangles), a cytosolic GAF domain (red diamond) and a histidine kinase domain (green rectangle). However, ETR2, ERS2 and EIN4 contain a degenerate histidine kinase domain but possess a functional serine/threonine kinase domain. Additionally, ETR1, ETR2 and EIN4 contain a C-terminal receiver domain.

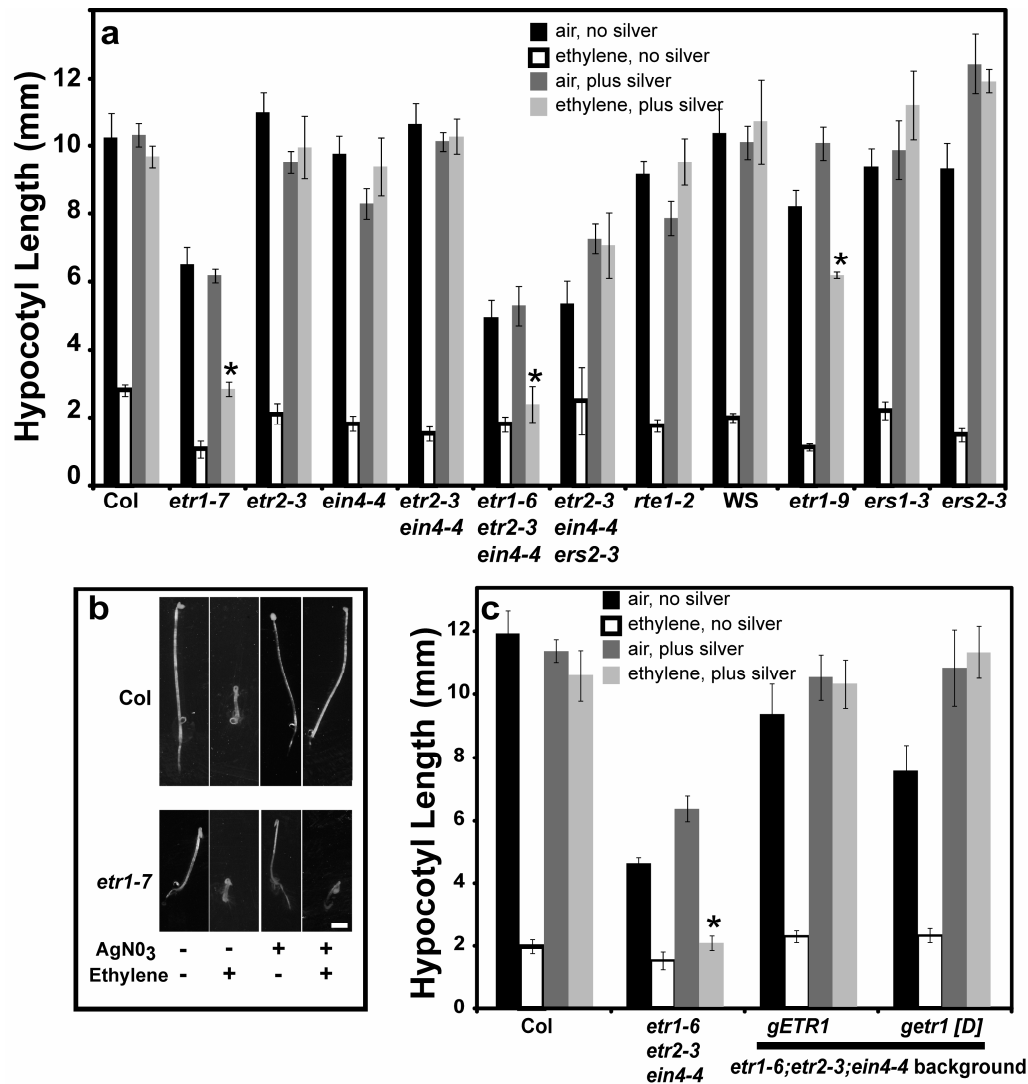


Figure 3. Growth effect of silver nitrate on ethylene receptor mutants in *Arabidopsis thaliana*. In all panels, seedlings were grown under the various conditions for 4 days. Ethylene-treated seedlings were grown in the presence of 100 ppm ethylene and silver-treated seedlings were grown on agar plates containing 100 μ M silver nitrate. (A) Hypocotyl measurements of ethylene receptor LOF mutants. (B) Columbia and *etr1-7* etiolated seedlings grown with and without silver nitrate and ethylene. Scale bar equals 2 mm. (C) Hypocotyl measurements of the *etr1-6;etr2-3;ein4-4* mutant transformed with wildtype ETR1 (*gETR1*) and the phosphotransfer mutant with the conserved aspartate residue mutated (*gETR1[D]*).

