Unique Functions of the Ethylene Receptors in Seed Germination

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Abstract

The endogenous phytohormone ethylene regulates many agroeconomically important aspects of plant development, including germination, fruit ripening, leaf and flower senescence, and organ abscission, as well as stress tolerance. In *Arabidopsis thaliana*, ethylene is perceived by a family of five membrane receptors known as ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2, and ETHYLENE INSENSITIVE4 (EIN4). Previous research has shown that these receptors have both overlapping and unique functions in mediating ethylene responses. We have investigated the role of individual ethylene receptors in seed germination during salt stress and following far-red light treatment. Both of these conditions are known to inhibit germination of wild-type seeds by enhancing and reducing production of the phytohormones abscisic acid (ABA) and gibberellic acid (GA) which are known to inhibit and promote germination respectively. We found that ETR1 inhibits while ETR2 promotes seed germination during both salt stress and far-red light treatment. During salt stress, ethylene was found to play only a minor role in the opposing actions of ETR1 and ETR2 on seed germination. Instead, differences in production and/or sensitivity to ABA played the major role in the opposing roles of ETR1 and ETR2 on seed germination during salt stress. Following far-red treatment, ethylene appeared to play a larger role than during salt stress, but ultimately ETR1 likely inhibits germination by affecting ABA and GA synthesis.

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List of Abbreviations

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	1-aminocyclopropane-1-carboxylase synthase
AHK	Arabidopsis histidine kinase
ARR	Arabidopsis response regulator
AVG	aminoethoxyvinylglycine
BAP	6-benzylaminopurine
CA	catalytic and ATP-binding domain
Col	Columbia
CTR	CONSTITUTIVE ETHYLENE RESPONSE
DMSO	dimethyl sulfoxide
EBD	ethylene binding domains
EBF	EIN3 binding F box protein
EIN	ETHYLENE INSENSITIVE
ERS	ETHYLENE RESPONSE SENSOR
ETP	EIN2 targeting protein
ETR	ETHYLENE RESPONSE
GA	gibberellic acid
GA3ox	GA 3-oxidase
GAI	GA-INSENSITIVE
GID1	GIBBERELLIN INSENSITIVE DWARF1
HPK	histidine protein kinase
LOF	loss-of-function
MS	Murashige and Skoog
NCED	9-cis epoxycarotenoid dioxygenase
NF	norflurazon
P450	cytochrome P450 monooxygenases

PAC	paclobutrazol
PHY	phytochrome
PP2C	Protein Phosphatase 2C
PYL	Pyrabactin Resistance 1–Like
PYR	Pyrabactin Resistance
RCAR	Regulatory Component of ABA Receptor
RGA	REPRESSOR OF ga1-3
RGL	RGA-LIKE
SnRK	Snf1-related protein kinases
WS	Wassilewskija
ZEP	zeaxanthin epoxidase

Chapter I
Introduction

Through the process of photosynthesis, plants convert light energy into chemical energy that they store in the bonds of sugar. In doing this, plants and other photosynthetic organisms, provide the energy needed to support all life on earth. In the process, they also produce the oxygen animals need to carry out aerobic respiration and more efficiently convert the energy stored in the sugar back into usable energy. In the human diet, approximately 78 percent of the world's daily per capita calorie consumption comes from plants (Bruinsma, 2003). In particular, cereal seeds such as wheat, rice and maize are a major source of food accounting for about 56 percent of the per capita total calorie consumption (Bruinsma, 2003).

In addition to providing us with a variety of food, seeds are also one of the major evolutionary advancements that have driven the success of the angiosperms or flowering plants. Seeds allow the next generation of plants to survive unfavorable conditions such as winter or drought and provide a mechanism with which to disperse or spread the plants to new locations. Fruit ripening, seed germination and plant growth are all regulated by the plant hormone ethylene (Abeles et al., 1992). The focus of this dissertation is on the unique role of the ethylene receptors in seed germination during two conditions known to inhibit germination: (1) salt stress and (2) far-red light. This research was done with the model flowering plant *Arabidopsis thaliana*.

Ethylene Biosynthesis

Ethylene (C_2H_4) is the simplest alkene and is a gas under normal physiological conditions (Yang and Hoffmann, 1984). Most plant tissues are capable of producing ethylene, but the amount of ethylene made varies greatly depending on the developmental stage and environmental conditions of the plant. On a daily basis, ethylene production peaks during the middle of the day (Rikin et al., 1985; Ievinsh and Kreicbergs, 1992; Finlayson et al., 1998; Thain et al., 2004). Developmentally, ethylene production is known to increase during germination (Meheriuk and Spencer, 1964; Spencer and Olson, 1965; Ketring and Morgan, 1969), leaf and flower senescence and fruit ripening (Yang and Hoffmann, 1984; Argueso et al., 2007). Environmental factors such as light, temperature and numerous stresses including pathogen attack, flooding,

drought, and wounding are all known to increase ethylene production (Yang and Hoffmann, 1984; Finlayson et al., 1998; Argueso et al., 2007). Other plant hormones such as, auxin, cytokinin, brassinosteroids, and abscisic acid (ABA) also affect ethylene production, however whether they increase or decrease ethylene production varies depending on the specific tissue, age of the plant and environmental conditions (Abeles, 1973; Wright, 1980; Mor et al., 1983; Arteca and Arteca, 2008).

In vascular plants, ethylene biosynthesis starts with the conversion of methionine to *S*-adenosyl-L-methionine by S-adenosylmethionine synthetase (Argueso et al., 2007). This is followed by the first committed step and in most cases, the rate-limiting step, the conversion of *S*-adenosyl-L-methionine to 1-aminocyclopropane-1-carboxylic acid (ACC) by 1-aminocyclopropane-1-carboxylase synthase (ACS) (Argueso et al., 2007). Ethylene, along with CO_2 and cyanide byproducts, is then produced from ACC by ACC oxidase (ACO) (Argueso et al., 2007).

Regulation of ethylene biosynthesis is thought to occur predominantly by transcriptional and posttranscriptional regulation of ACS. Arabidopsis contains a family of nine *ACS* genes that have unique and overlapping developmental and tissue expression and are differentially expressed in response to various hormones and stresses (Tsuchisaka and Theologis, 2004a; Wang et al., 2004). Eight of these genes encode proteins that function as active homodimers (ACS2, ACS4-9, ACS11) and one encodes a protein that is enzymatically inactive as a homodimer (ACS1) (Yamagami et al., 2003; Tsuchisaka and Theologis, 2004b). Posttranscriptional regulation of the ACS proteins is mediated through rapid degradation by the 26S proteosome, which is inhibited by phosphorylation of the C-terminal domain (Argueso et al., 2007). Arabidopsis contains a family of five *ACO* genes, also known as *Ethylene Forming Enzyme*, however, they have not been studied in detail.

Ethylene Perception and Signaling

In Arabidopsis, ethylene is perceived by a family of five receptors located in the endoplasmic reticulum (Chen et al., 2002; Ma et al., 2006; Grefen et al., 2008) called ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2, and

ETHYLENE INSENSITIVE4 (EIN4) (Figure I-1) (Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998). These receptors resemble bacterial two-component sensor histidine kinases (Chang et al., 1993). Each of the receptors contains an ethylene binding domain, a GAF domain, and a kinase domain. Three of the receptors (ETR1, ETR2 and EIN4) contain an additional C-terminal receiver domain. Each of these domains and their role in ethylene perception or signal output will be discussed in the following section.

In addition to the family of ethylene receptors, a number of ethylene signaling components have been identified in forward genetic screens and more recently with reverse genetic approaches. Three of the earliest components of ethylene signaling to be identified were CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), EIN2 and EIN3. As their names suggest, in the absence of ethylene, CTR1 loss-of-function mutants phenotypically resemble wild-type plants grown in ethylene while, in the presence of ethylene, EIN2 and EIN3 loss-of-function mutants look like wild-type plants grown in air (Guzman and Ecker, 1990; Kieber et al., 1993; Roman et al., 1995). CTR1 shares similarity to Raf-like serine/threonine kinases and acts as a negative regulator of ethylene signaling (Kieber et al., 1993). ETR1, ETR2 and ERS1 have been shown to interact with CTR1 in a yeast two-hybrid assay and all of the ethylene receptors are thought to be involved in localization of CTR1 to the ER membrane (Clark et al., 1998; Cancel and Larsen, 2002; Gao et al., 2003). EIN2 is a critical positive regulator of ethylene signaling that, like the ethylene receptors, is located in the endoplasmic reticulum (Alonso et al., 1999; Bisson et al., 2009). The N-terminus of EIN2 is composed of twelve transmembrane domains and shares sequence similarity with the Nramp family of metal transporters (Alonso et al., 1999). It also contains a large soluble C-terminal domain of 833 amino acid residues that when overexpressed leads to constitutive ethylene signaling (Alonso et al., 1999). Recently, EIN2 has been shown to be negatively regulated by the two F-box proteins EIN2 TARGETING PROTEIN1 and 2 (ETP1 and ETP2) (Qiao et al., 2009). EIN3 and its homolog, EIN3 LIKE1 (EIL1) are nuclear located transcription factors that are required for most ethylene responses (Chao et al., 1997; Binder et al., 2004a). They are positive regulators of ethylene signaling and are negatively regulated by the F-box proteins EIN3 BINDING F BOX PROTEIN1 and 2 (EBF1 and 2) (Chao et al., 1997; Guo and Ecker, 2003; Gagne et al., 2004; An et al., 2010).

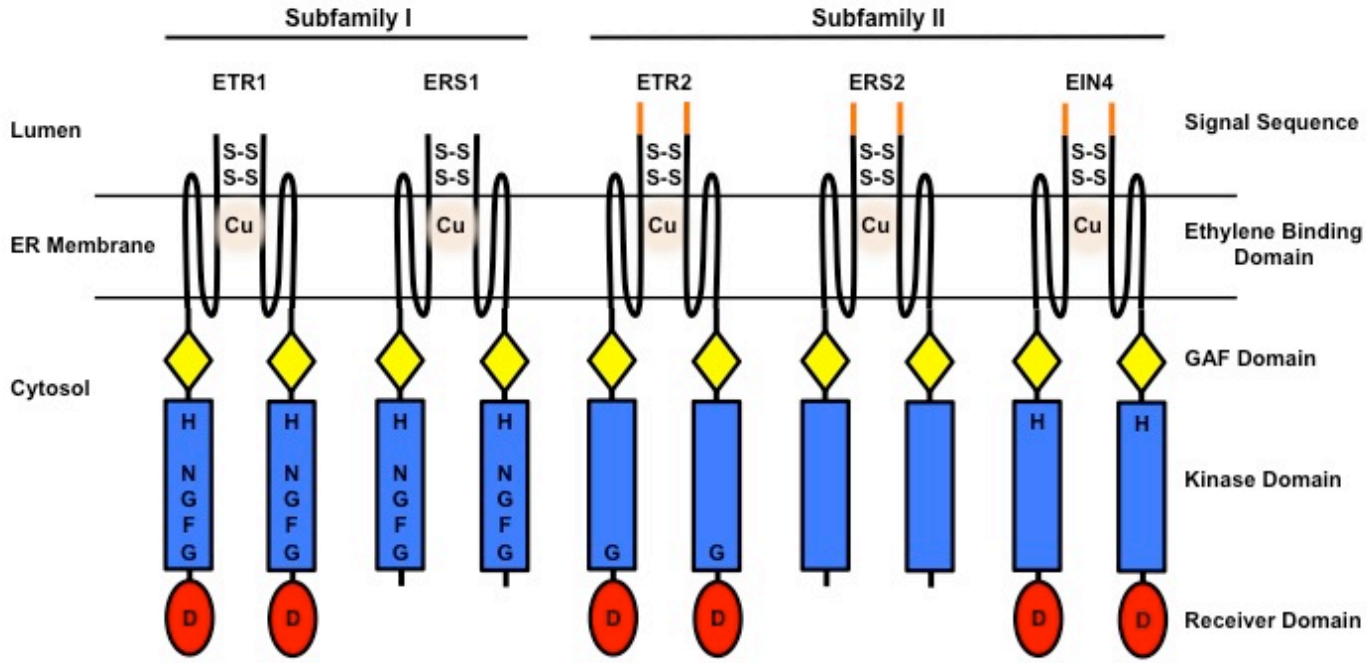


Figure I-1. Structure of Arabidopsis Ethylene Receptors.

The Arabidopsis ethylene receptors are located in the endoplasmic reticulum (ER) where they are thought to function as homodimers. The receptors can be divided into two subfamilies based on the sequence alignment of their ethylene binding domains. Each of the receptors contain an ethylene binding domain that is thought to chelate at least one copper(I) ion giving the dimer the ability to bind at least one molecule of ethylene. They each also contain a GAF domain and a kinase domain. ETR1, ETR2 and EIN4 contain an additional C-terminal Receiver domain. The Subfamily II members contain a hydrophobic N-terminal sequence that may be a fourth membrane spanning region or a signal sequence. The conserved histidine (H) and motifs (NGFG) in the kinase domain and the aspartic acid (D) in the receiver domain are shown if present. This figure is modified from Hall et al. (2007) and Lacey and Binder (2014)

An inverse-agonist model for ethylene signaling has been developed based on data that shows loss of multiple ethylene receptors lead to constitutive ethylene responses while mutations known to prevent ethylene binding lead to ethylene insensitivity (Bleecker et al., 1988; Hua et al., 1998; Rodríguez et al., 1999). Although incomplete, continued epistasis and biochemical analysis of the proteins involved in ethylene signal transduction have led to a detailed model of ethylene signaling (Figure I-2). In the absence of ethylene, the ethylene receptors promote activity of CTR1 and active CTR1 phosphorylates the C-terminus of EIN2 (EIN2-CEND) (Clark et al., 1998; Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). Phosphorylation of EIN2 by CTR1 prevents the nuclear accumulation of EIN2-CEND, which prevents accumulation of the downstream transcription factor EIN3 (Ju et al., 2012; Wen et al., 2012). Degradation of EIN3 and EIL1 by the 26S proteasome and failure of its accumulation represses ethylene responses (Guo and Ecker, 2003; Gagne et al., 2004; An et al., 2010). Upon ethylene binding, the ethylene receptor-CTR1 complex is inactivated leading to EIN2-CEND translocation to and accumulation in the nucleus (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). This results in the accumulation of EIN3 and EIL1, which leads to most ethylene responses (Guo and Ecker, 2003; Gagne et al., 2004; An et al., 2010).

The Ethylene Receptors

The ethylene receptors resemble bacterial two-component sensor histidine kinases (Chang et al., 1993). They can be split into two subfamilies based on sequence alignment of their ethylene binding domains (Wang et al., 2006). Subfamily I contains ETR1 and ERS1 while subfamily II contains ETR2, ERS2 and EIN4 (Figure I-1). Subfamily II members contain a hydrophobic N-terminal sequence that may be a fourth membrane spanning region or a signal sequence. Each of the receptors contain an ethylene binding domain, a GAF domain, and a histidine kinase domain. Three of the receptors (ETR1, ETR2 and EIN4) contain an additional C-terminal receiver domain. Each of these domains and their role in ethylene perception or signal output will be discussed below.

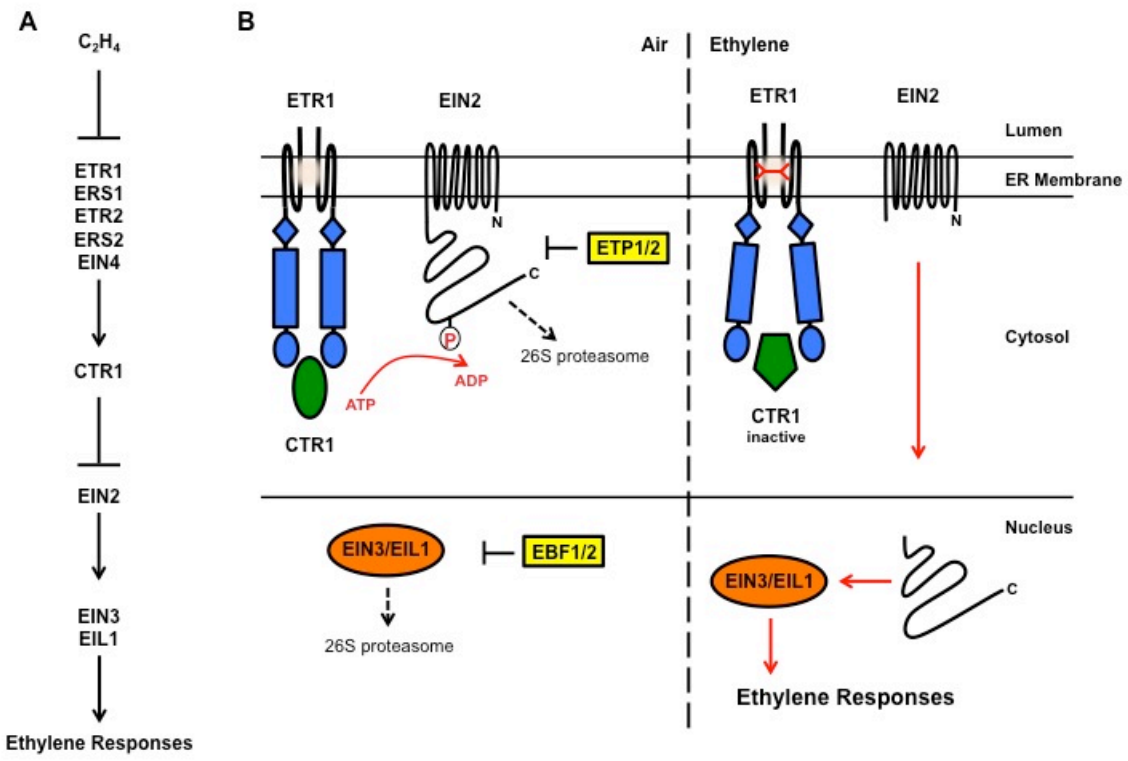


Figure I-2 Ethylene Signal Transduction.

(A) Genetic model of ethylene signaling. (B) Biochemical model of ethylene signaling. In the absence of ethylene, the ethylene receptors promote CTR1 activity and CTR1 phosphorylates the C-terminus of EIN2. ETP1/2 promote the degradation of EIN2 and EBF1/2 promote the degradation of EIN3 and EIL1 thereby repressing ethylene responses. Upon ethylene binding, the ethylene receptor-CTR1 complex is inactivated leading to cleavage of the EIN2 C-terminus and the translocation and accumulation of it in the nucleus. This results in the accumulation of EIN3 and EIL1, which leads to most ethylene responses. This figure is modified from Shakeel et al. (2013) and Ju et al. (2012).

Ethylene Binding Domain

Ethylene binding domains (EBD) are predicted to be composed of three transmembrane spanning α-helices (Rodríguez et al., 1999). They have been identified in all sequenced plants and in cyanobacteria suggesting they have a plastid origin (Rodríguez et al., 1999; Mount and Chang, 2002; Wang et al., 2006). Although no crystal structure is available for this domain, the ETR1 EBD has been studied in great detail both biochemically and genetically. Long before the ethylene receptors were identified, they were predicted to bind ethylene via a metal ion. Aside from the reactive nature of alkenes with metals, a metal cofactor was thought to be required for ethylene binding because carbon monoxide, which is known to require a metal ion for binding, was shown to produce ethylene responses in peas (Burg and Burg, 1967; Abeles, 1973). RESPONSIVE TO AGONIST1, a protein similar to the yeast copper transporter Ccc2, is now known to provide the receptors with the copper necessary for ethylene binding (Hirayama et al., 1999). Each receptor homodimer is thought to chelate at least one copper(I) ion giving the dimer the ability to bind at least one molecule of ethylene (Schaller and Bleecker, 1995; Rodríguez et al., 1999). Mutational studies show that aspartic acid^{25}, tyrosine^{32}, isoleucine^{35} and proline^{36} in the first alpha helix and isoleucine^{62}, cysteine^{65} and histidine^{69} in the second alpha helix of the ETR1 EBD are conserved among the ethylene receptors and, are required for ethylene binding (Hall et al., 1999; Rodríguez et al., 1999; Wang et al., 2006). Cysteine^{65} is also required to bind copper. When expressed in yeast, Arabidopsis ETR1 binds ethylene with a K_d of 2.4 nM (Schaller and Bleecker, 1995). Higher order heteromeric interactions between the ethylene receptors are thought to amplify receptor signaling allowing Arabidopsis to respond to ethylene concentrations 300 times less than their K_d (Binder et al., 2004b; Gao et al., 2008; Chen et al., 2010).