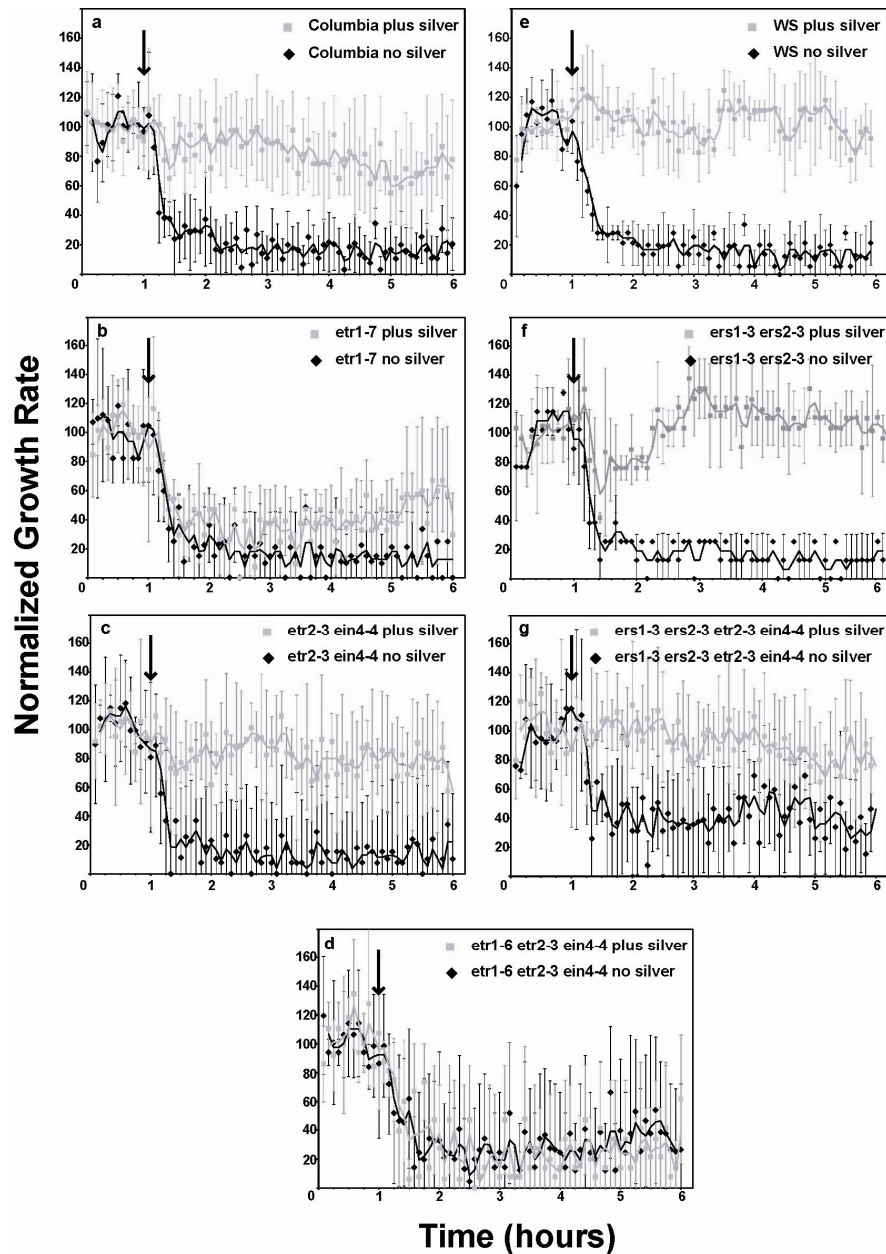


Figure 4. Effect of silver nitrate on rapid growth response to ethylene in single and combinatorial LOF receptor mutants. Seedlings were grown in air for 1 hour followed by the addition of 1 ppm ethylene (arrow) for 5 hours. The growth response of seedlings grown on plates containing 100 μ M silver nitrate (grey line) was compared to seedlings grown in the absence of silver nitrate (black line). Panels a through g represent the growth of the respective receptor mutant. Panels b, c and d are in the Col background and f and g are in the WS background. The bold grey and black lines represent the moving average of the growth rate. The Y-axis represents the growth rate after the addition of ethylene normalized to the growth rate in air. Data represent the mean $\pm$ SD from at least 5 seedlings total from at least 4 separate experiments.

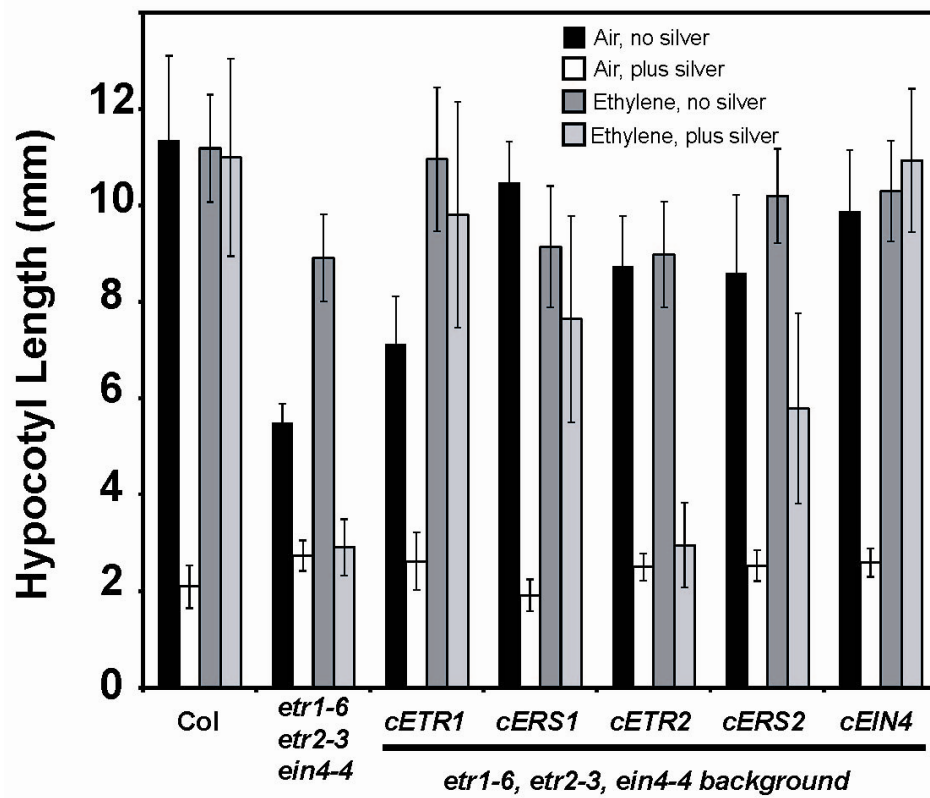


Figure 5. Ability of receptor transgenes to rescue silver's effect in *etr1-6;etr2-3;ein4-4* background. Seedlings were grown on agar plates with and without 100 μ M silver nitrate in chambers with continuous flow of either air or 1 ppm ethylene. Hypocotyl measurements were taken.