Two other group 11 transition metals, silver and gold, are also able to support ethylene binding to ETR1 (Rodríguez et al., 1999; Binder et al., 2007). However, whereas copper and gold support ethylene responses, silver inhibits ethylene responses (Beyer, 1976; Binder et al., 2007). Recently, silver has also been shown to support ethylene binding to ERS1, but not to the subfamily II receptors ETR2, EIN4 and ERS2 (McDaniel and Binder, 2012). Interestingly, although silver only supports ethylene binding to the subfamily I receptors ETR1 and ERS1, the

subfamily II receptors EIN4 and ERS2, but not ETR2 are able to partially mediate the effects of silver (McDaniel and Binder, 2012).

In addition to identifying residues in the EBD required for coordinating the copper cofactor and ethylene binding, mutational studies have also defined residues that do not affect ethylene binding, but either result in ethylene insensitive plants or cause constitutive ethylene signaling (Wang et al., 2006). This detailed biochemical and genetic analysis of the ETR1 EBD has led to a three state model for ethylene receptor output (Wang et al., 2006). As expected, this model suggests that the absence of ethylene results in active receptors that prevent ethylene responses (state I) while ethylene binding to the receptors results in inactive receptors that promote ethylene responses (state III). However, it predicts an intermediate state (state II) in which ethylene is bound, but the receptors still prevent ethylene responses.

GAF Domain

All of the ethylene receptors, including those found in cyanobacteria, contain a GAF domain. GAF domains were first described as non-catalytic cGMP binding sites found in a phosphodiesterase in bovine rod photoreceptors (Charbonneau et al., 1990) and have since been found to bind a diversity of small molecules (Kanacher et al., 2002; Sardiwal et al., 2005; Tucker et al., 2006; Cann, 2007; Levdikov et al., 2009; Ulijasz et al., 2009). In most cases, however, the ligand remains unknown and in some cases there is no evidence of ligand binding. In the latter case, the GAF domain is thought to serve a structural role in the protein (Levdikov et al., 2009). The GAF domain in the *Synechocystis* ethylene receptor, SynETR1, has been shown *in vitro* to bind the chromophore phycocyanobilin and to be capable of blue green photoconversion (Ulijasz et al., 2009). However, the GAF domain of ETR1 has not been shown to bind a ligand and is missing the cysteine found in the GAF domain of the Arabidopsis phytochromes required for chromophore binding (Aravind and Ponting, 1997). Instead, the GAF domain in plant ethylene receptors has been suggested to mediate higher order heteromeric interactions between the receptor homodimers (Gao et al., 2008; Grefen et al., 2008). As mentioned above, these higher order heteromeric interactions are thought to amplify receptor signaling and allow Arabidopsis to respond to ethylene concentrations 300 times less than the receptor K_d (Chen et al., 2010). In addition, the ETR1 GAF domain appears to be capable of signaling independent of the kinase

and receiver domains (Xie et al., 2006; Qiu et al., 2012). This so called "N-terminal signaling" is independent of CTR1, but is promoted by REVERSION TO SENSITIVITY1 (Qiu et al., 2012).

Kinase Domain

The ethylene receptors all contain a histidine protein kinase (HPK)-like domain. In bacteria, HPK domains are known to have autokinase activity, phosphotransfer activity and, in some cases, additional phosphatase activity (Stewart, 2010). This domain can be split into two subdomains: a dimerization and histidine phosphotransfer domain (DHp) that is characterized by an H box, and a catalytic and ATP-binding domain (CA) that is characterized by a N, G1 (D), F, and G2 (G) box (Wolanin et al., 2002; Stewart, 2010). As the name suggests, the DHp subdomain is required for HPK homodimer formation. Homodimerization is necessary for autophosphorylation which is thought to occur in *trans* to the H box histidine (Parkinson and Kofoid, 1992; Wolanin et al., 2002). This phospho accepting histidine serves as an intermediate for transfer of the phosporyl group to an aspartate in the response regulator's receiver domain (Parkinson and Kofoid, 1992). Mutation of the phospho accepting histidine has been shown to eliminate both autophosphorylation and phosphatase activity, while mutation of other residues in the H box can individually affect either autophosphorylation or phosphatase activity (Parkinson and Kofoid, 1992). Within the CA domain, the N, G1, F and G2 boxes are involved in ATP binding (Wolanin et al., 2002). Residues in the N box coordinate the divalent metal cofactor required for ATP binding while the F box makes up part of the ATP lid that is flanked by the G1 and G2 boxes with the G1 box forming a flexible hinge at the end of the ATP lid (Parkinson and Kofoid, 1992; Wolanin et al., 2002). Mutation of the N, G1, or G2 boxes has been shown to eliminate autokinase activity, however it is not uncommon for a HPK CA domain to lack one of these boxes (Parkinson and Kofoid, 1992; Wolanin et al., 2002).

As mentioned above, the ethylene receptors have been subdivided into two families based on their ethylene binding domain (Wang et al., 2006). In Arabidopsis, subfamily I members contain all of the conserved motifs of the HPK domain required for histidine kinase activity, while subfamily II members lack one or more of these features (Moussatche and Klee, 2004; Wang et al., 2006). Consistent with their domain features, *in vitro* analysis of the Arabidopsis subfamily I ethylene receptors, ERS1 and ETR1, suggests they have histidine kinase activity while analysis

of subfamily II members, ETR2, ERS2 and EIN4, suggests they lost their histidine kinase activity and together with ERS1 acquired serine/threonine kinase activity (Gamble et al., 1998; Moussatche and Klee, 2004). Although in Arabidopsis histidine kinase activity appears to be restricted to subfamily I members, this does not appear to be the case in tobacco (*Nicotiana tabacum*). Of the four ethylene receptors identified in tobacco, NtETR1, a subfamily I member, and NTHK1 and NTHK2, both subfamily II receptors, were examined for kinase activity. As with Arabidopsis, NtETR1 was shown to have histidine kinase activity and both of the subfamily II proteins were shown to have serine/threonine kinase activity *in vitro* in the presence of Mn^{2+} (Xie et al., 2003; Zhang et al., 2004; Chen et al., 2009b). However, the subfamily II member NTHK2 was shown to have both serine/threonine and histidine kinase activity (Zhang et al., 2004). Whereas ETR1, ERS1, and NtETR1 have histidine kinase activity in the presence of Mn^{2+}, NTHK2 has histidine kinase activity in the presence of Ca^{2+}. It is not known whether any of the Arabidopsis subfamily II members have histidine kinase activity when Ca^{2+} is supplied as the metal cofactor, however this seems unlikely given their divergent HPK domain (Gamble et al., 1998; Moussatche and Klee, 2004; Zhang et al., 2004; Chen et al., 2009b). Although all of the ethylene receptors examined to date show serine/threonine and/or histidine kinase activity *in vitro*, direct biochemical evidence for kinase activity *in vivo* and whether they phosphorylate other proteins has not been shown for any of the ethylene receptors. In fact, whether or not ETR1 and ERS1 have histidine kinase activity *in vivo* has been called to question based on the finding that under physiologically relevant cellular ratios of Mg^{2+} to Mn^{2+}, where Mg^{2+} concentrations are 50-100 fold higher than that of Mn^{2+}, ERS1 only shows autophosphorylation on serine residues and ETR1 shows no autophosphorylation (Moussatche and Klee, 2004).

In bacterial two-component histidine kinases, binding of ligand to the N-terminal domain modulates activity of the kinase domain. Binding of ethylene to the ethylene receptors also appears to modulate activity of their kinase domain. As discussed above, ETR1 is capable of autophosphorylation *in vitro* in the presence of manganese (Gamble et al., 1998; Moussatche and Klee, 2004). Ethylene and the structurally similar compound cyanide (interestingly a byproduct of ethylene biosynthesis) have both been shown to reduce autophosphorylation of ETR1 *in vitro* (Voet-van-Vormizeele and Groth, 2008). Reduction of ETR1 autophosphorylation by cyanide requires both the ethylene binding N-terminal domain and the metal cofactor copper (Voet-van-

Vormizeele and Groth, 2008; Bisson and Groth, 2012). Silver nitrate is known to facilitate ethylene (Rodríguez et al., 1999) and cyanide (Bisson and Groth, 2012) binding to the N-terminal domain of ETR1, but to block ethylene responses (Beyer, 1976; Binder et al., 2007). In the presence of silver nitrate, cyanide fails to reduce autophosphorylation of ETR1 (Bisson and Groth, 2012). Additionally, the ethylene antagonist 1-methylcyclopropene, known to inhibit fruit ripening and senescence, blocks ETR1 autophosphorylation in the presence of cyanide (Voet-van-Vormizeele and Groth, 2008). Interestingly, an *in vivo* study on two tomato ethylene receptors, LeETR4 and NEVER-RIPE, shows that both receptors are highly and multiply phosphorylated in the absence of ethylene and have reduced and minimal phosphorylation in the presence of ethylene (Kamiyoshihara et al., 2012). However, it remains to be determined whether this represents autophosphorylation or phosphorylation by another protein kinase.

Even though the *in vitro* studies mentioned above suggest that the ethylene receptors have kinase activity that is modulated by ligand binding and genetic complementation studies with truncated ETR1 show the importance of the kinase domain in ethylene signal output, complementation studies with kinase deficient versions of ETR1 suggest that kinase activity is not required for signaling (Gamble et al., 2002; Wang et al., 2003; Binder et al., 2004b; Qu and Schaller, 2004; Xie et al., 2006; Hall et al., 2012). Instead ETR1 kinase activity appears to modulate responsiveness and sensitivity to ethylene as well as recovery from ethylene after its removal (Binder et al., 2004b; Qu and Schaller, 2004; Hall et al., 2012). Whether or not the kinase activity of other ethylene receptors, particularly serine/threonine activity, is required for signaling or modulates the plant's responsiveness and/or sensitivity to ethylene has not been directly tested. However, when overexpressed in *Arabidopsis*, the subfamily II ethylene receptor NTHK1 caused increased sensitivity of etiolated seedlings to the ethylene precursor ACC, while the kinase deficient version of NTHK1 maintained wild-type sensitivity to ACC (Chen et al., 2009b). This suggests that serine/threonine kinase activity may also be required for wild-type sensitivity to ethylene.

Receiver Domain

Some of the ethylene receptors are hybrid kinases and contain a C-terminal receiver domain. In eudicots, receiver domains have been found in both subfamily I and subfamily II members, but

in all monocots studied to date, receiver domains have only been identified in subfamily II members (Binder et al., 2012). Receiver domains contain six conserved residues: three aspartic acid residues (two of which can also be a glutamic acid residue), a lysine, a serine/threonine and a phenylalanine/tyrosine residue (Bourret, 2010). The three aspartic acid residues form an acidic pocket and, along with the lysine, coordinate a metal cofactor in the active site. One of these aspartic acid residues also serves as the site of phosphorylation (Bourret, 2010). The serine/threonine, and phenylalanine/tyrosine residues are involved in conformational changes of the receiver domain and signal output (Bourret, 2010). The *Arabidopsis* ETR1 receiver domain was crystalized and despite showing low sequence similarity to the well studied *E. coli* CheY receiver domain, they showed high structural conservation (Müller-Dieckmann et al., 1999). The most interesting difference between ETR1 and CheY receiver domains is the orientation of their γ loops. The γ loop is thought to be involved in molecular recognition and discrimination (Müller-Dieckmann et al., 1999). In CheY, the backbone carbonyl of an asparagine residue in the γ loop participates in cation ligation, however the backbone carbonyl of the corresponding cysteine in ETR1 is facing away from the acidic pocket and would not be able to participate in cation ligation unless the γ loop underwent a major conformational change (Müller-Dieckmann et al., 1999). The orientation of the γ loop in the other ethylene receptors and the biological implication of their orientation is not currently known.

In prokaryotes, receiver domains are usually attached to an effector domain where they act as a phospho mediated on/off switch for controlling the output of their effector domain (Bourret, 2010). Most of these effector domains regulate transcription and in these cases, phosphorylation of the receiver domain is thought to result in its dimerization which promotes DNA binding and transcriptional activation by the effector domain (Bourret, 2010; Gao and Stock, 2010). The *Arabidopsis* ETR1 receiver domain is found as a dimer both in solution and in the crystal form (Müller-Dieckmann et al., 1999). Based on comparison to bacterial CheY and CheB receiver domains, phosphorylation of the ETR1 receiver domain is predicted to result in release of its dimerization (Müller-Dieckmann et al., 1999).

In prokaryotes, receiver domains in hybrid kinases often participate in multistep phosphorelays where the phospho group is passed from the phospho accepting histidine in the kinase domain to

the aspartic acid of the receiver domain and then to the phospho accepting histidine of a histidine phosphotransfer protein and then to the aspartic acid of another receiver domain containing protein (Bourret, 2010). Although some of the ethylene receptors are hybrid kinases containing both a sensor histidine kinase and a receiver domain, they do not appear to participate in a canonical multistep phosphorelay. In fact, of all of the ethylene receptors tested for kinase activity to date, only one, rice ETR2, has been shown to phosphorylate its receiver domain *in vitro* and whether or not the conserved aspartic acid was phosphorylated was not tested (Wuriyanghan et al., 2009).

Instead of being involved in a phosphorelay, as with kinase activity, the receiver domain appears to be required for wild-type sensitivity to ethylene rather than direct signal output. For example, ETR1(1-603) that lacks its receiver domain is able to rescue the constitutive ethylene phenotype of etiolated *etr1 etr2 ein4* triple mutants grown in air however, it is hypersensitive to low concentrations of ethylene (Qu and Schaller, 2004). Although, *in vitro*, ETR1 fails to phosphorylate the conserved aspartate in the receiver domain (Moussatche and Klee, 2004), it is possible that phosphotransfer to the conserved aspartic acid in the receiver domain occurs *in vivo* and may be required for wild-type sensitivity to ethylene. This is seen in the growth recovery of the etiolated triple *etr1 etr2 ein4* mutant after removal of ethylene. Although both wild-type gETR1 and getr1-[D] lacking the conserved aspartate in the receiver domain are able to rescue growth of the etiolated triple *etr1 etr2 ein4* mutant in air, gETR1 rescues normal growth recovery after ethylene removal to that of the *etr2 ein4* double mutant while getr1-[D] only partially rescues this phenotype (Binder et al., 2004b). Additionally, the receiver domain, but not necessarily phosphotransfer, appears to be required for ETR1 specific phenotypes. For example, ETR1 was shown to be both necessary and sufficient for ethylene stimulated hypocotyl nutations and while the receiver domain of ETR1 was required for ethylene stimulated nutations, the phosphor accepting aspartic acid in the receiver domain was not required (Binder et al., 2006; Kim et al., 2011). Furthermore, the receiver domain of EIN4 was not able to substitute for the ETR1 receiver domain (Kim et al., 2011). All of this data suggest that the receiver domain has multiple functions in signal output.

Unique Functions of the Ethylene Receptors

All five of the Arabidopsis ethylene receptors function redundantly in ethylene signaling to regulate growth (Hua and Meyerowitz, 1998; Hall and Bleecker, 2003). However, it is becoming clear that the receptors also have unique functions, and in some cases, opposing functions to one another (Table I-1). Although the mechanism for relaying these differences to downstream signaling components is not known, there is often a common feature shared by receptors that function similarly for a given phenotype. For example, the subfamily I members ETR1 and ERS1 have a more prominent role in regulating growth than subfamily two members (Hall and Bleecker, 2003; Qu et al., 2007). Likewise, ETR1, ETR2 and EIN4, which all have receiver domains, are more important than ERS1 and ERS2 for rapid growth recovery of etiolated seedlings after ethylene removal (Binder et al., 2004b). Similarly, ETR1, which stands out as the only receptor to have both Histidine kinase activity and a receiver domain is both necessary and sufficient for ethylene stimulated nutations (bending of the hypocotyl) and plays the predominant role in mediating the inhibitory effect of silver on ethylene responses (Binder et al., 2006; Kim et al., 2011; McDaniel and Binder, 2012). In other cases, the receptor with a unique function doesn't have an obvious known distinguishing feature. For example, EIN4 is the only receptor involved in suppressing root bulging in a mutant lacking a functional UDP-glucose 4-epimerase gene (Seifert et al., 2004) while ETR2 is the only receptor involved in trichome development (Plett et al., 2009b). Perhaps most interesting is evidence suggesting that the receptors have opposite functions to one another. In one case, ETR1 and EIN4 were shown to have opposite roles with respect to cell death caused by the fungal toxin Fumonisin B_1 (Plett et al., 2009a). ETR1 also functions oppositely to the other receptors with respect to nutations (Kim et al., 2011) and in some cases, ERS1 functions opposite to the other receptors in the growth of etiolated seedlings (Liu et al., 2010).

Seed Germination

Increasing data highlighting the unique and opposing roles the ethylene receptors have in various traits known to involve ethylene signaling has led us to evaluate the role of individual ethylene receptors in seed germination. Seeds are structurally simple. In the case of Arabidopsis, they are composed of an embryo with a root called a radicle and two cotyledons which are embryonic

Table I-1 Unique Functions of the Ethylene Receptors

Trait	ETR1	ERS1	ETR2	EIN4	ERS2
Growth	++	++/-	+	+	+
Growth Recovery	+		+	+	
Nutations	+	-	-	-	-
Inhibition by Silver	++	+		+	+
Fumonisin B_1 Resistance	-			+	
Trichome Development			+		

A plus sign (+) indicates that the relative degree to which the receptor promotes the response. A minus sign (-) that indicates that the receptor inhibits the response. No sign indicates that the receptor was not observed to affect the response. This table is modified from Shakeel et al. (2013).

leaves that store energy and nutrients to aid in germination and development of the young seedling. The mature embryo is surrounded by a single cell layer of endosperm, which is surrounded by the testa or seed coat (Bentsink and Koornneef, 2008).

Seed germination begins with the uptake of water known as imbibition and in Arabidopsis is a two step process starting with testa rupture followed by endosperm rupture (Bentsink and Koornneef, 2008). Germination is controlled by endogenous and exogenous cues that act to inhibit germination in unfavorable conditions and promote germination in favorable conditions. Absence of stresses, such as high salt concentration, along with sufficient water, oxygen, appropriate temperature, and in some plant species, light are required for germination (Seo et al., 2009). The phytohormones abscisic acid (ABA) and gibberellic acid (GA) act antagonistically to inhibit and promote germination respectively (Weitbrecht et al., 2011). In some species, ethylene is also known to promote germination (Kępczyński and Kępczyński, 1997).

Abscisic acid

The plant hormone ABA regulates many aspects of plant growth and development, including embryo maturation, promotion of seed dormancy and inhibition of seed germination. It also promotes tolerance to environmental stresses such as cold, drought and salinity (Finkelstein, 2013). Endogenous ABA levels are determined by regulation of both its biosynthesis and catabolism (Finkelstein, 2013). A number of ABA biosynthesis enzymes have been identified in forward and reverse genetic screens and include zeaxanthin epoxidase (ZEP) or ABA1, ABA2, ABA3, ABA4 and a family of 9-cis epoxycarotenoid dioxygenase (NCED) (Koornneef et al., 1982; Léon-Kloosterziel et al., 1996; Tan et al., 2003; North et al., 2007). A family of four cytochrome P450 monooxygenases (P450) enzymes (CYP707As) have been identified in Arabidopsis that are responsible for ABA catabolism (Saito et al., 2004). CYP707A2 is the most important during germination and is responsible for the decrease in ABA levels upon seed imbibition (Kushiro et al., 2004).

ABA signal transduction has been difficult to study because of the very large gene families involved and is complicated by the identification of at least three completely different classes of putative ABA receptors (Cutler et al., 2010). However, the recent identification of the

PYR/PYL/RCAR (Pyrabactin Resistance/Pyrabactin Resistance 1–Like/ Regulatory Component of ABA Receptor) family of START proteins has helped elucidate what is being called the "core" ABA signaling pathway (Cutler et al., 2010). This family of fourteen PYR/PYL/RCAR proteins appear to be the major class of ABA receptors (Ma et al., 2009; Park et al., 2009). Upon binding ABA, these receptors have been shown to interact with and inhibit the phosphatase activity of the Protein Phosphatase 2C (PP2C) proteins ABI1, ABI2 and HAB1, which are negative regulators of ABA signaling (Ma et al., 2009; Park et al., 2009; Nishimura et al., 2010). Inactivation of the PP2C proteins allows ABA signaling to proceed through the Snf1-related protein kinases (SnRK)s. The SnRK2 protein kinases in particular are thought to phosphorylate and activate the bZIP transcription factors, which are positive regulators of ABA signaling, including ABI5, a key repressor of seed germination (Piskurewicz et al., 2008; Nakashima et al., 2009; Cutler et al., 2010). Two other transcription factors that are important positive regulators of ABA signaling in seeds are ABI3 and ABI4, which are B3 and AP2/ERF domain containing transcription factors respectively (Cutler et al., 2010). In the absence of ABA, the PP2Cs have been shown to dephosphorylate and inactivate a family of three SnRK2 proteins (Umezawa et al., 2009). However, other SnRK proteins, including SALT OVERLY SENSITIVE2, a kinase important for salt tolerance, are also involved in ABA signaling (Ohta et al., 2003).

Further complicating ABA signaling is its extensive cross-talk with other hormone signaling pathways including ethylene signaling. Two key proteins in ethylene signaling, EIN2 and CTR1, were discovered in forward genetic screens aimed at identifying enhancers or repressors of ABA signaling during seed germination (Cutler et al., 1996; Beaudoin et al., 2000; Ghassemian et al., 2000). EIN2 loss-of-function (LOF) mutants and ethylene insensitive *etr1-1* mutants are hypersensitive to ABA or NaCl with respect to seed germination, whereas the constitutive ethylene signaling mutant *ctr1* is less sensitive to ABA or NaCl (Beaudoin et al., 2000; Ghassemian et al., 2000; Wang et al., 2007; Cheng et al., 2009; Subbiah and Reddy, 2010). Part of the increased sensitivity of *ein2* and *etr1-1* mutants to ABA is due to the increased ABA production of these mutants. EIN2 LOF mutants produce almost twice as much ABA as wild-type while the ethylene insensitive *etr1-1* mutant produces about 20% more ABA than wild-type (Cheng et al., 2009). However, this increase in ABA production alone does not explain the ten-fold increase in sensitivity to ABA of *ein2* mutants compared to wild-type seeds during

germination and suggests that in addition to affecting ABA synthesis, EIN2 decreases sensitivity to ABA (Beaudoin et al., 2000).

Gibberellic Acid

The plant hormone GA is another important regulator of plant growth and development. It promotes stem elongation, flowering, and seed development, and functions antagonistically with ABA to promote seed germination (Yamaguchi, 2008). Over 100 different GAs have been identified in plants, but only four (GA_1, GA_3, GA_4 and GA_7) are known to be bioactive (Yamaguchi, 2008). The synthesis of bioactive GA requires many steps, but there are only three different classes of enzymes involved in this process: terpene synthases, P450s, and 2-oxoglutarate-dependent dioxygenases (2ODDs). Two of the enzymes that are important for the synthesis of the final steps of bioactive GAs in Arabidopsis are 2ODDs called GA 3-oxidase1 (GA3ox1) and GA3ox2 (Yamaguchi, 2008). Deactivation of GA by a class of GA2ox enzymes is important for regulating the available bioactive GA (Yamaguchi, 2008).

GA is perceived by soluble receptors called GIBBERELLIN INSENSITIVE DWARF1 (GID1). Arabidopsis has three GID1 receptors (GID1A, GID1B, and GID1C) which have both overlapping and unique functions in plant growth and development (Griffiths et al., 2006; Iuchi et al., 2007). When GID1 binds GA, it is thought to undergo a conformational change that allows it to interact with the DELLA family of transcriptional repressors that are negative regulators of GA responses (Griffiths et al., 2006; Nakajima et al., 2006; Ueguchi-Tanaka et al., 2007). This GA-GID1-DELLA complex results in the recognition of DELLA by the SCF F-box protein SLEEPY1 or SNEEZY which targets it for degradation by the 26S proteasome (Dill et al., 2004; Griffiths et al., 2006; Ariizumi et al., 2011). There is also evidence for non-proteolytic DELLA regulation in which the GA-GID1-DELLA complex is thought to inhibit DELLA induced gene expression by preventing interaction with its target proteins (Ariizumi et al., 2013). Arabidopsis has a family of five DELLA proteins called REPRESSOR OF *ga1-3* (RGA), RGA-LIKE1 (RGL1), RGL2, RGL3, and GA-INSENSITIVE (GAI) (Wen and Chang, 2002). RGL2 is the predominant DELLA responsible for inhibiting seed germination although RGA, RGL1 and GAI are thought to enhance RGL2 function (Lee et al., 2002; Tyler et al., 2004; Cao et al., 2005). In the absence of GA, the DELLA proteins are thought to interact with bHLH transcription factors

and prevent them from activating transcription of their target genes (Gao et al., 2011), but they are also known to promote transcription of some genes by competing for interaction with their transcriptional repressors (Hou et al., 2010).

Although GA signaling appears simple, it is complicated by crosstalk with other hormone signaling pathways and environmental stimuli. Most notably is its extensive crosstalk with ABA biosynthesis and signaling. GA and ABA levels appear to be regulated such that when either hormone level decreases the other increases. This is seen in the GA deficient mutant *ga1* which has an increased expression of the ABA biosynthesis genes ABA1 and NCED6 and decreased expression of the ABA catabolic gene CYP707A2 that presumably lead to its increased endogenous ABA level (Oh et al., 2007). Similarly, the ABA deficient mutant *aba2-2* has increased expression of the GA biosynthesis genes GA3ox1 and GA3ox2 and produces more GA_4 than wild-type after treatment with FR light (Seo et al., 2006). Although the mechanism is not known, the DELLA proteins appear to be key regulators of this crosstalk. For example, in seeds, ABA promotes *RGL2* mRNA accumulation and RGL2 increases ABA accumulation and promotes expression of *ABI5*, a key negative regulator of seed germination (Piskurewicz et al., 2008).

Light

Light is required for seed germination of certain species including Arabidopsis and is intricately connected to GA and ABA levels and signaling. Red light is known to promote seed germination while far-red light reversibly inhibits the effect of red light on germination (Borthwick et al., 1952). In Arabidopsis, red and far-red light are sensed by the phytochrome family of photoreceptors (PHYA – E) that covalently bind to a tetrapyrrole chromophore (Clack et al., 1994; Li et al., 2011). These phytochromes, like the ethylene receptors, resemble bacterial two-component sensor histidine kinases (Hwang et al., 2002). However, like the subfamily II ethylene receptors, they lack histidine kinase activity and are thought instead to have serine/threonine kinase activity (Yeh and Lagarias, 1998; Fankhauser et al., 1999). The phytochromes exist in two states: (1) inactive Pr and (2) active Pfr. In the dark, they are synthesized in the inactive Pr form and red light converts this inactive Pr form to the active Pfr form while far-red light converts the Pfr form back to the Pr form (Li et al., 2011). There are two

types of phytochromes: type I and type II. Type I (PHYA) are light labile and are abundant in dark-grown tissue, but not in light grown tissue because the Pfr form is unstable. Type II phytochromes (PHYB-E) are light stable and are constitutively present at low but equal levels in both dark and light grown tissue (Li et al., 2011).

The phytochromes are required for seed germination in light. Arabidopsis lacking all five of the phytochromes fail to germinate unless supplemented with exogenous GA (Strasser et al., 2010). PHYB and to a lesser extent PHYA promote germination in red light (Reed et al., 1994; Shinomura et al., 1994). PHYB is required for germination in the dark (Reed et al., 1994; Shinomura et al., 1994). In far-red light PHYA and PHYB have opposite roles; PHYA promotes while PHYB inhibits germination (Reed et al., 1994). One of the ways the phytochromes promote seed germination is by enhancing degradation of the negative regulator of seed germination PIL5 (PIF3 LIKE 5 also known as PIF1). PIL5 is a bHLH transcription factor that inhibits germination by reducing the sensitivity of seeds to GA and by promoting expression of ABA synthesis and GA catabolism genes while also inhibiting GA synthesis genes and ABA catabolism genes (Oh et al., 2004; Oh et al., 2006; Oh et al., 2007). Interestingly, the DELLA proteins are thought to interact with PIF3 to repress activation of its target genes, but it is not known whether RGL2 or any of the other DELLAs interact with PIL5 during seed germination.

Aims of the dissertation

As discussed above, all five of the Arabidopsis ethylene receptors function redundantly in ethylene signaling to regulate growth (Hua and Meyerowitz, 1998; Hall and Bleecker, 2003), but it is becoming increasingly evident that they also have unique functions, and in some cases, opposing functions to one another (Binder et al., 2004b; Seifert et al., 2004; Binder et al., 2006; Plett et al., 2009b; Liu et al., 2010; Kim et al., 2011; McDaniel and Binder, 2012). The aim of this dissertation is to examine the role of individual ethylene receptors in seed germination under two conditions known to inhibit seed germination: (1) salt stress, a condition known to involve ethylene signaling in Arabidopsis (Wang et al., 2007; Wang et al., 2008; Lin et al., 2012) and (2) far-red light which to this point has not been clearly implicated in ethylene signaling. Chapter two will present data and discuss the opposing roles of ETR1 and ETR2 in germination under

salt stress while chapter three will discuss the unique role of the ETR1 in germination following far-red treatment.

Chapter II
Unique Roles of the Ethylene Receptors in Seed Germination Under Salt Stress

The research presented in this chapter was published in Plant Physiology under the title "The Ethylene Receptors ETHYLENE RESPONSE1 and ETHYLENE RESPONSE2 Have Contrasting Roles in Seed Germination of Arabidopsis during Salt Stress" (Wilson et al., 2014).

Introduction

Abiotic stress is a major agricultural problem. Worldwide it is the predominant cause of crop failure, reducing average crop yields by more than 50% and leading to hundreds of millions of dollars in economic losses (Mahajan and Tuteja, 2005). Specifically, accumulation of salt in cultivated land is a major concern. It is estimated that 20% of arable land is affected by salt stress and that this could increase to 30% in the next 25 years (Mahajan and Tuteja, 2005). Many economically important crops, including rice and maize, are glycophytes (salt sensitive). High salt concentrations in the soil negatively affect plant growth and development through water stress and nutritional deficit (Pasternak, 1987). Even when water is readily available, high concentrations of salt in the soil cause drought-like conditions for the plant because of a reduction in the osmotic potential of the soil which hampers the ability of roots to absorb water (Pasternak, 1987). The salt ions can also be toxic to the plant by competing for uptake into cells with essential nutrients and causing nutritional deficiencies. For example, sodium competes with potassium, calcium and magnesium for cellular uptake and chloride reduces the uptake of nitrate (Pasternak, 1987; Mahajan and Tuteja, 2005). Ultimately, this leads to slower growth, reduced reproduction and possibly even death of the plant.

Not surprisingly, given the adverse effects of salt stress on plant growth and development, high salt concentrations inhibit seed germination. Salt is thought to inhibit germination due to ion toxicity and by preventing imbibition (Saleki et al., 1993). The phytohormone abscisic acid (ABA) which is known to promote seed dormancy is thought to play a role in delaying germination during salt stress. In support of this, Arabidopsis mutants deficient in ABA production and sensitivity germinate sooner than wild-type during NaCl stress (Koornneef et al.,

1982). On the hand, ethylene signaling is thought to promote germination during salt stress in Arabidopsis.

Ethylene is a gaseous plant hormone that regulates many important aspects of plant development, including germination, fruit ripening, leaf and flower senescence, and organ abscission, as well as abiotic and biotic stress tolerance (Abeles et al., 1992). In Arabidopsis, ethylene is perceived by a family of five receptors called ETR1, ERS1, ETR2, ERS2 and EIN4 (Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998). Genetic and biochemical studies suggest that in the absence of ethylene, the ethylene receptors promote activity of CTR1, a negative regulator of ethylene signaling, and that active CTR1 phosphorylates the C-terminus of EIN2 (Clark et al., 1998; Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). When phosphorylated by CTR1, nuclear localization of the EIN2 C-terminus is prevented and the downstream transcription factor EIN3 fails to accumulate (Ju et al., 2012; Wen et al., 2012). Degradation of EIN3 and EIL1 by the 26S proteasome represses ethylene responses (Guo and Ecker, 2003; Gagne et al., 2004; An et al., 2010). Upon ethylene binding, the ethylene receptor-CTR1 complex is inactivated leading to translocation and accumulation of the EIN2 C-terminus into the nucleus (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). This results in the accumulation of EIN3 and EIL1, which leads to most ethylene responses (Guo and Ecker, 2003; Gagne et al., 2004; An et al., 2010).

All five of the Arabidopsis ethylene receptors function redundantly in ethylene signaling to regulate growth (Hua and Meyerowitz, 1998; Hall and Bleecker, 2003), but they also have unique and/or opposing roles in various traits known to involve ethylene signaling (Shakeel et al., 2013). It is already known that in the presence of salt stress, the ethylene insensitive mutants *etr1-1*, *ein2*, and *ein3* germinate poorly compared to wild-type while the constitutive ethylene signaling mutant *ctr1* germinates better than wild-type (Wang et al., 2007; Wang et al., 2008; Lin et al., 2012). However it is not know known if all of the ethylene receptors function redundantly in this condition. This led us to examine the role of the individual ethylene receptors in seed germination under salt stress.

We found that ETR1 and EIN4 inhibit germination during salt stress while ETR2 promotes germination during salt stress. Surprisingly, although high concentrations of ethylene are able to

almost completely eliminate the difference in germination between wild-type and the receptor loss-of-function (LOF) mutants, abscisic acid (ABA) appears to be the major physiologically relevant hormone involved in the difference in germination between ETR1 and ETR2 LOF mutants.

Results

Opposing roles for ethylene receptors in seed germination under NaCl stress

In order to determine the individual contribution of each ethylene receptor to seed germination in the presence of salt stress, the germination time course of each receptor loss-of-function (LOF) mutant and their corresponding wild-type was observed in the presence and absence of 150 mM NaCl. In the absence of NaCl, all seed lines germinated to at least 90% and the germination time-course of each LOF mutant was similar to its respective wild-type (Figure II-1). Imbibition in the presence of 150 mM NaCl delayed the germination of both Columbia (Col) and Wassilewskija (WS) seeds and, consistent with previous results (Quesada et al., 2000), WS was only able to germinate to about 22% under these conditions in the time course of this experiment Figure II-1. Interestingly, in the presence of 150 mM NaCl, all three *etr1* LOF mutants started to germinate sooner than their respective wild-type while *etr2-3* started to germinate later than wild-type (Figure II-1 and Figure II-2). Additionally, all three *etr1* LOF mutants and *ein4-4* germinated faster than wild-type, while *etr2-3* germinated slower than wild-type (Figure II-1). Under these conditions, germination of the *ers1-3* and *ers2-3* LOF mutants in the presence of 150 mM NaCl was similar to wild-type (Figure II-1). These data suggest that ETR2 promotes while ETR1 and to a lesser extent EIN4 inhibit germination in the presence of NaCl stress.

In order to gain insight into the opposing effects ETR1 and EIN4 have compared to ETR2 on seed germination during salt stress, we conducted an epistasis analysis to examine the genetic interaction between these receptors. In the absence of NaCl, all of the single and double mutants (*etr1-6 etr2-3*, *etr1-7 ein4-4*, and *ein4-4 etr2-3*) and the triple mutant (*etr1-6 ein4-4 etr2-3*) germinated to at least 90% with similar time-courses (data not shown). In the presence of 150 mM NaCl, *etr1-6 etr2-3*, and *ein4-4 etr2-3* had germination time-courses that were faster than the *etr2-3* single mutant, but slower than *etr1-6* or *ein4-4* respectively (Figure II-3). Similarly, it

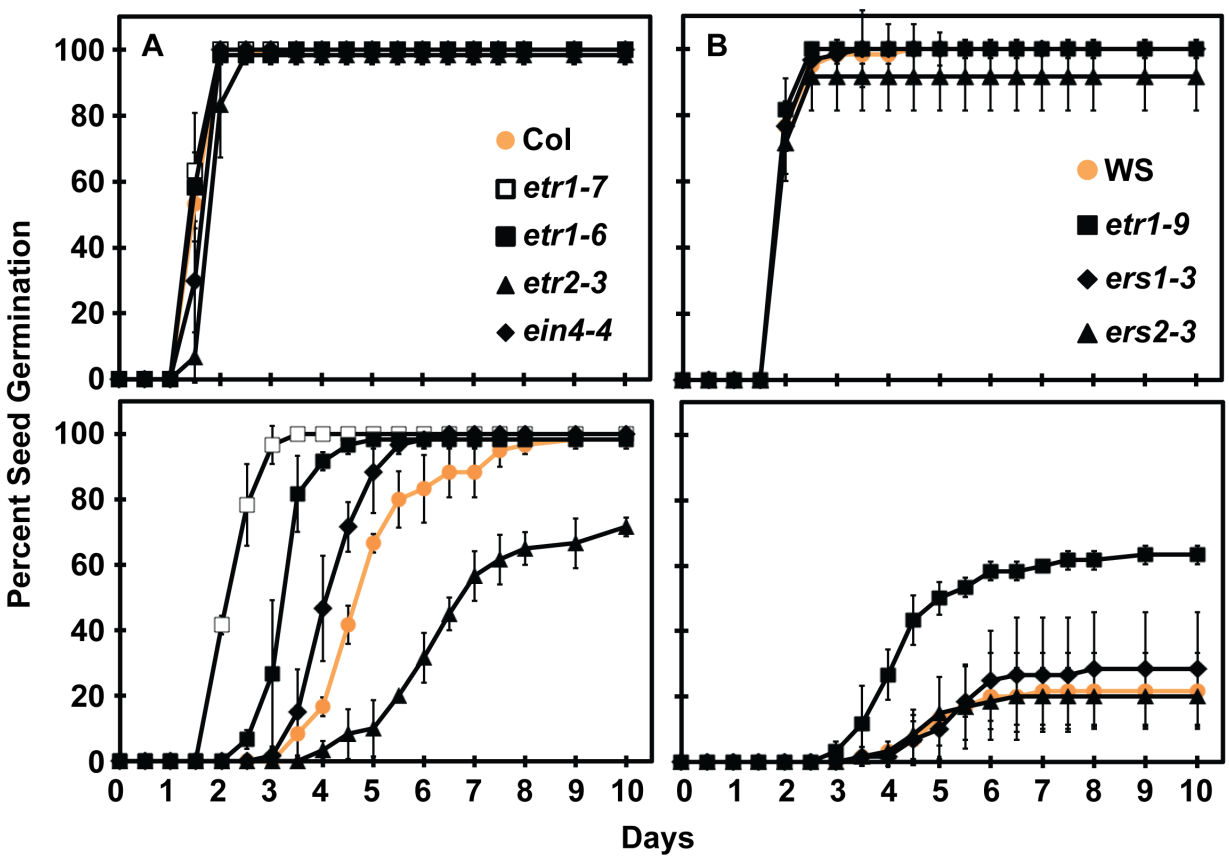


Figure II-1. Germination time course of ethylene receptor LOF mutants under NaCl stress. Germination time course of (A) *etr1-7*, *etr1-6*, *etr2-3*, and *ein4-4* compared to the Col wild-type and (B) *etr1-9*, *ers1-3*, and *ers2-3* compared to the WS wild-type in the absence (top) and presence (bottom) of 150 mM NaCl. The percent of germinated seeds was determined every 12-24 h. Each experiment was done in triplicate and the average percent seed germination ± SD at each time point is plotted for each seed line.