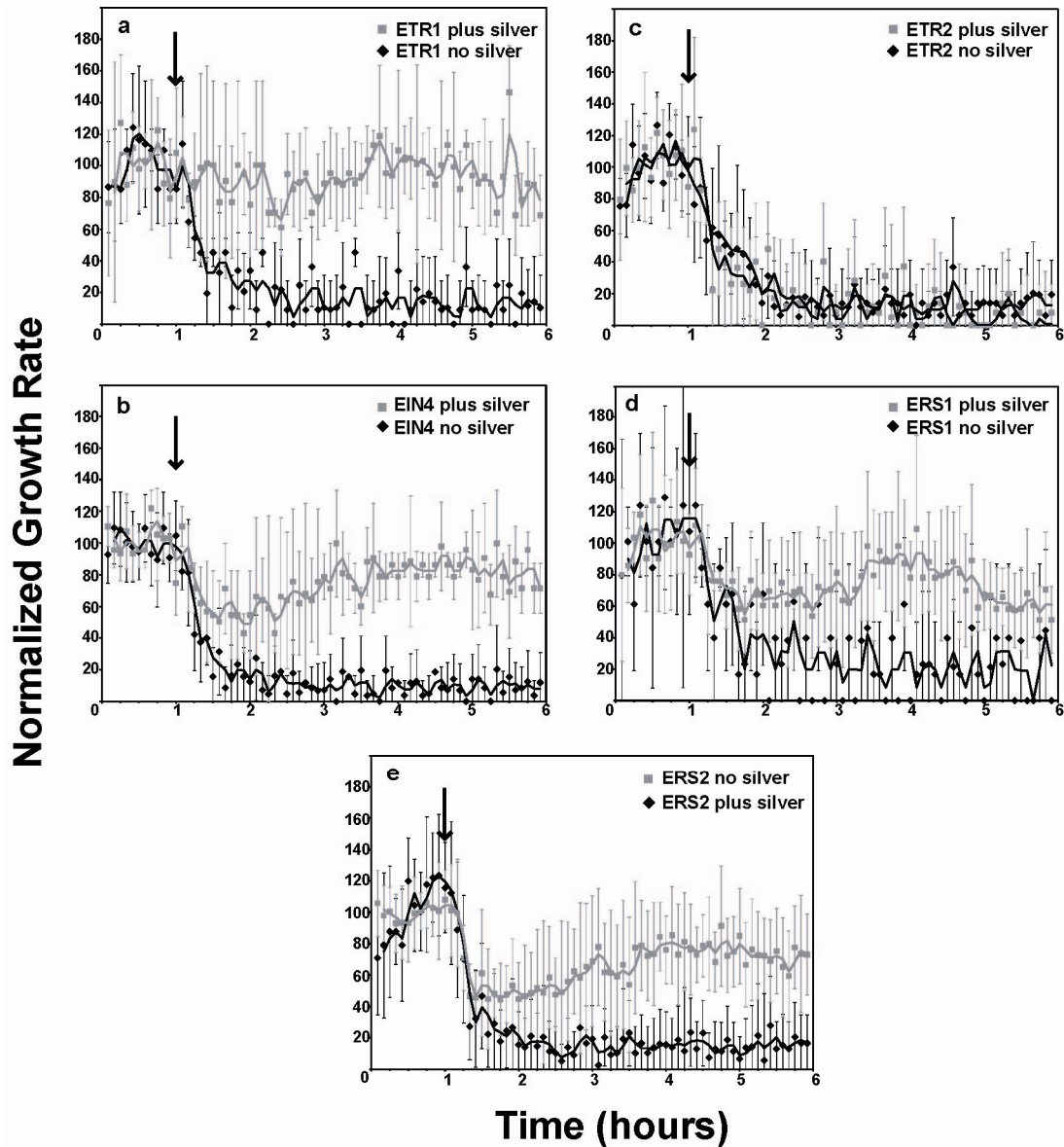


Figure 6. Rapid growth analysis of the ability of receptor transgenes to rescue silver's effect in *etr1-6;etr2-3;ein4-4* background. Seedlings were grown in air for 1 hour followed by the addition of 1 ppm ethylene (arrow) for 5 hours. The growth response of seedlings grown on plates containing 100μM silver nitrate (grey line) was compared to seedlings grown in the absence of silver nitrate (black line). Panels a through e represent the growth of the respective receptor mutant. Data represent the mean $\pm$ SD from at least 5 seedlings total from at least 4 separate experiments.

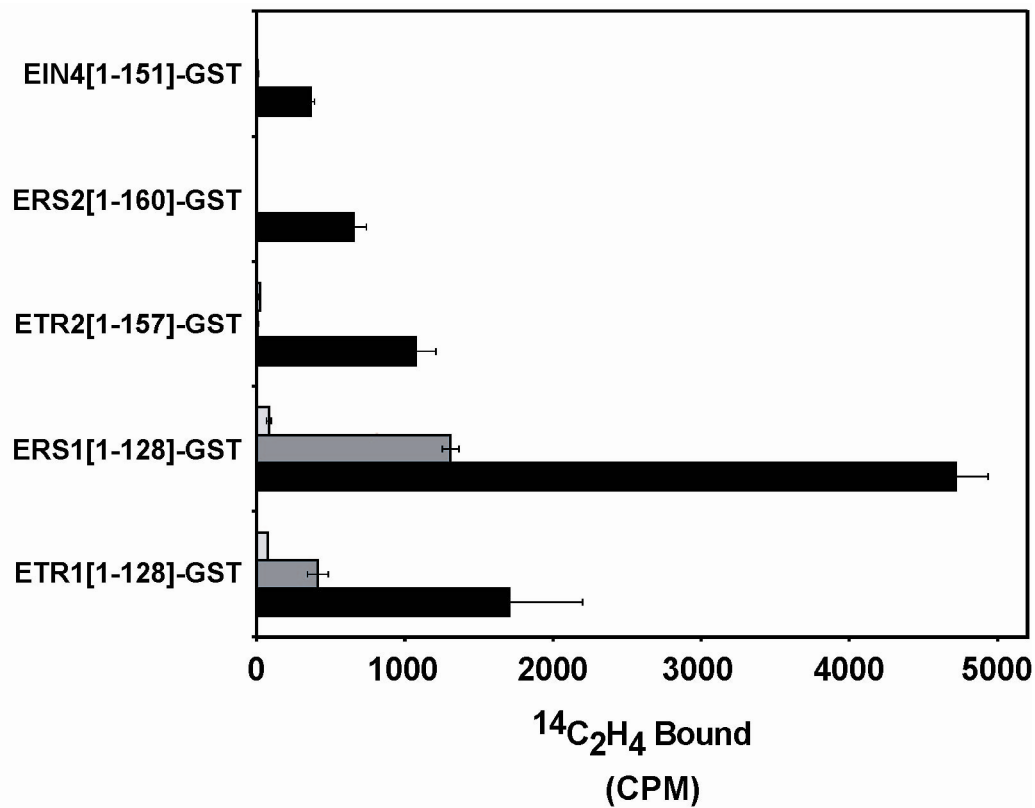


Figure 7. Comparison of ethylene binding to receptor isoforms between copper and silver. The binding domain of each receptor isoform was expressed and isolated from yeast cells. Samples were incubated for 30 minutes with 300 μ M silver nitrate (grey bars), copper sulfate (dark grey bars) or neither (light grey bars). Samples were then treated with [14 C]ethylene (0.1 ppm) and identical samples treated with [14 C]ethylene (0.1 ppm) plus [12 C]ethylene (1000 ppm). Subsequently, samples were analyzed for [14 C]ethylene bound. Specific ethylene binding was calculated by subtracting with [14 C]ethylene (0.1 ppm) plus [12 C]ethylene (1000 ppm) from [14 C]ethylene (0.1 ppm). Data represent the mean $\pm$ SD from 3 replicates per condition.