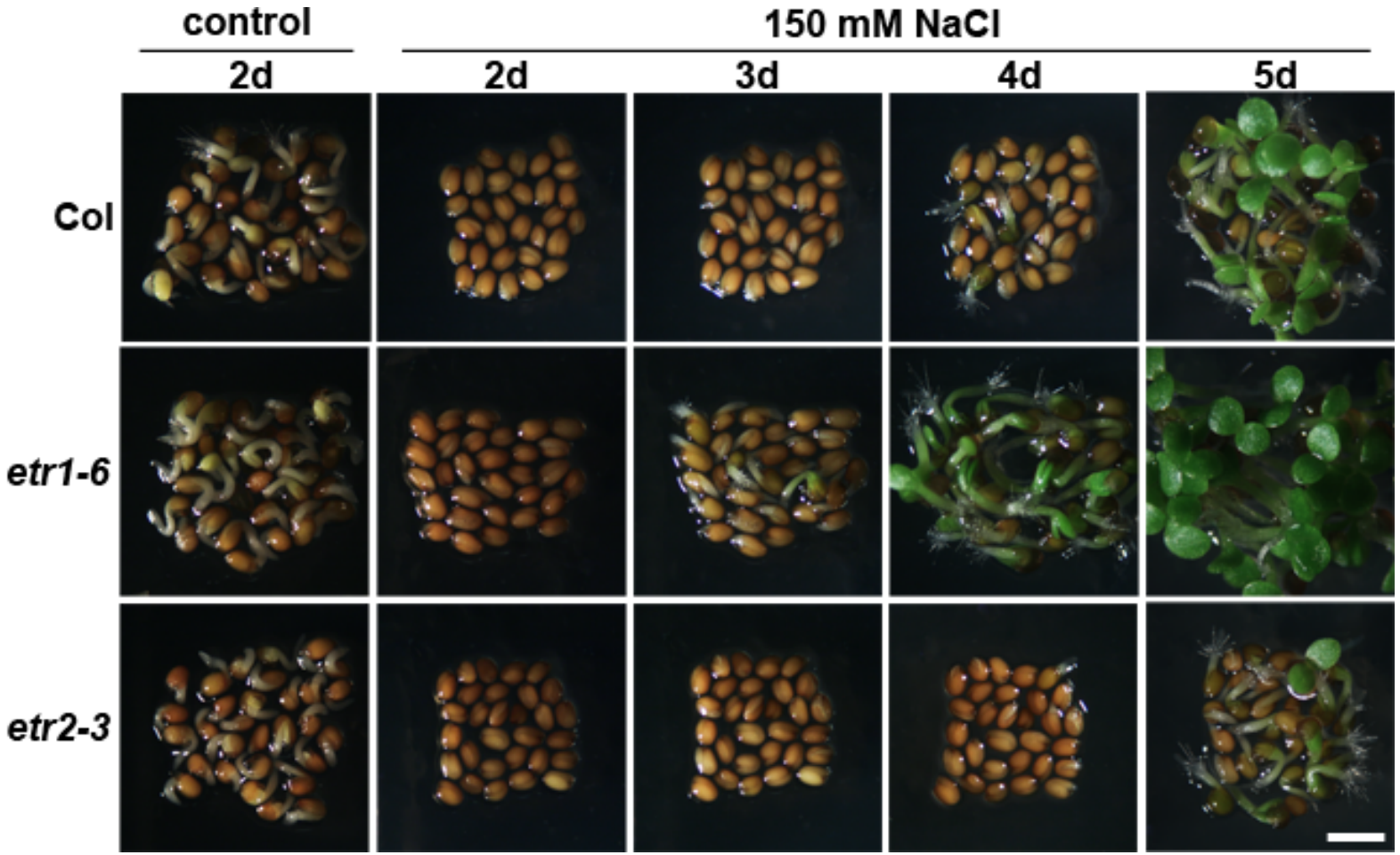


Figure II-2. Germinating Seeds

Col, *etr1-6* and *etr2-3* seeds were germinated on agar plates containing 150 mM NaCl for the indicated times. Germination at two days in the absence of NaCl is shown as a control. The scale bar is 1 mm.

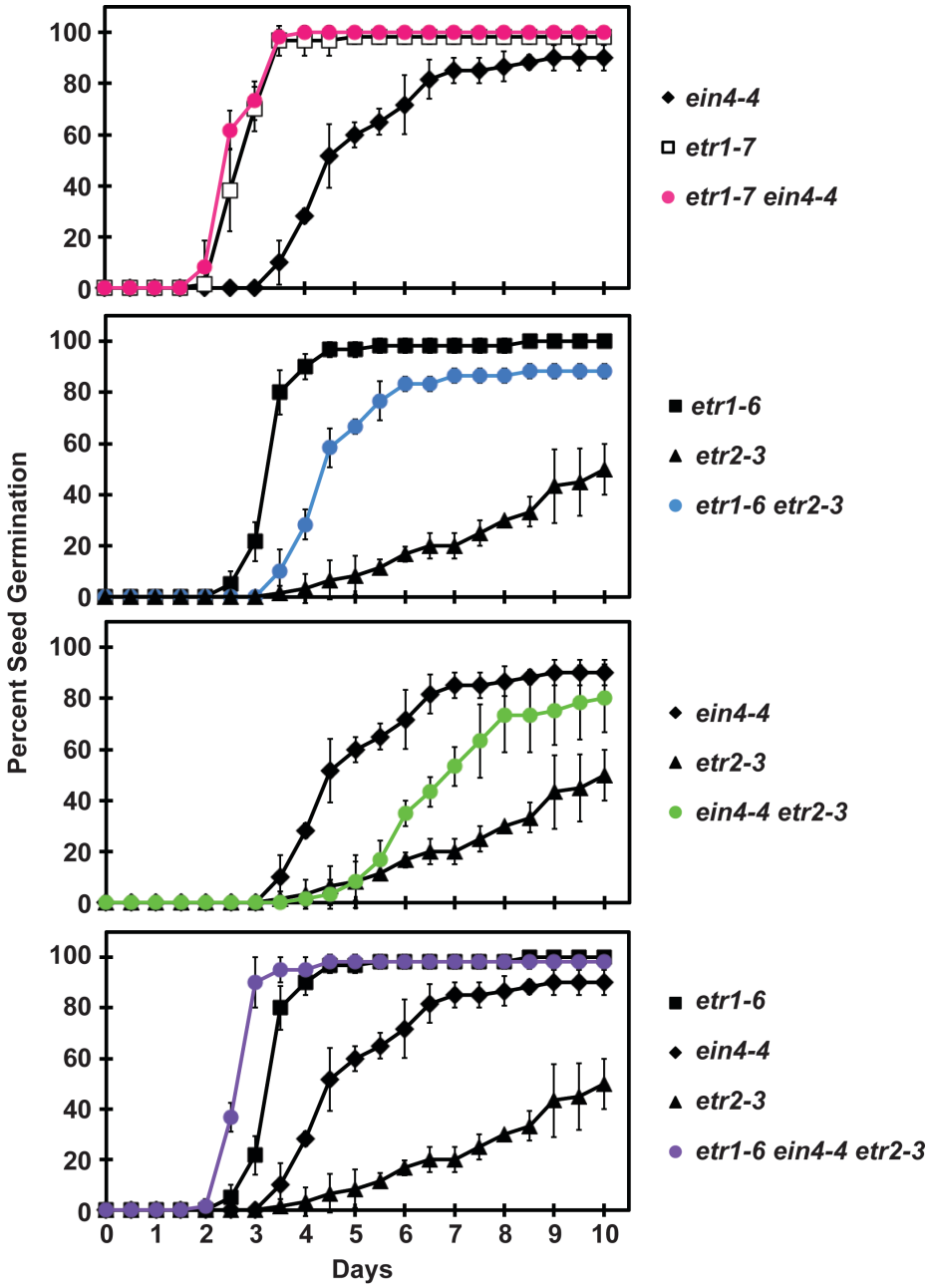


Figure II-3. Germination time course of ethylene receptor double mutants under salt stress.

Germination time course of *etr1-7 ein4-4*, *etr1-6 etr2-3*, and *ein4-4 etr2-3* double mutants and the *etr1-6 ein4-4 etr2-3* triple mutant compared to their respective single mutants in the presence of 150 mM NaCl. The percent of germinated seeds was determined every 12-24 h. Each experiment was done in triplicate and the average percent seed germination ± SD at each time point is plotted for each seed line.

took less time for 50% of the *etr1-6 etr2-3* and *ein4-4 etr2-3* double mutants to germinate than for *etr2-3* to germinate, but it took longer for the double mutants to germinate than it took *etr1-6* and *ein4-4* respectively (Figure II-4). Although similar, the *etr1-6 etr2-3* double mutant reached 50% germination faster than the *ein4-4 etr2-3* double mutant at 4.4 and 6.8 days respectively. This is consistent with a larger role for ETR1 compared to EIN4 as suggested above. The *etr1-7 ein4-4* double mutant had a germination time course similar to *etr1-7*, but faster than *ein4-4* (Figure II-3). Likewise, the *etr1-7 ein4-4* double mutant germinated statistically faster than *ein4-4*, but not statistically faster than *etr1-7* (Figure II-4).

Triple mutants were also examined. The triple *etr1-6 ein4-4 etr2-3* mutant, germinated faster and reached 50% germination faster than all three single mutants (Figure II-3 and Figure II-4). Germination on NaCl was affected the most by loss of either ETR1 or ETR2; therefore, we focused on investigating the contrasting role of these two receptors in this phenotype.

Effect of ionic and osmotic stress on germination of *etr1-6* and *etr2-3*

In order to investigate whether the difference in germination observed between the *etr1* and *etr2* LOF mutants in the presence of NaCl is due to ion toxicity, osmotic stress or both, germination time-courses were examined in the presence of increasing concentrations of NaCl and their osmotic equivalents of mannitol. All of the seeds germinated slower on NaCl or mannitol than in control conditions (Figure II-5 and Figure II-6). As observed above, *etr1-6* germinated sooner and statistically faster than wild-type; whereas, *etr2-3* germinated later and statistically slower than wild-type at all concentrations NaCl tested (Figure II-5 and Figure II-6). The *etr2-3* mutant showed extreme sensitivity to NaCl, taking more than twice as long to reach 50% germination as *etr1-6* at all concentrations of NaCl tested and reaching only about 10% germination at 200 mM NaCl (Figure II-5 and Figure II-6). Mannitol had similar, but less profound effects on the germination of *etr1* and *etr2-3*. These data suggest that ion toxicity plays a bigger role than osmotic stress in the difference in germination observed between the *etr1* and *etr2* mutants.

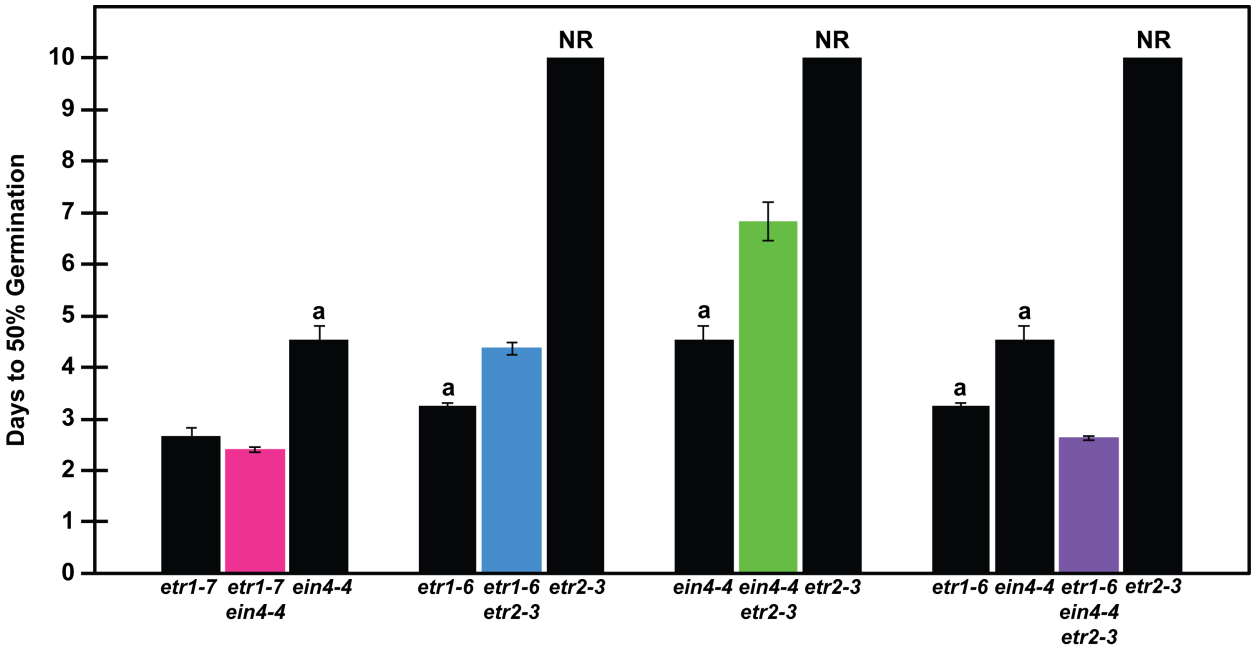


Figure II-4. Time for 50% of the ethylene receptor double and triple mutant seeds to germinate under salt stress.

The time for *etr1-7 ein4-4*, *etr1-6 etr2-3*, *ein4-4 etr2-3*, and *etr1-6 ein4-4 etr2-3* and their respective single mutants to reach 50% germination in the presence of 150 mM NaCl was calculated from the data in figure II-3. The average ± SD for each line is plotted. NR denotes that 50% germination was not reached by all samples during the ten day time period of the experiment (two of the three samples reached 50% in an average of 9.4 days, but the third sample only reached 40% by day ten). [a]Denotes a statistical difference between the single mutant and its respective double mutant where $p < 0.05$ with a t-test.

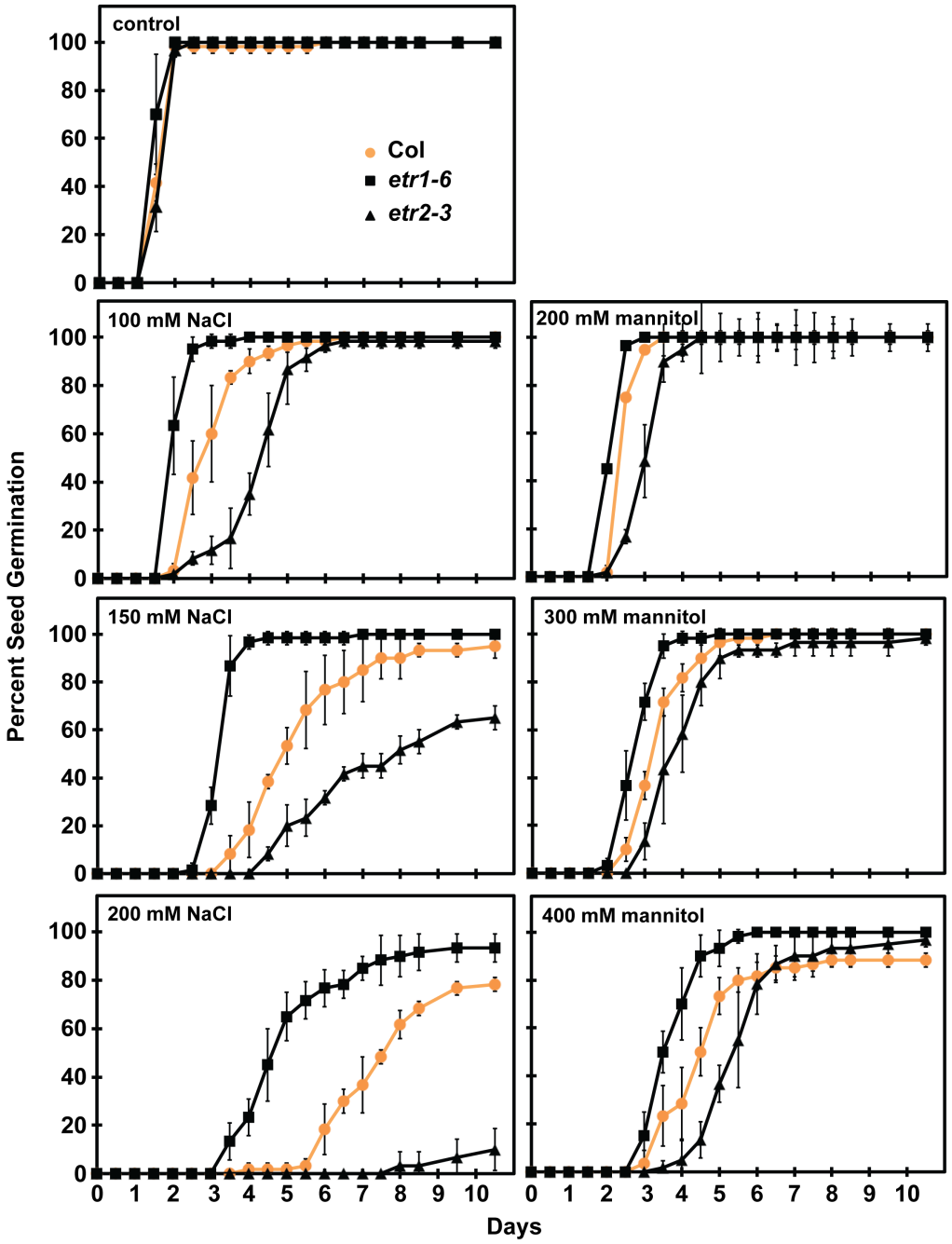


Figure II-5. Germination time course of *etr1* and *etr2* LOF mutants under salt and mannitol stress.

Germination time course of *etr1-6* and *etr2-3* compared to the Col wild-type in the presence of increasing concentrations of NaCl and their osmotic equivalent of mannitol. The percent of germinated seeds was determined every 12-24 h. Each experiment was done in triplicate and the average percent seed germination ± SD at each time point is plotted for each seed line.

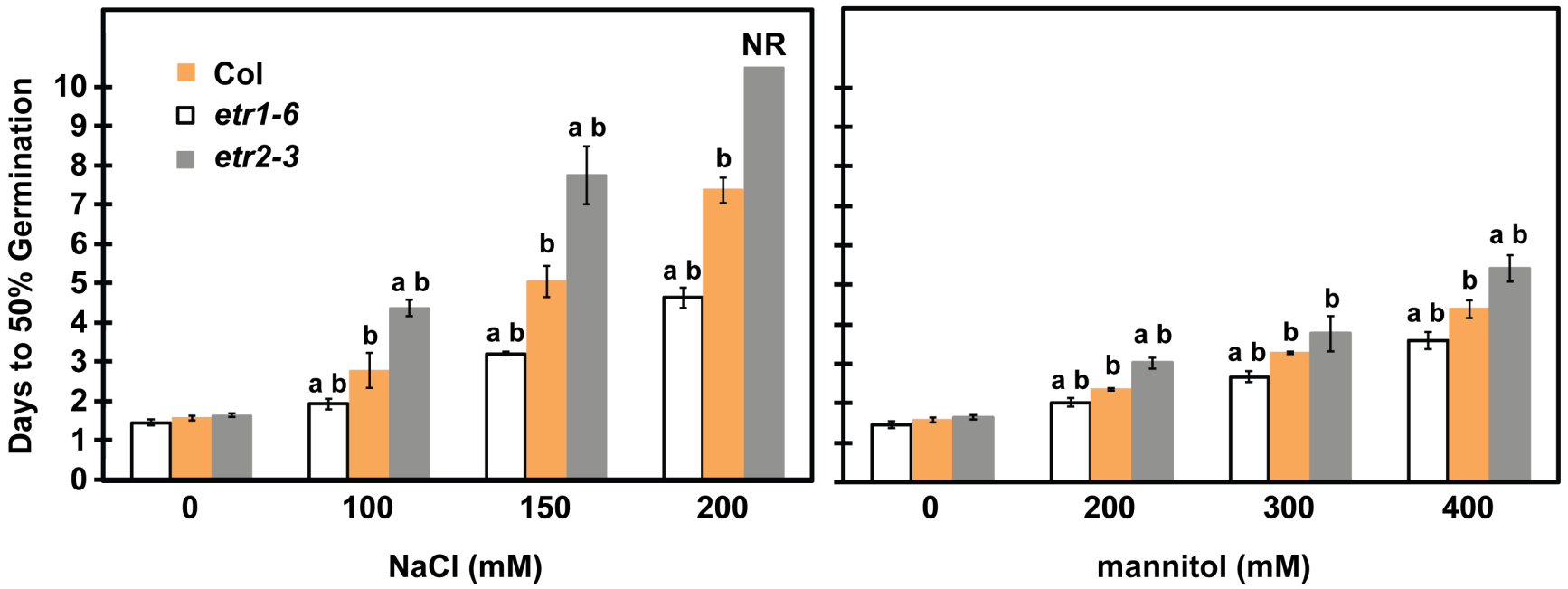


Figure II-6. Time for 50% of *etr1-6*, *etr2-3* and Col to germinate under increasing concentrations of NaCl and mannitol.

The time for *etr1-6*, *etr2-3* and the Col control to reach 50% germination in the presence of increasing concentrations of NaCl and their osmotic equivalent of mannitol was calculated from the data in Figure II-5. The average ± SD for each line is plotted. NR denotes that 50% germination was not reached during the 10.5 day time period of the experiment. [a]Denotes a statistical difference compared to Col under that treatment and [b]denotes a statistical difference compared to the untreated control of that seed line where $p < 0.05$ with a t-test.

Effect of ethylene on germination of *etr1-6* and *etr2-3*

Exogenous ethylene is known to stimulate germination of Arabidopsis seeds (Bleecker et al., 1988); therefore it is possible that differences in ethylene production or sensitivity are responsible for the difference in germination observed between *etr1* and *etr2* LOF mutants. Specifically, if ethylene is involved, the *etr1* LOF mutants should either produce more ethylene or be more sensitive to ethylene and the *etr2* LOF mutants should either produce less ethylene or be less sensitive to ethylene compared to wild-type. Consistent with this hypothesis, loss of ETR1 is known to lead to slightly increased sensitivity and responsiveness to ethylene in seedling growth (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002).

In order to investigate whether the difference in germination between *etr1-6* and *etr2-3* during salt stress is a consequence of increased ethylene production by *etr1-6* and a decreased ethylene production by *etr2-3*, we measured the ethylene produced by these two mutants during germination in the presence and absence of 150 mM NaCl. During germination in the absence of NaCl, *etr1-6* and *etr2-3* produced similar amounts of ethylene at a similar time-course as wild-type (Figure II-7). On the other hand, during germination on 150 mM NaCl, *etr1-6* produced more ethylene sooner than wild-type, while *etr2-3* produced less ethylene than wild-type (Figure II-7). Although the increase in ethylene produced by *etr1-6* and the reduced ethylene production by *etr2-3* correlates with their germination phenotypes, ethylene was not detected prior to radicle emergence. This link between radicle emergence and ethylene production and the question as to whether ethylene is produced coincident with or prior to radicle emergence has been noted by others (Meheriuk and Spencer, 1964; Spencer and Olson, 1965; Ketring and Morgan, 1969; Woeste et al., 1999). In order to address whether ethylene is being trapped in the seeds prior to germination and released upon radicle emergence, we allowed the seeds to germinate for 24 hours in a sealed vial and then either heated the seeds to drive ethylene from the seed interior (Ramonell et al., 2002) or crushed the seeds to release any trapped gas. No ethylene was detected following either of these treatments suggesting that either ethylene is not produced prior to seed germination or that it is produced at a level below the detection limit of our equipment (data not shown). While we can not rule out the possibility that ethylene is produced prior to germination, the difference in ethylene production following germination is very small, representing an accumulation of less than 0.01 ppm ethylene in the headspace of the vial.

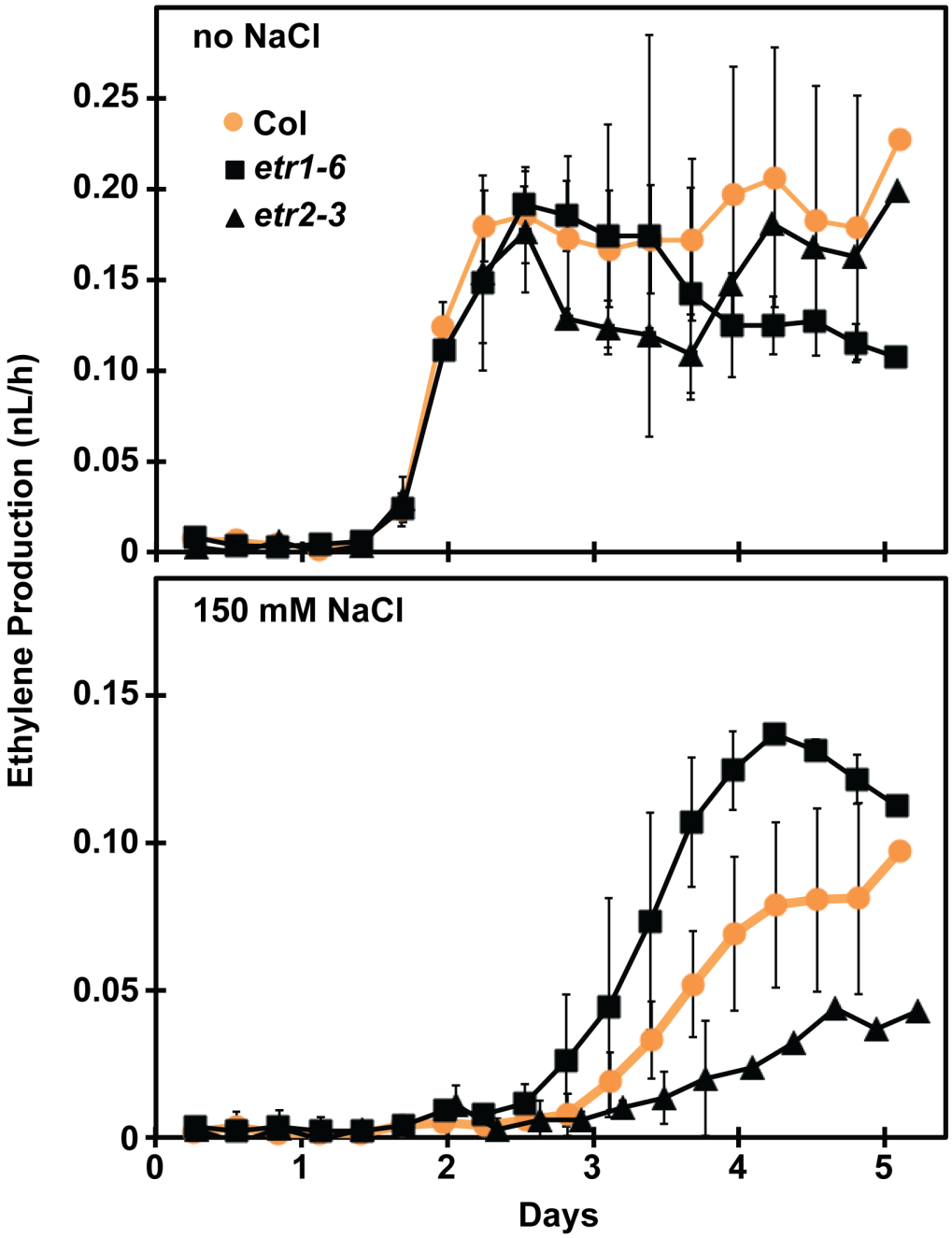


Figure II-7. Ethylene production by *etr1-6*, *etr2-3* and Col during seed germination.

The concentration of ethylene produced by *etr1-6*, *etr2-3* and the Col control during germination in the absence (top) and presence (bottom) of 150 mM NaCl was measured every 6h. The average of at least two experiments is plotted. Error bars represent the SD from at least three experiments.

In order to determine what, if any, concentration of ethylene is able to eliminate the difference in germination observed between *etr1* and *etr2* during salt stress and whether this concentration could account for the germination differences, we treated seeds germinating on 150 mM NaCl with 0.01, 0.1 and 1 ppm ethylene. When treated with 0.01 ppm ethylene, the time-course of *etr2-3* germination was similar to wild-type (Figure II-8). However, at this concentration of the ethylene, the time to reach 50% germination was not statistically reduced in any of the seed lines (Figure II-9). When treated with 0.1 or 1 ppm ethylene, the time-course of *etr1-6* germination was slightly accelerated while the time courses of wild-type and *etr2-3* were clearly accelerated, almost completely eliminating any difference in germination between the three seed lines at the highest concentration tested (Figure II-8). Both of these concentrations of ethylene also significantly decreased the time to reach 50% germination compared to the untreated control of that seed line (Figure II-9). However, the concentration of ethylene needed to have these effects is much higher than the concentration of ethylene we measured during germination of these seed lines. Additionally, despite the improved germination observed in all of the seed lines, it is clear that the *etr2-3* germination improved the most in response to the ethylene treatment, with a reduction in the time to reach 50% germination of about 3.1 days versus only a half a day improvement in *etr1-6* (Figure II-9). Although we can not rule out the possibility that *etr1-6* contains a higher, saturating subcellular concentration of ethylene than *etr2-3*, these data suggest that *etr2-3* is more sensitive to ethylene than *etr1-6*. This is opposite to what would be predicted if ethylene sensitivity were causing the differences in germination observed between *etr1-6* and *etr2-3*.

Although 0.01 ppm ethylene (the concentration closest to that produced by germinating seeds) failed to eliminate the difference in germination between *etr2-3* and *etr1-6*, as mentioned above, we can not rule out the possibility that *etr1-6* contains a higher subcellular concentration of ethylene than either *etr2-3* or wild-type. In order to further explore this possibility, we treated germinating seeds in the presence and absence of 150 mM NaCl with 5 μM aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis. This concentration of AVG, which reduced the ethylene production of seedlings by about 50% (data not shown), had no statistical effect on the time to 50% germination of either wild-type or *etr1-6* in the presence

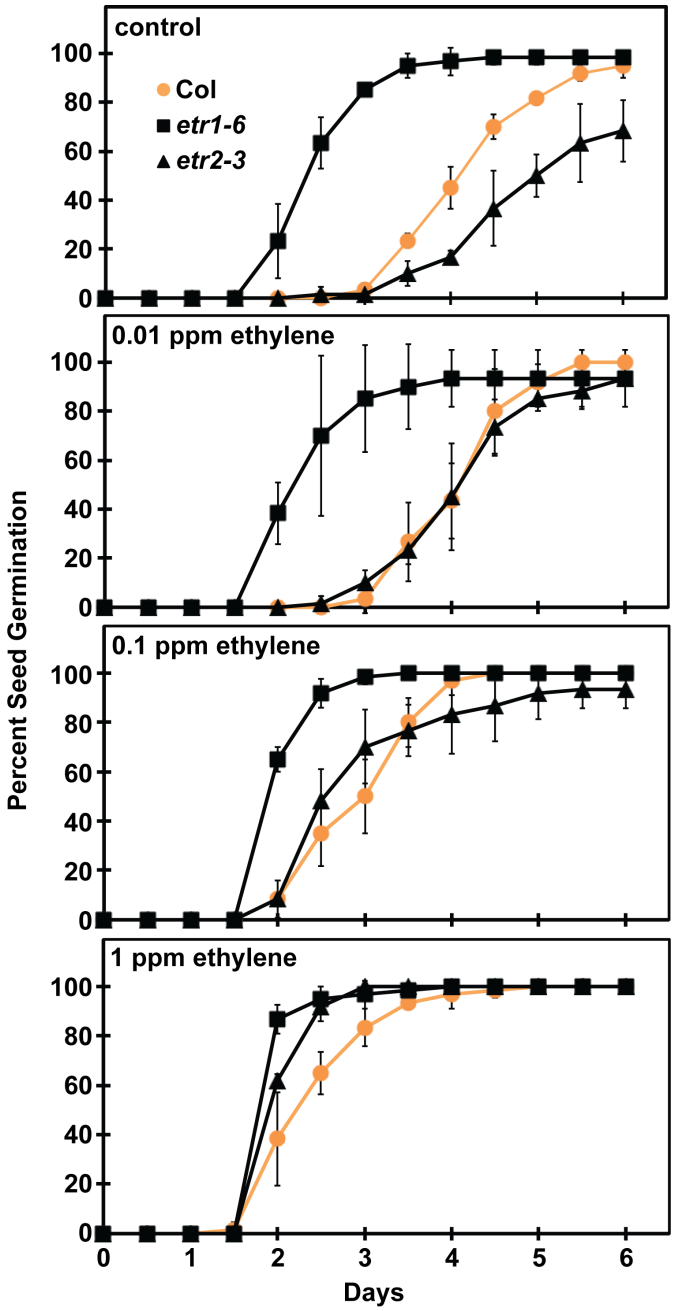


Figure II-8. The effect of ethylene on germination time course of *etr1-6*, *etr2-3* and Col under salt stress.

Germination time course of *etr1-6* and *etr2-3* compared to the Col wild-type in the presence of 150 mM NaCl and increasing concentrations of ethylene. The percent of germinated seeds was determined every 12 h. Each experiment was done in triplicate and the average percent seed germination ± SD at each time point is plotted for each seed line.

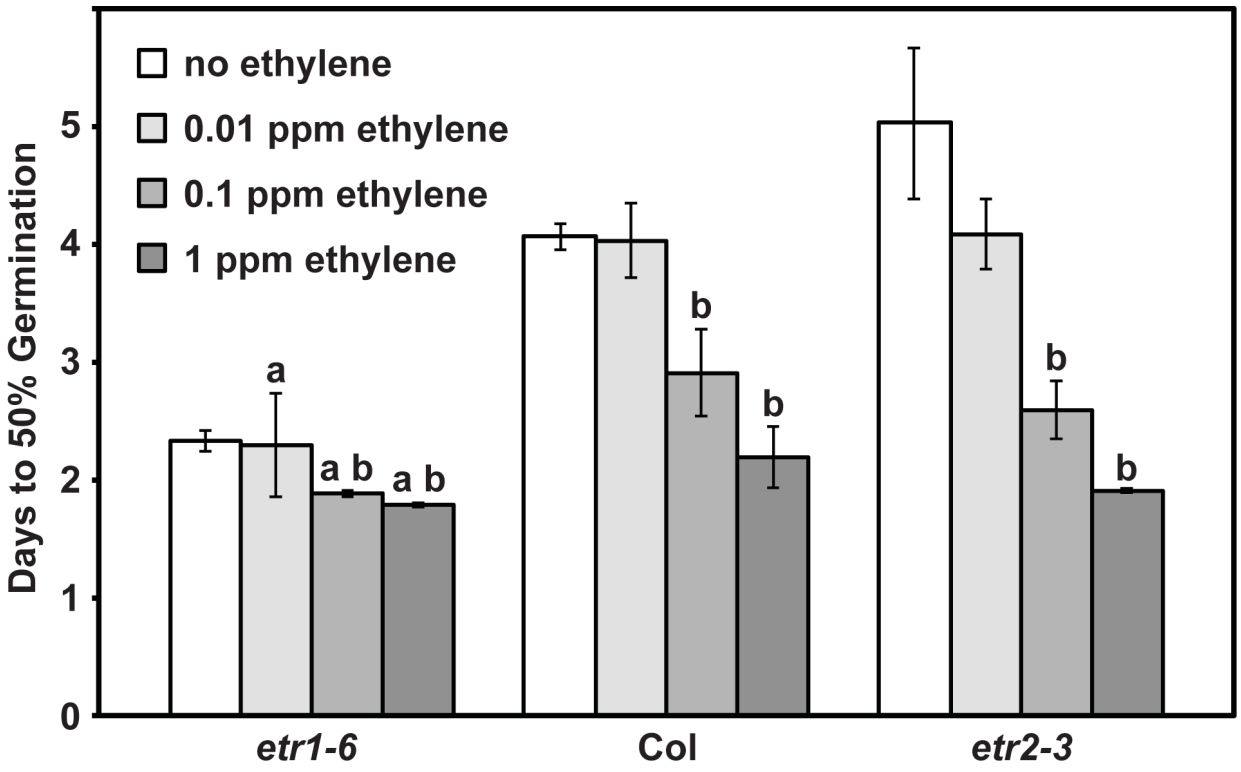


Figure II-9. Time for 50% of *etr1-6*, *etr2-3* and Col to germinate under salt stress in the presence of increasing concentrations of ethylene.

The time for *etr1-6*, *etr2-3* and the Col control to reach 50% germination in the presence of 150 mM NaCl and increasing concentrations of ethylene was calculated from the data in Figure II-8. The average ± SD for each line is plotted. [a]Denotes a statistical difference compared to Col under that treatment and [b]denotes a statistical difference compared to the untreated control of that seed line where $p < 0.05$ with a t-test.

or absence of 150 mM NaCl (Figure II-10). These data suggest that the faster germination time of *etr1-6* mutants is not caused by increased ethylene production in this mutant.

Analysis of *etr1-6* and *etr2-3* for differences in ethylene production or sensitivity that could account for their opposite germination phenotypes suggest that differences in ethylene production or sensitivity are not the major factor, therefore we analyzed the role of other hormones known to be involved in germination in this phenotype.

Effect of GA on germination of *etr1-6* and *etr2-3*

The plant hormone gibberellic acid (GA) is known to promote seed germination (Bentsink and Koornneef, 2008). In order to test whether difference in GA sensitivity or synthesis are responsible for the difference in germination between *etr1* and *etr2* LOF mutants, we treated seeds germinating in the presence of 150 mM NaCl with 1 and 10 μM GA. These concentrations of GA had little effect on the germination time-course of *etr1-6* or wild-type and only slightly improved the germination of *etr2-3* (Figure II-11). The highest concentration of GA did statistically improve the time to 50% germination of both wild-type and *etr2-3*, but despite this improvement, GA failed to eliminate the difference in germination observed between the three lines (Figure II-12). These data suggest that differences in GA sensitivity are not likely to be responsible for the difference in germination observed between the *etr1-6* and *etr2-3* mutants and that GA may already be being produced at a saturating level in these seeds.

To determine the role of GA biosynthesis, we examined the effect of the GA biosynthesis inhibitor paclobutrazol (PAC) on germination of the mutants. As observed with NaCl, *etr1-6* was less sensitive to PAC than wild-type or *etr2-3*. However, PAC had a very different effect on germination than NaCl. Although PAC did slightly delay the onset of germination, its prominent effect was to reduce the percent of seeds that germinated (Figure II-13). This is in contrast to NaCl, in which the primary effect was to delay the onset of germination and only at higher concentrations did this lead to a reduction in the percent of germinated seeds (Figure II-5). Addition of GA to PAC treated seeds, restored the percent of germinated seeds back to at least 95%, but was less effective at restoring the difference in the germination time-course, which suggests that PAC may be having a GA-independent effect on germination. Overall, these data

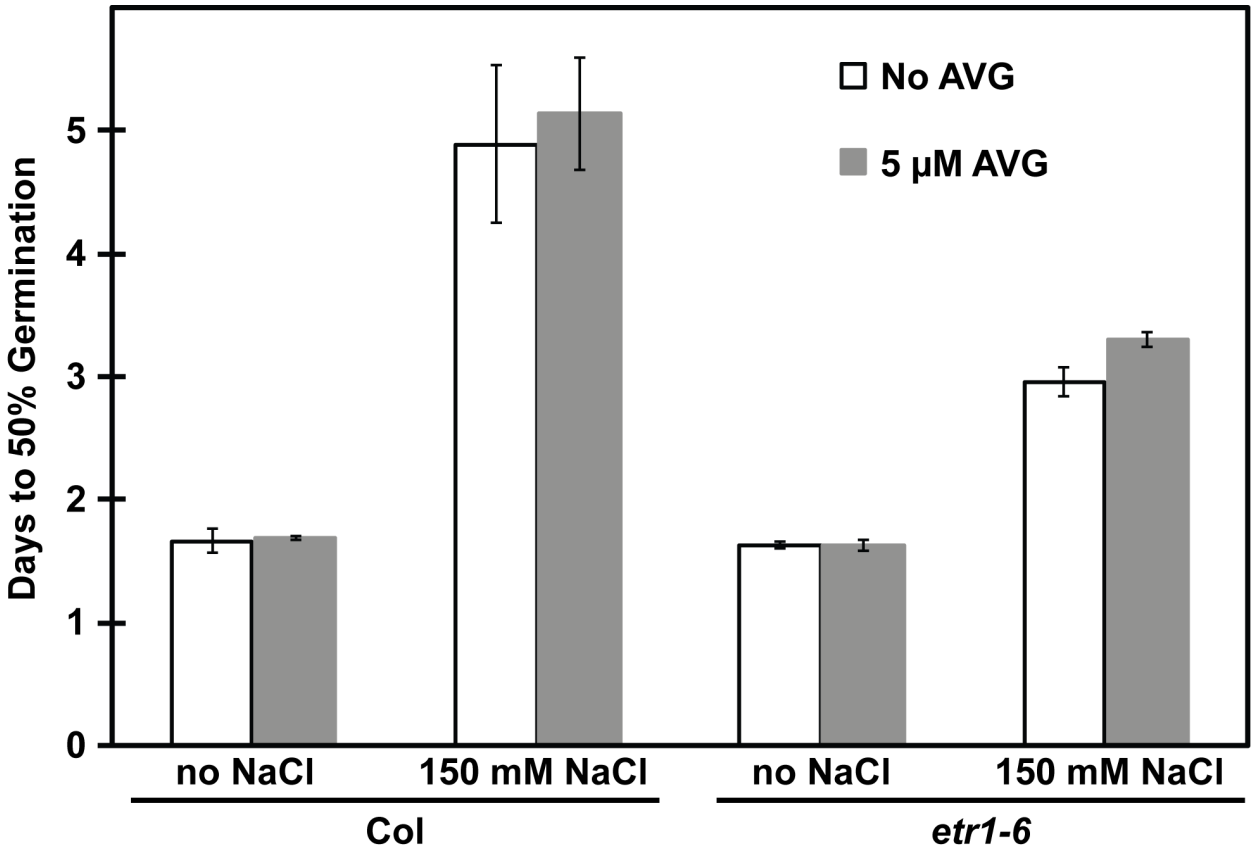


Figure II-10. Time for 50% of *etr1-6* and Col to germinate in the presence of the ethylene biosynthesis inhibitor AVG.