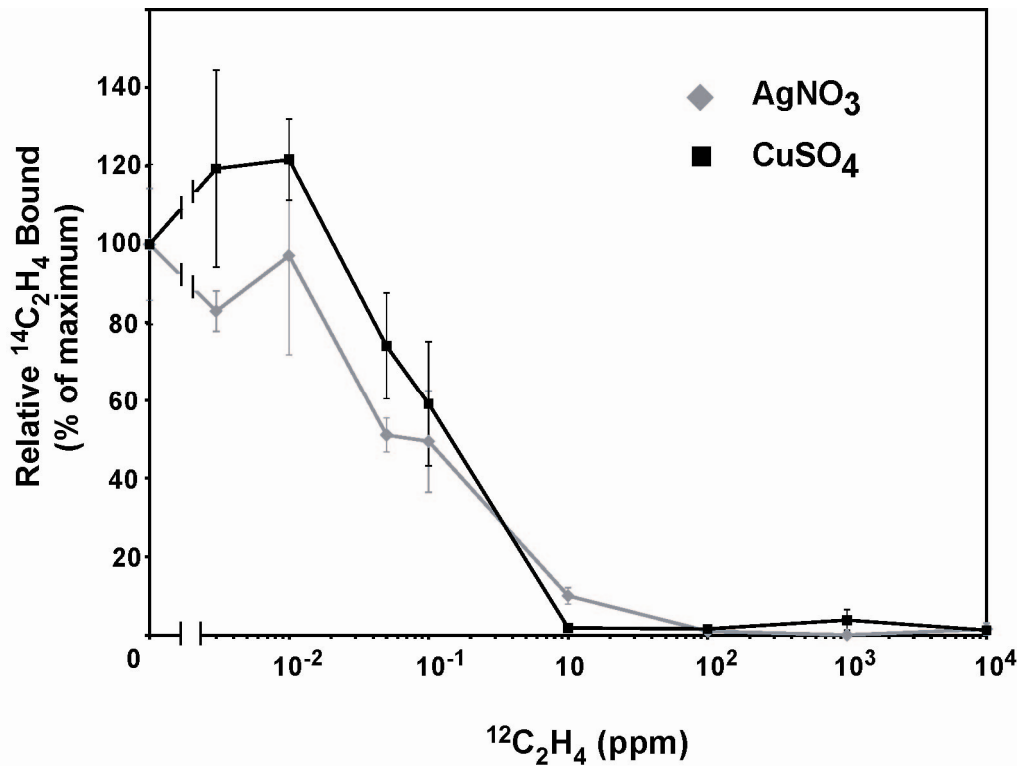


Figure 8. A comparison of ethylene's affinity to ETR1[1-128]-GST with copper and silver. Membranes isolated from yeast cells expressing ETR1[1-128]-GST were incubated with either 300 μM copper sulfate (black squares) or silver nitrate (grey diamonds). Samples were treated with ^{14}C ethylene (1.0 ppm) and 0, 0.1, 0.3, 0.5, 1, 10, 100, 1000, and 10000 ppm of ^{12}C ethylene. Samples were analyzed for ^{14}C ethylene bound. Data represent the average levels of binding for each condition $\pm$ SD from 3 replicates per condition normalized to binding in the absence of ^{12}C ethylene added.

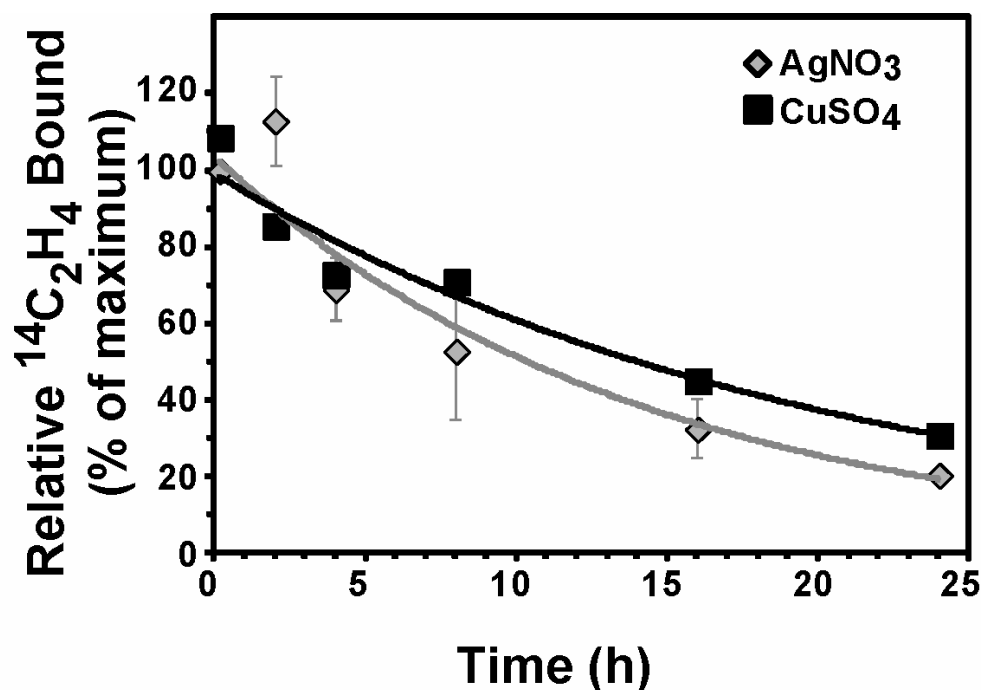


Figure 9. Time-course of dissociation of ethylene to ETR1 in the presence of copper and silver. ETR1[1-128]-GST was exogenously expressed in yeast and isolated from yeast membranes. Samples were incubated with 300 μ M copper sulfate (black squares) or silver nitrate (grey diamonds) then treated with [14 C]ethylene (0.1 ppm) and identical samples treated with [14 C]ethylene (0.1 ppm) plus [12 C]ethylene (1000 ppm). Samples were aired out for the indicated times in a chamber with a continuous flow of humidified air and analyzed for [14 C]ethylene bound. Data for both conditions were normalized to the level of ethylene binding after airing for 10 min. Data represent the mean $\pm$ SD from 3 replicates per condition.