The time for *etr1-6* and the Col control to reach 50% germination in the absence or presence of 150 mM NaCl with and without the ethylene biosynthesis inhibitor AVG was determined. Each experiment was done in triplicate and the average ± SD for each line is plotted.

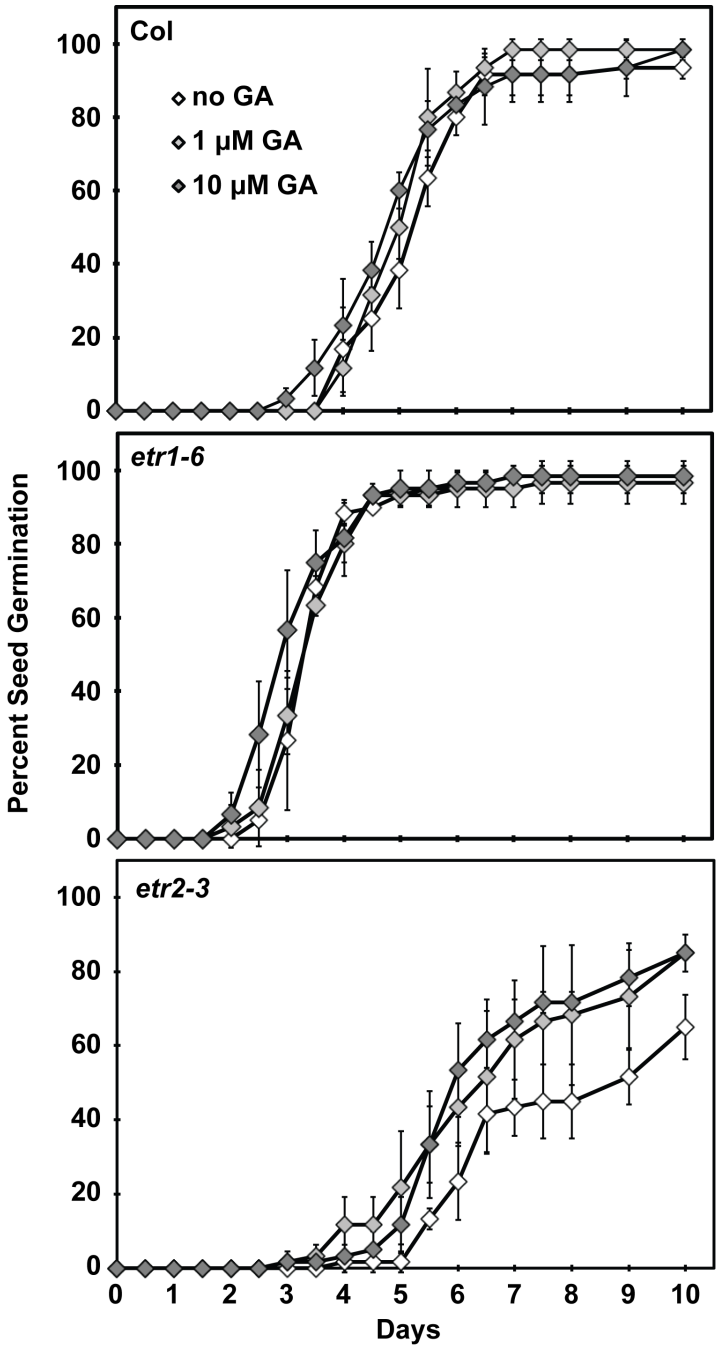


Figure II-11. The effect of GA on the germination time course of *etr1-6*, *etr2-3* and Col under salt stress.

Germination time course of *etr1-6* and *etr2-3* compared to the Col wild-type in the presence of 150 mM NaCl with the indicated concentrations of GA. The percent of germinated seeds was determined every 12-24 h. Each experiment was done in triplicate and the average percent seed germination ± SD at each time point is plotted for each seed line.

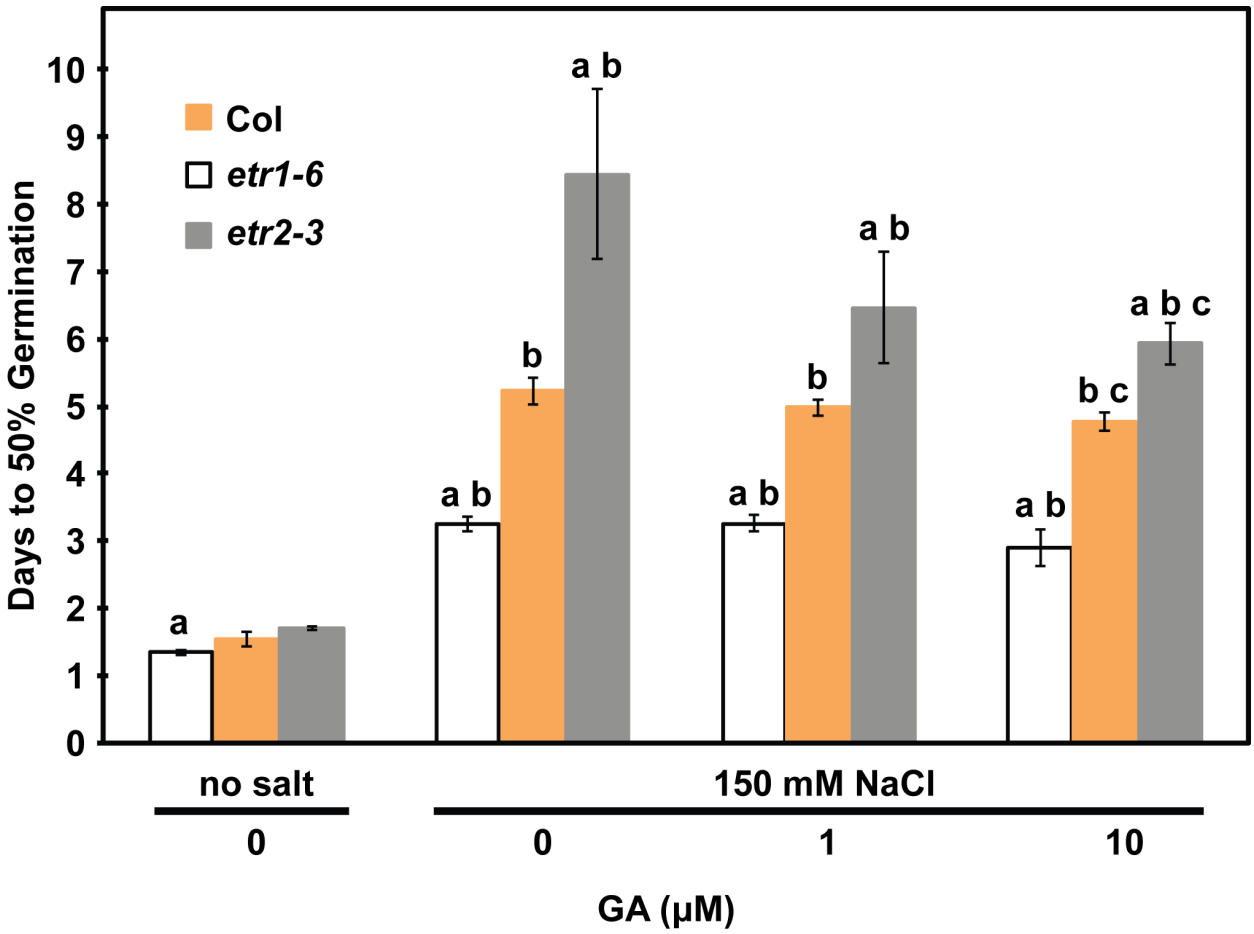


Figure II-12. Time for 50% of *etr1-6*, *etr2-3* and Col to germinate in the presence of GA.

The time for *etr1-6*, *etr2-3* and the Col control to reach 50% germination in the presence of the indicated concentrations of GA was calculated from the data in Figure II-11. The average ± SD for each line is plotted. [a]Denotes a statistical difference compared to Col under that treatment, [b]denotes a statistical difference compared to the no salt control of that seed line and [c]denotes a statistical difference compared to the salt without GA control of that seed line where $p < 0.05$ with a t-test.

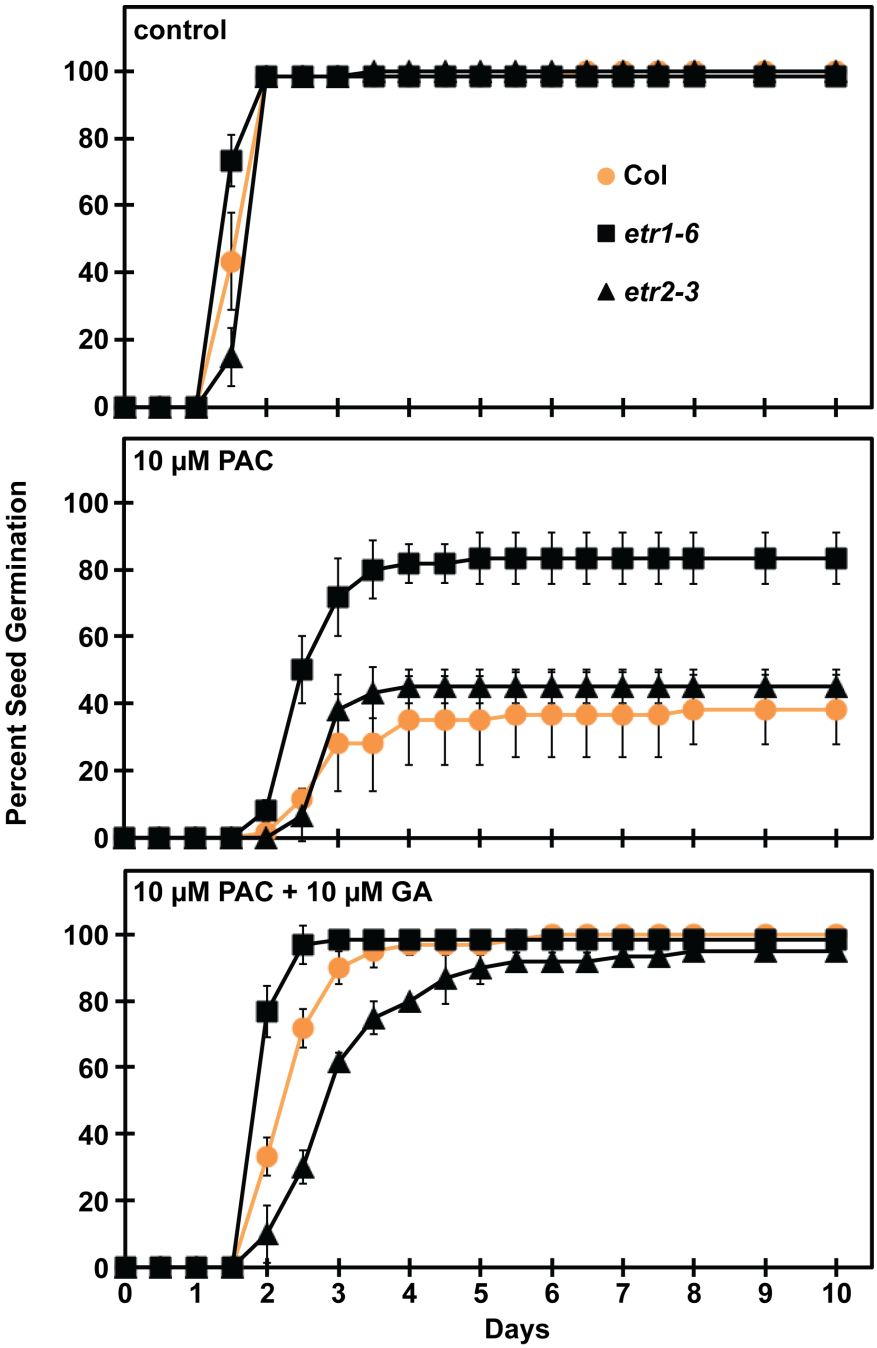


Figure II-13. The effect of PAC on the germination time course of *etr1-6, etr2-3* and Col.

Germination time course of *etr1-6* and *etr2-3* compared to the Col wild-type in the presence of 10 µM PAC or 10 µM PAC with 10 µM GA. The percent of germinated seeds was determined every 12-24 h. Each experiment was done in triplicate and the average percent seed germination ± SD at each time point is plotted for each seed line.

suggest that difference in GA biosynthesis or sensitivity are not likely the cause of the difference in germination between the *etr1* and *etr2* LOF mutants.

Effect of cytokinin on germination of *etr1-6* and *etr2-3*

Like GA, cytokinin also promotes seed germination in Arabidopsis (Wang et al., 2011). In order to determine whether differences in cytokinin synthesis or sensitivity are responsible for the difference in germination between etr1 and etr2 LOF mutants, we treated seeds germinating in the presence of 150 mM NaCl with 0.1 or 1 μM of the synthetic cytokinin, 6-benzylaminopurine (BAP). Treatment with BAP had little effect on the germination of etr1-6 or wild-type, but 1 μM BAP improved the germination time-course and time to 50% germination of etr2-3 seeds (Figure II-14 and Figure II-15). Despite this improvement, BAP was not able to eliminate the difference in germination between *etr1-6* and *etr2-3*. Furthermore, treatment with lovastatin, an inhibitor of cytokinin synthesis had no obvious effect on the germination of wild-type or either mutant (data not shown). These data suggest that difference in cytokinin sensitivity or synthesis are not likely the cause of the altered germination of *etr1-6* and *etr2-3* during salt stress.

Effect of ABA on germination of *etr1-6* and *etr2-3*

The hormone ABA is known to promote seed dormancy and is thought to delay germination during salt stress (Koornneef et al., 1982). In order to determine whether differences in ABA synthesis or sensitivity are responsible for the difference in germination between *etr1* and *etr2* LOF mutants, we germinated seeds on increasing concentrations of ABA. ABA phenocopied the effect of NaCl on germination and delayed the germination of wild-type seeds (Figure II-16). Compared to wild-type, *etr1-6* was less sensitive and *etr2-3* was more sensitive to ABA (Figure II-16 and Figure II-17). These data suggest that the difference in germination of *etr1-6* and *etr2-3* mutants during NaCl stress might be due to differences in ABA production or sensitivity. To further examine this possibility, we examined the effect of increasing concentrations of the ABA biosynthesis inhibitor norflurazon on germination during NaCl stress. Treatment with 1 μM norflurazon improved the germination time-course and time to 50% germination of *etr2*-3 and to a lesser extent wild-type (Figure II-18 and Figure II-19). Higher concentrations of norflurazon improved the germination of all three seeds lines and almost completely eliminated the

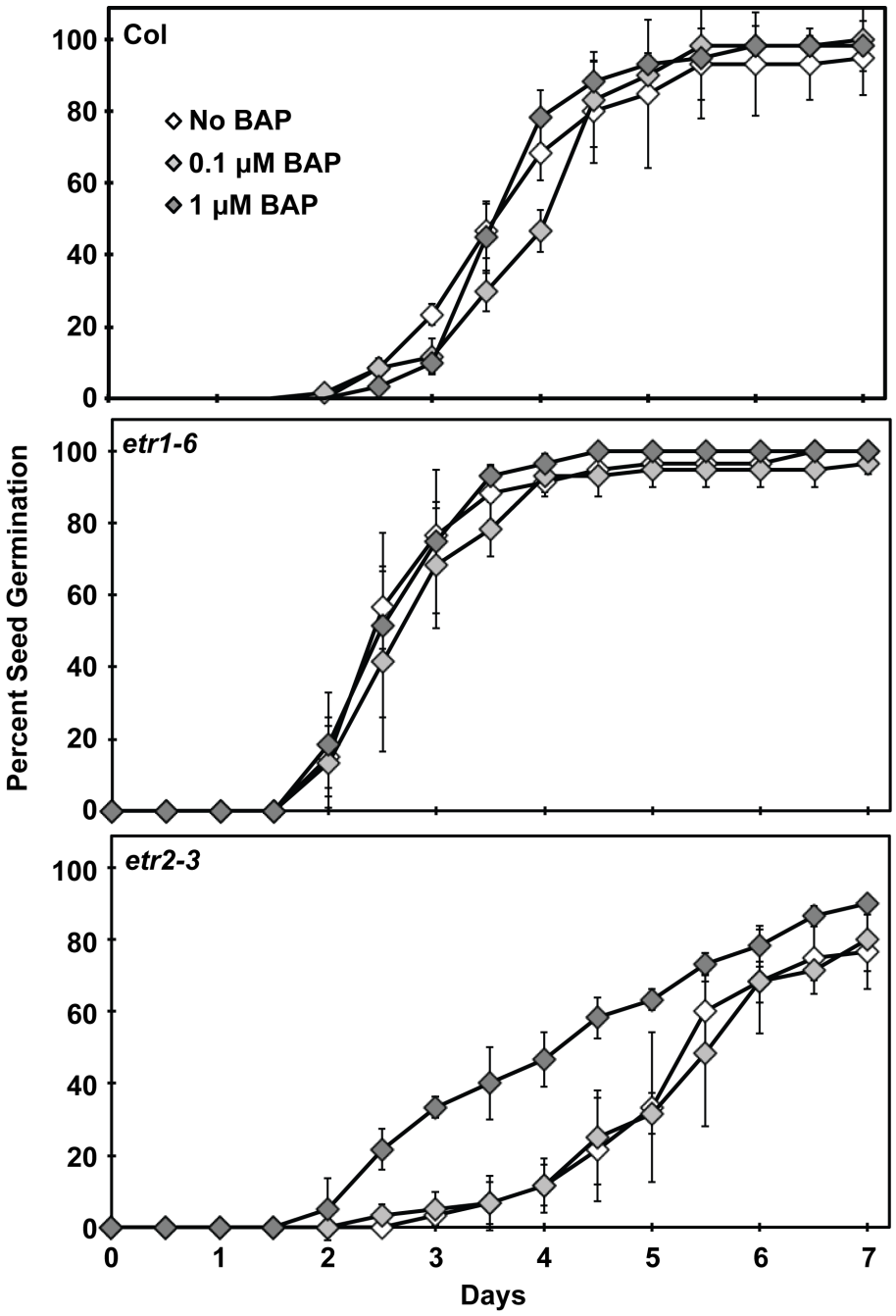


Figure II-14. The effect of cytokinin on the germination time course of *etr1-6*, *etr2-3* and Col under salt stress.

Germination time course of *etr1-6* and *etr2-3* compared to the Col wild-type in the presence of 150 mM NaCl with the indicated concentrations of BAP. The percent of germinated seeds was determined every 12 h. Each experiment was done in triplicate and the average percent seed germination ± SD at each time point is plotted for each seed line.

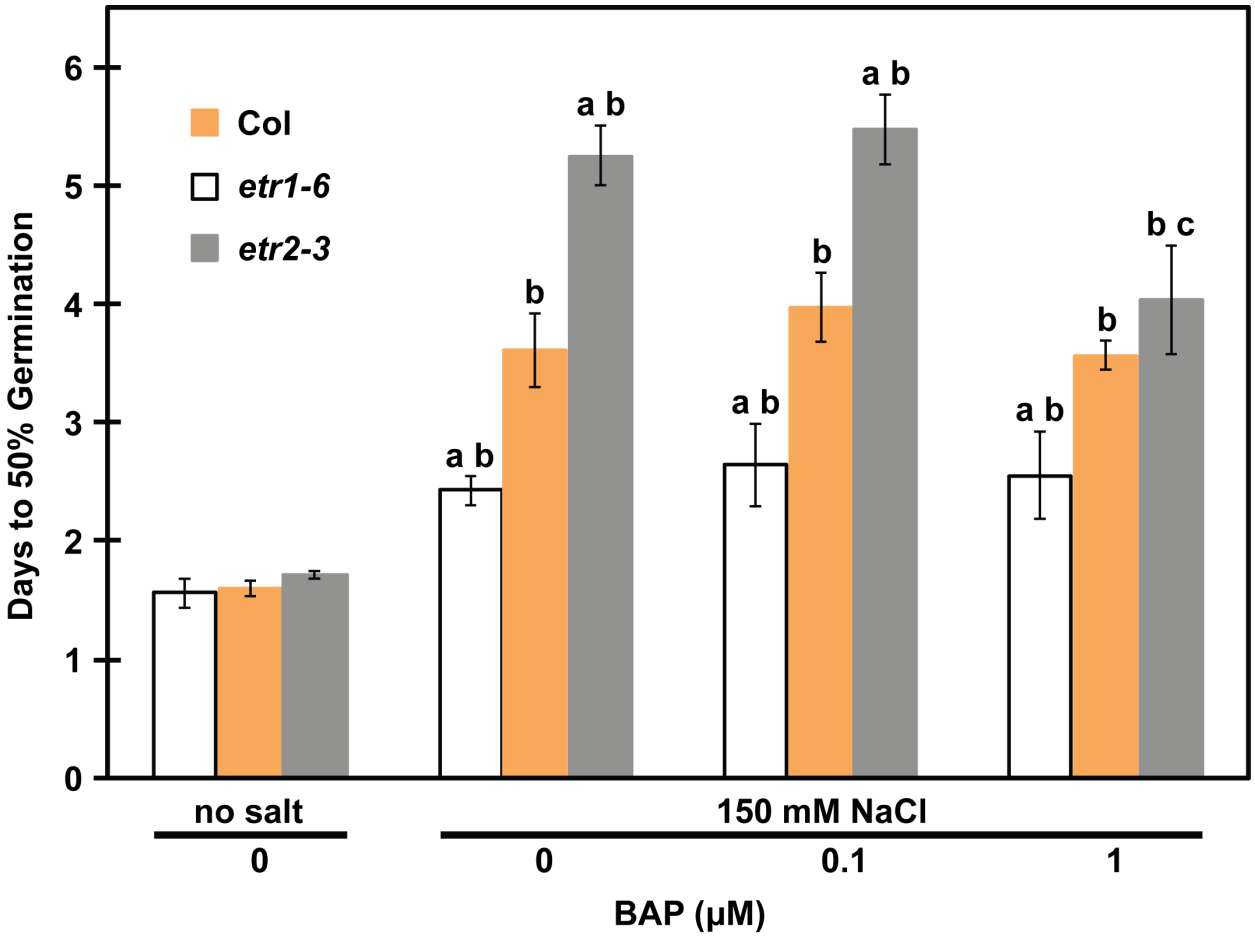


Figure II-15. Time for 50% of *etr1-6*, *etr2-3* and Col to germinate in the presence of cytokinin.

The time for etr1-6, etr2-3 and the Col control to reach 50% germination in the presence of the indicated concentrations of BAP was calculated from the data in Figure II-14. The average ± SD for each line is plotted. [a]Denotes a statistical difference compared to Col under that treatment, [b]denotes a statistical difference compared to the no salt control of that seed line and [c]denotes a statistical difference compared to the salt without BAP control of that seed line where $p < 0.05$ with a t-test.

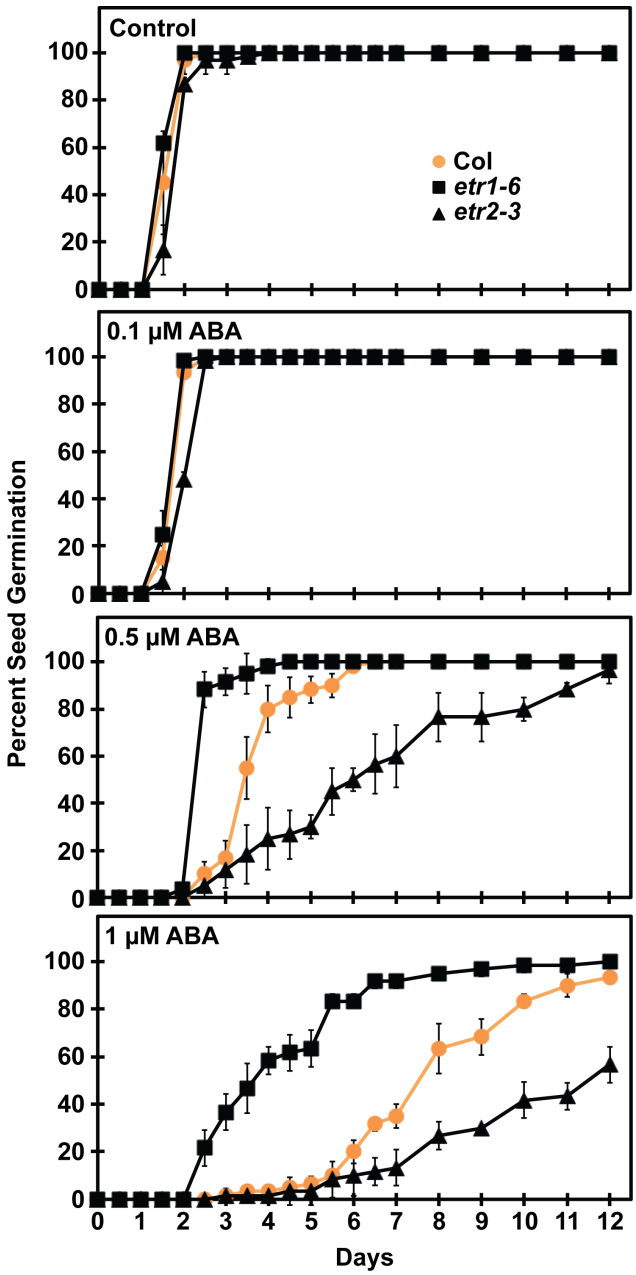


Figure II-16. The effect of increasing concentrations of ABA on the germination time course of *etr1-6*, *etr2-3* and Col.

Germination time course of *etr1-6* and *etr2-3* compared to the Col wild-type in the presence of increasing concentrations of ABA. The percent of germinated seeds was determined every 12-24 h. Each experiment was done in triplicate and the average percent seed germination ± SD at each time point is plotted for each seed line.

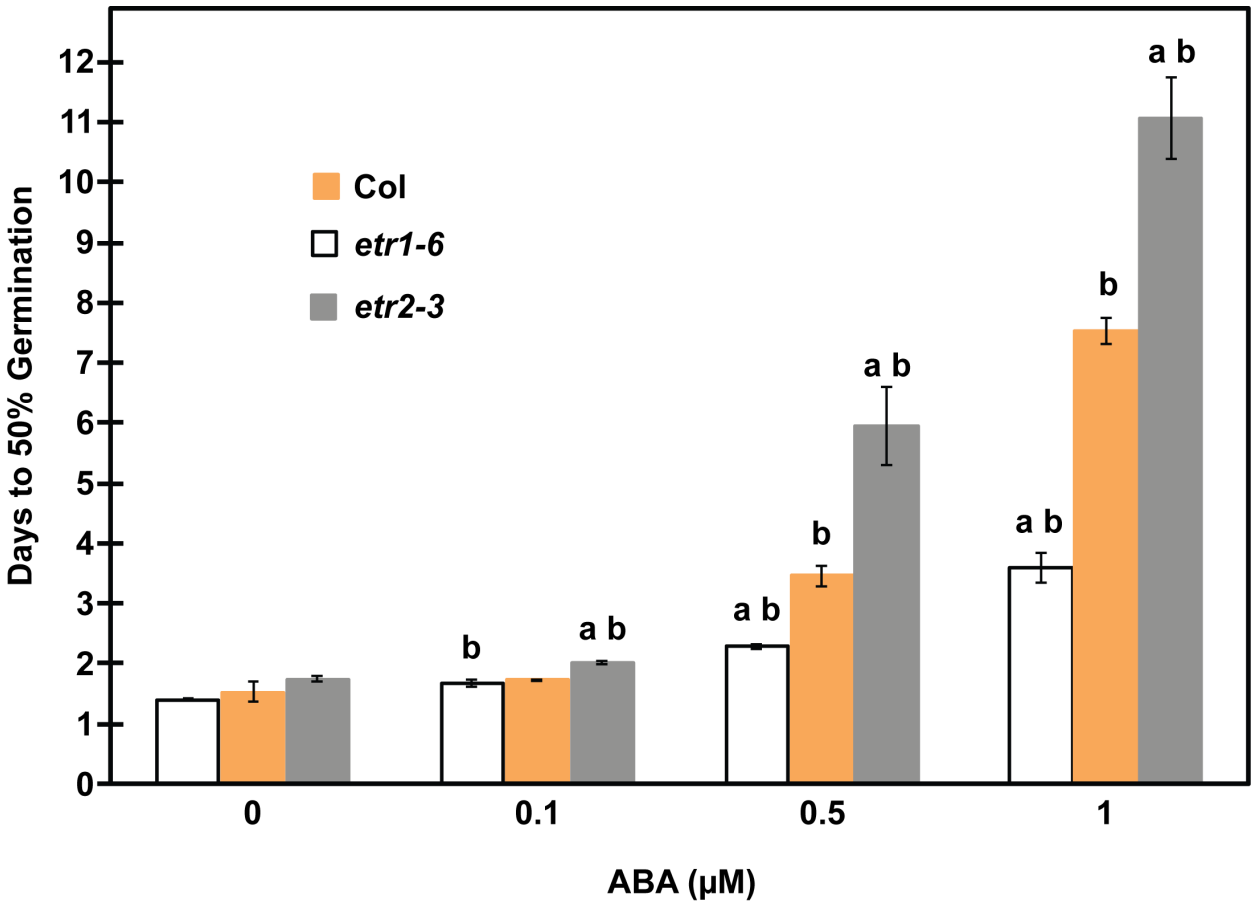


Figure II-17. Time for 50% of *etr1-6, etr2-3* and Col to germinate in the presence of ABA.

The time for *etr1-6*, *etr2-3* and the Col control to reach 50% germination in the presence of increasing concentrations of ABA was calculated from the data in Figure II-16. The average ± SD for each line is plotted. [a]Denotes a statistical difference compared to Col under that treatment and [b]denotes a statistical difference compared to the untreated control of that seed line where $p < 0.05$ with a t-test.

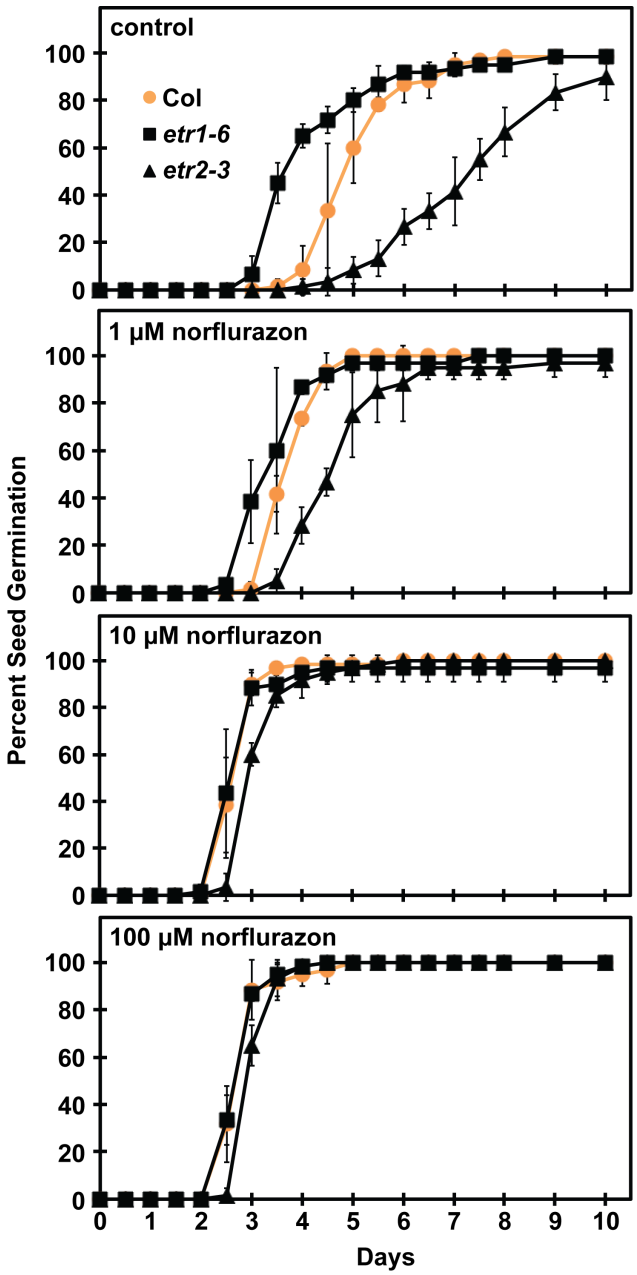


Figure II-18. The effect of increasing concentrations of norflurazon on the germination time course of *etr1-6*, *etr2-3* and Col under salt stress.

Germination time course of *etr1-6* and *etr2-3* compared to the Col wild-type in the presence of 150 mM NaCl with increasing concentrations of norflurazon. The percent of germinated seeds was determined every 12-24 h. Each experiment was done in triplicate and the average percent seed germination ± SD at each time point is plotted for each seed line.

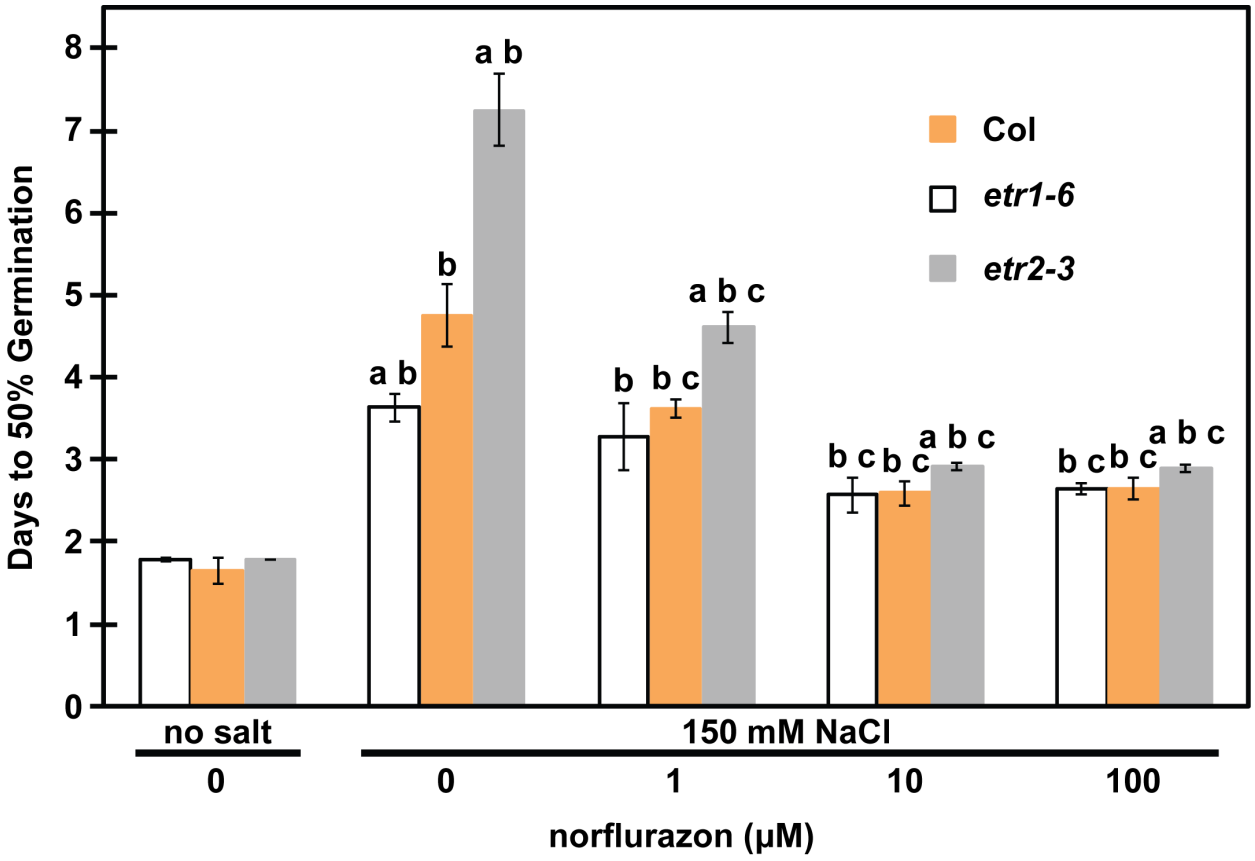


Figure II-19.Time for 50% of *etr1-6*, *etr2-3* and Col to germinate in the presence of norflurazon.

The time for *etr1-6*, *etr2-3* and the Col control to reach 50% germination in the presence of the indicated concentrations of norflurazon was calculated from the data in Figure II-18. The average ± SD for each line is plotted. [a]Denotes a statistical difference compared to Col under that treatment, [b]denotes a statistical difference compared to the no salt control of that seed line and [c]denotes a statistical difference compared to the salt without norflurazon control of that seed line where $p < 0.05$ with a t-test.

difference in germination during salt stress of *etr1-6* and *etr2-3* (Figure II-18 and Figure II-19). These data suggest that the difference in ABA sensitivity or production can account for the difference in germination observed between *etr1-6* and *etr2-3* seeds during salt stress.

Discussion

Ethylene receptors have been shown to have unique and sometimes opposite functions to one another (Shakeel et al., 2013). Previous research showed that ethylene signaling promotes germination in the presence of salt stress and that the gain-of-function ethylene insensitive mutant *etr1-1* germinates poorly during salt stress compared to wild-type (Wang et al., 2007; Wang et al., 2008; Lin et al., 2012), but the role of the other ethylene receptors in germination during salt stress was not studied. We examined the role of individual ethylene receptors in seed germination under salt stress and found that ETR2 promotes while ETR1, and to a lesser extent EIN4, inhibits germination in these conditions.

It is known that *etr1* LOF mutants are slightly more sensitive and responsive to ethylene (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002). Therefore, we tested whether differences in ethylene sensitivity or production could account for the difference in germination observed between *etr1* and *etr2* LOF mutants during salt stress. In contrast to previous experiments showing that etr1 mutants are more sensitive to ethylene (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002), we found that *etr1-6* was less sensitive and *etr2-3* was more sensitive to ethylene than wild-type during germination under salt stress. This is opposite to what one would predict given that ethylene promotes germination during salt stress and the *etr2-3* mutant shows delayed germination in these conditions. One possible explanation for the reduced sensitivity towards ethylene observed in *etr1-6* is that it overproduces ethylene and is saturated in its response to ethylene. However, when we measured ethylene production during germination, we were unable to detect ethylene production prior to radicle emergence. Following germination on 150 mM NaCl, *etr1-6* produced more and *etr2-3* produced less ethylene. Although this is consistent with and could explain their germination phenotypes, it could also simply be a reflection of the different percent of germinated seeds between the genotypes. In order to further investigate this, we treated *etr1-6* and Col seeds germinating in the presence and absence of 150

mM NaCl with the ethylene biosynthesis inhibitor, AVG. Although this concentration of AVG reduced ethylene production in seedlings by about 50%, it had no effect on the germination of Col or *etr1-6*. These data suggest that differences in ethylene sensitivity or production are not the underlying cause for the difference in germination observed between *etr1-6* and *etr2-3*. However, the poor germination in general and during salt stress of ethylene insensitive mutants, including *etr1-1*, suggests that ethylene is important for seed germination in these conditions (Bleecker et al., 1988; Wang et al., 2007; Wang et al., 2008; Lin et al., 2012). Furthermore, exogenous application of ethylene was capable of eliminating the difference in germination between *etr1*-6 and *etr2-3* and we can not rule out the possibility that *etr1-6* and *etr2-3* have increased and decreased subcellular concentrations of ethylene respectively that are responsible for their opposite germination phenotypes during salt stress or that *etr1-6* is hypersensitive to very small concentrations of ethylene.

As with ethylene, differences in production or sensitivity towards GA or cytokinin, both positive regulators of seed germination, are not able to explain the contrasting roles of ETR1 and ETR2 in seed germination during salt stress. On the other hand, treatment of wild-type, *etr1-6* and *etr2-3* seeds with ABA, a negative regulator of seed germination, phenocopied the effect of NaCl on their germination time courses. As with NaCl, *etr1-6* was less sensitive and *etr2-3* was more sensitive to ABA than wild-type. Treatment with norflurazon, an inhibitor of ABA biosynthesis, almost completely eliminated the difference in germination between wild-type, *etr1-6* and *etr2-3* germination on 150 mM NaCl. This data and the observation that salt stress increases endogenous ABA levels (Zhu, 2002) suggests that differences in ABA sensitivity or biosynthesis are likely to be the major factor underlying the difference in germination between *etr1-6* and *etr2-3* germination during salt stress.