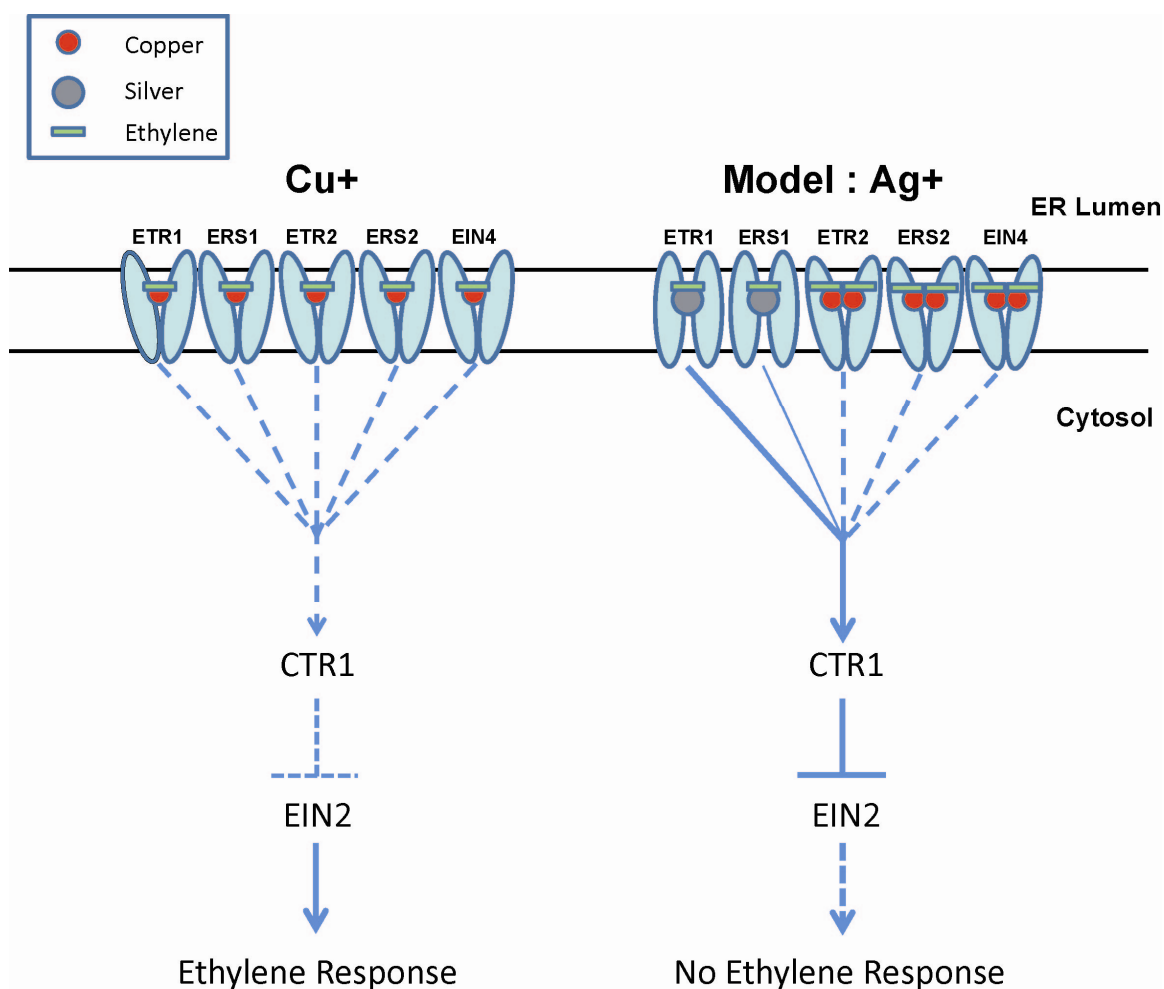


Figure 10. A proposed model to describe silver's effect on ethylene signaling in *Arabidopsis thaliana*. The current paradigm for ethylene signaling is that a copper ion coordinates into the ethylene binding pocket, which elicits a conformational change and leads to inactivation of the receptor. As a result, CTR1 activity is diminished, which leads to the accumulation of EIN2 and thus an ethylene response. Our model for signaling in the presence of silver is that only ETR1 and ERS1 are able to coordinate silver into the ethylene binding pocket. Ethylene is able to bind to ETR1 and ERS1, but because of the bulky nature, silver inhibits the conformational change, which maintains the receptor in the active conformation. Therefore, negative regulation on ethylene responses persists. It is unclear why ERS1 does not have as large a role in mediating the effects of silver. One explanation is that ETR1 levels are higher than ERS1 in dark-grown seedlings. It is also unclear why EIN4 and ERS2 transgenes can partially support the silver phenotype. It is possible that silver is affecting these receptors outside of the ethylene-binding pocket.

VITA

Brittany Kathleen McDaniel was born in Houston, Texas on July 17th, 1987, the fourth daughter of Barbara A. McDaniel and James L. McDaniel. After completing her work at Jersey Village High School in Houston, Texas she enrolled in the fall of 2009 at Texas State University at San Marcos in San Marcos, Texas. There she received her Bachelor of Science in Biology with a minor in Chemistry. She then entered graduate school in 2009 at The University of Tennessee at Knoxville.