That ABA may be playing a major role in the germination differences between *etr1-6* and *etr2-3* mutants during salt stress is not very surprising given the considerable amount of data showing crosstalk between ethylene and ABA signaling and biosynthesis. For instance, EIN2 and CTR1 mutants have turned up as repressors and enhancers respectively of ABA signaling in multiple forward genetic screens (Cutler et al., 1996; Beaudoin et al., 2000; Ghassemian et al., 2000). EIN2 LOF mutants produce almost twice as much ABA as wild-type and the ethylene insensitive

etr1-1 mutant produces about 20% more ABA than wild-type (Cheng et al., 2009). Seeds of both mutants are hypersensitive to NaCl and exogenous ABA whereas seeds of the constitutive ethylene signaling mutant, *ctr1* are less sensitive to NaCl and ABA (Beaudoin et al., 2000; Ghassemian et al., 2000; Wang et al., 2007; Cheng et al., 2009; Subbiah and Reddy, 2010). These data suggest that certain ethylene receptors affect both sensitivity to and biosynthesis of ABA; however, our data suggests that this may occur independent of ethylene perception.

Opposite roles for ethylene receptors have been observed in other traits known to involve ethylene signaling, however the mechanism for this unknown (Binder et al., 2006; Plett et al., 2009a; Kim et al., 2011). Based on the analysis of the double and triple LOF mutants during salt stress along with analysis of ABA and norflurazon, we propose a genetic model in which ETR1 and to a lesser extent EIN4 promote seed germination in parallel during salt stress by increasing ABA production and/or sensitivity and in which EIN2 functions by inhibiting ETR1 and EIN4 (Figure II-20). More specifically, analysis of the double *etr1 ein4*, *etr1 etr2*, and *etr2 ein4* LOF mutants suggests that ETR1 and EIN4 function in parallel to inhibit seed germination during salt stress and that ETR1 plays a greater role in this inhibition than EIN4. The observation that the double *etr1 ein4* and triple *etr1 ein4 etr2* mutants germinate with a similar time-course suggests that ETR2 promotes germination by inhibiting the function of ETR1 and EIN4.

Materials and Methods

Chemicals

The plant hormones gibberellic acid (GA), abscisic acid (ABA) and the synthetic cytokinin, 6-benzylaminopurine (BAP), were obtained from ACROS Organics (Belgium). Norflurazon (NF), an inhibitor of ABA biosynthesis, and lovastatin, an inhibitor of cytokinin biosynthesis were obtained from Fluka (Switzerland). Paclobutrazol (PAC), an inhibitor of GA biosynthesis was a gift from Elena Shpak (University of Tennessee). Aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis was a gift from Rohm Haas (Philadelphia).

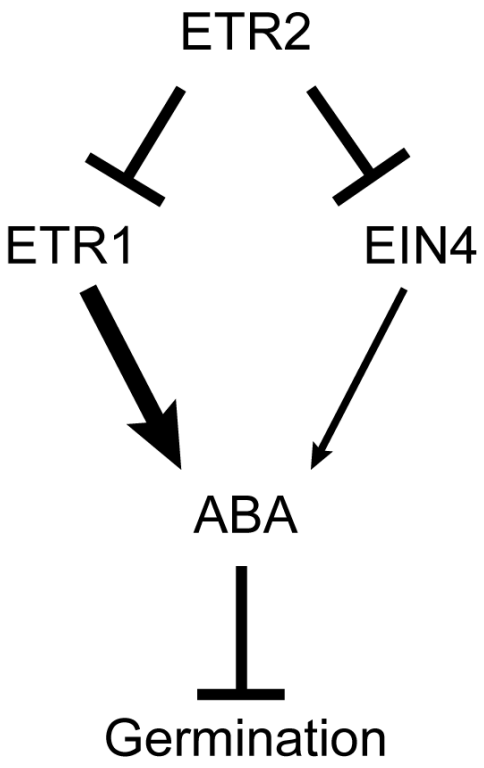


Figure II-20. Model for the roles of ETR1, ETR2, and EIN4 in seed germination under salt stress.

In this model, in the presence of salt stress, ETR1 and EIN4 stimulate ABA synthesis or signaling leading to reduced germination. ETR2 functions to promote germination by inhibiting ETR1 and EIN4. The width of the lines denotes the relative signal strength

Plants

All of the plants used in this chapter were described previously (Hua and Meyerowitz, 1998; Qu and Schaller, 2004; Kim et al., 2011). The *etr1-6*, *etr1-7*, *etr2-3*, and *ein4-4* mutants are in the Columbia (Col) background and the *etr1-9*, *ers1-3*, and *ers2-3* mutants are in the Wassilewskija (WS) background.

Germination Assays

In order to minimize biological variation, each experiment used a batch of age matched seeds between 250 and 300 μm in size from plants that were grown together in long day conditions as previously described (Hensel et al., 1993). Prior to use, the seeds were allowed to after-ripen in a room temperature desiccator for at least three weeks and were then sorted by size using sieves (Elwell et al., 2011). The seeds were surface sterilized in 70% ethanol for 30 seconds and allowed to dry on filter paper prior to imbibition on 0.8% (w/v) agar plates containing half-strength Murashige and Skoog (MS) basal medium with Gamborg's vitamins (Sigma, St. Louis, MO) at pH 5.7 with no added sugar. Where specified, NaCl or mannitol was added to the media at the indicated concentration. Additionally, GA, ABA, and NF were prepared as 1000x stocks in ethanol, filter sterilized and added to the media at the indicated concentrations after autoclaving (the solvent control plates contained 0.1% ethanol). PAC was prepared as above as a 10,000x stock in ethanol (the solvent control plates contained 0.01% ethanol). Lovastatin and BAP were prepared, as above, in dimethyl sulfoxide (DMSO) as 4000x and 5000x stocks respectively (the solvent control plates contained 0.025% and 0.02% DMSO respectively). AVG was prepared in water, filter sterilized and added to the media after autoclaving. The seeds were exposed to ethylene in flow through chambers as previously described (Chen and Bleecker, 1995). Twenty seeds of one genotype were placed on the agar plates in two rows of ten seeds with 5 mm space between the seeds. The plates were sealed with micropore surgical tape (3M, St. Paul, MN) to allow for gas exchange and prevent the accumulation of ethylene while also preventing water loss (Buer et al., 2003). The seeds were not stratified. Instead, the plates were placed directly into an environmentally controlled chamber and grown vertically at 20-21 °C in long-day conditions (16h of 12-13 μmol m^{-2} s^{-1} white light/8h dark). Germination was scored as a rupture of the testa (seed coat) at the indicated times.

Ethylene Measurements

The concentration of ethylene produced by 22 mg (dry weight) of seeds was measured every 6h using an ETD-300 photoacoustic ethylene detector (Sensor Sense, The Netherlands). The seeds were placed in a 6 mL glass vial on 1 mL half strength MS media prepared as described above and sealed with a rubber septum. This weight of seeds is equivalent to 1236 ±15 Col seeds, 1287 ±35 etr1-6 seeds and 1220 ±24 etr2-3 seeds. The concentration of ethylene produced by 22 mg of seeds 24h post imbibition in the sealed 6 mL glass vial was also measured. In this case, the seeds were placed on filter paper wetted with half strength MS media and prior to the measurement the vials were either heated to 80°C to drive ethylene from the interior of the seeds (Ramonell et al., 2002) or steel bearings were placed in the vials along with the seeds and the seeds were crushed by shaking the vials to release trapped ethylene. Data is an average of 2-3 experiments. Standard deviation is shown where three experiments were done.

Chapter III
Unique Role of ETR1 in Seed Germination Following Far-red Treatment

The research presented in this chapter has been submitted for review to Frontiers in Plant Science.

Introduction

Light is required for seed germination of certain species including Arabidopsis. For these plants, red light promotes seed germination while far-red light reversibly inhibits the effect of red light on germination (Borthwick et al., 1952). In Arabidopsis, red and far-red light are sensed by the phytochrome family of photoreceptors (PHYA – E) (Clack et al., 1994). These receptors are required for seed germination in light. Arabidopsis lacking all five of the phytochromes fail to germinate unless supplemented with exogenous GA (Strasser et al., 2010). PHYB and to a lesser extent PHYA promote germination in red light (Reed et al., 1994; Shinomura et al., 1994). PHYB is required for germination in the dark (Reed et al., 1994; Shinomura et al., 1994). In far-red light PHYA and PHYB have opposite roles; PHYA promotes while PHYB inhibits germination (Reed et al., 1994).

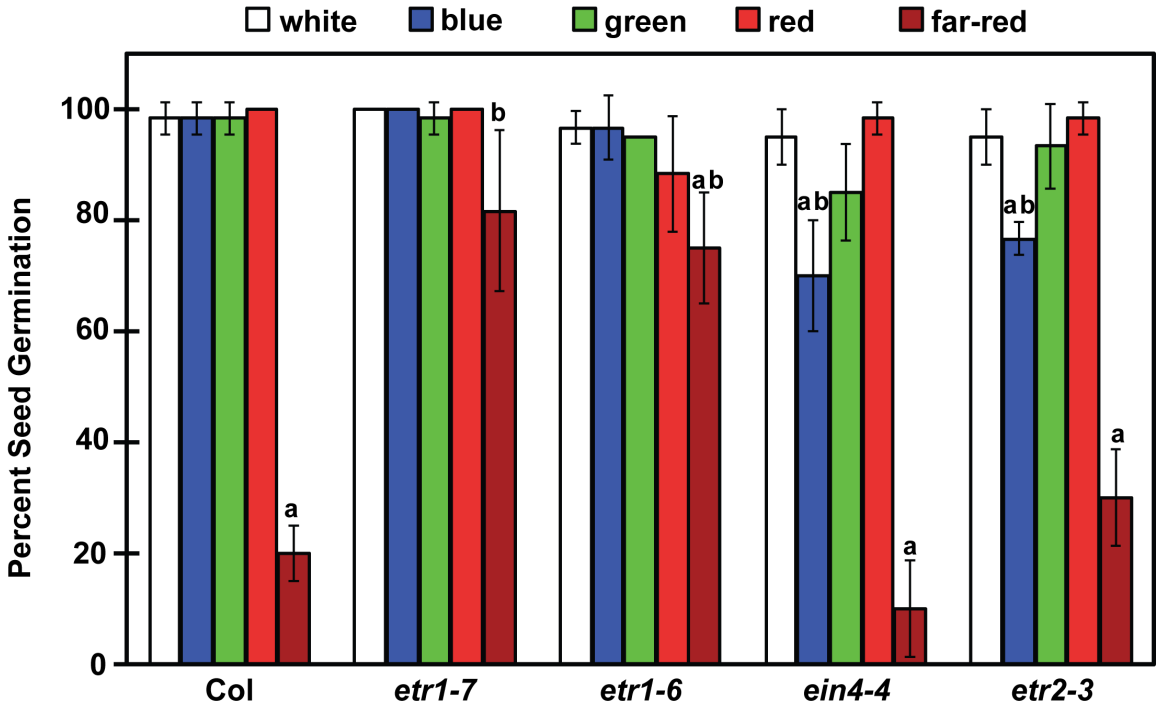
The phytochromes, like the ethylene receptors, resemble bacterial two-component sensor histidine kinases (Hwang et al., 2002). However, like the subfamily II ethylene receptors, they lack histidine kinase activity and are thought instead to have serine/threonine kinase activity (Yeh and Lagarias, 1998; Fankhauser et al., 1999). The phytochromes covalently bind a tetrapyrrole chromophore and exist in two states (Li et al., 2011). In the dark, they are synthesized in the inactive Pr form and red light converts this inactive Pr form to the active Pfr form while far-red light converts the Pfr form back to the Pr form (Li et al., 2011). One of the ways the phytochromes promote seed germination is by enhancing degradation of the negative regulator of seed germination PIL5 (PIF3 LIKE 5). PIL5 is a bHLH transcription factor that inhibits germination by reducing the sensitivity of seeds to GA and by promoting expression of ABA synthesis and GA catabolism genes while also inhibiting GA synthesis genes and ABA catabolism genes (Oh et al., 2004; Oh et al., 2006; Oh et al., 2007).

There are five ethylene receptors in Arabidopsis. The ethylene receptors have unique and/or opposing roles in various traits known to involve ethylene signaling including germination during salt stress (Shakeel et al., 2013; Wilson et al., 2014). This led us to examine the role of individual ethylene receptors in seed germination following far-red light, another condition known to inhibit seed germination. We also examined the role of the ethylene receptors in germination following red, blue and green light. We found that ETR1 inhibits seed germination following far-red light treatment while ETR2 and EIN4 promote germination following treatment with blue light. Following far-red light treatment, loss of ETR1 resulted in a decrease and increase of ABA and GA biosynthesis gene expression respectively. Epistasis analysis suggests that ETR1 and PhyA/PhyB function in parallel to inhibit and promote germination respectively.

Results

ETR1 inhibits germination in far-red light

In order to determine the contribution of each receptor isoform to seed germination under various light conditions, the percent of seed germination for each receptor loss-of-function mutant was determined seven days following a three hour treatment with white, red, blue, green or far-red light as described in Materials and Methods. Following white light treatment, all seed lines reached at least 95 % germination and germination after treatment with red or green light was not statistically different from that of white light for any of the seed lines tested (Figure III-1). Treatment with blue light did not affect seed germination of wild-type or *etr1* mutants, however the percent of *etr2-3* and *ein4-4* seeds that germinated following blue light treatment was statistically less than their germination following white light treatment (Figure III-1). Consistent with prior results (Shinomura et al., 1994), treatment with far-red light inhibited germination of wild-type seeds (Figure III-1). Germination of *etr2-3* and *ein4-4* seeds following far-red light treatment was not statistically different from that of wild-type (Figure III-1). Interestingly however, far-red light had little effect on seed germination of the *etr1* LOF mutants. Both *etr1-7* and *etr1-6* germinated statistically better than wild-type following far-red light treatment and germination of the *etr1-7* allele was not statistically different from its germination following



white light (Figure III-1). Loss of ERS1 or ERS2 had no measurable effect on germination in any of the light conditions tested (data not shown). These data suggest that ETR2 and EIN4 promote

Figure III-1. Effect of different wavelengths of light on ethylene receptor loss-of-function mutants.

Seed germination of ethylene receptor loss-of-function mutants was compared to wild-type seeds 7 days following treatment with different wavelengths of light. Seeds were treated with 4 h of white light followed by a 3 h treatment with the stated monochromatic light. The seeds were then placed in the dark for 7 days after which the number of germinated seeds was determined. The average percent of germinated seeds ± SD from at least three biological replicates is shown. [a]Denotes a statistical difference from the white light treated control of that seed line and [b]denotes a statistical difference from wild-type treated with the same wavelength of light where $p < 0.05$ with a t-test.

seed germination in blue light and that ETR1 inhibits germination in far-red light. Loss of ETR1 significantly improved seed germination following far-red light, therefore we focused on investigating this effect.

In order to further test whether the improved seed germination following far-red light is a consequence of the ETR1 LOF mutation, a germination time-course for three *etr1* LOF alleles and their respective wild-type was determined in continuous far-red light. As a control, seeds were also germinated in continuous white light. In the white light control, all seed lines had similar germination time-courses and reached at least 98% germination by 2.5 days (Figure III-2 top). In continuous far-red light, where the percent of seed germination was determined every 12-24 h, Col reached 83% germination by day 7 (Figure III-2). This was not statistically different from germination of the *etr1* alleles. However, as evidenced by a statistically ($p < 0.05$) higher percent germination of *etr1-6* or *etr1-7* compared to Col between 1.5 and 4 days or 1.5 and 6 days respectively, *etr1-6* and *etr1-7* both germinate faster than Col in these conditions (Figure III-2A bottom). WS only reached 33% in these conditions (Figure III-2B top) and *etr1-9* germinated to a statistically higher percent than WS starting at 3.5 days (Figure III-2B bottom). These data suggest that ETR1 inhibits germination following exposure to far-red light.

ETR1 and ETR2

ETR1 and ETR2 where shown to have opposite effects on seed germination during salt stress (Figure II-1). Although *etr2-3* germinated statistically similar to wild-type following far-red light (Figure III-1), it is possible that a difference in germination between *etr2-3* and wild-type would be masked by the poor germination of wild-type in these conditions. Therefore, we examined the genetic interaction of ETR1 and ETR2 by comparing germination of the *etr1-6 etr2-3* LOF mutant to their single LOF mutants following far-red light treatment. Following white light, germination of the single *etr1* and *etr2* LOF mutants was similar to the double LOF mutant and all three lines germinated to at least 88% by day seven (Figure III-3). Following far-red treatment, the *etr1* and *etr2* single mutants germinated to a statistically higher and lower percent than the double mutant respectively (Figure III-3). These data suggest that ETR1 and ETR2 have opposite effects on germination following far-red light treatment.

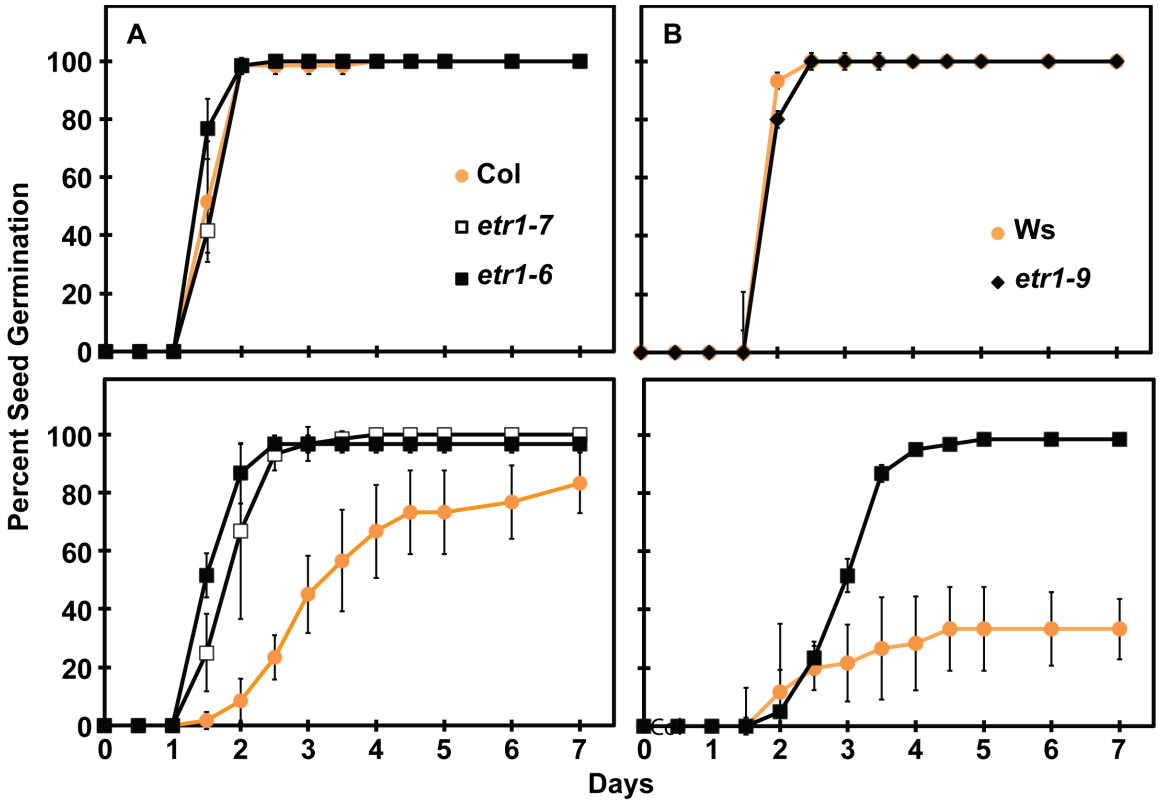


Figure III-2. Germination time-course of *etr1* loss-of-function mutants during far-red treatment.

Germination time course of *etr1* loss-of-function alleles (A) *etr1-7* and *etr1-6* and (B) *etr1-9* compared to their respective wild-type during treatment with continuous white light (top) or continuous far-red light (bottom) following a 2 h white light treatment. The percent of germinated seeds was determined every 12-24 h. Each experiment was done in triplicate and the average percent seed germination ± SD at each time point is plotted for each seed line.

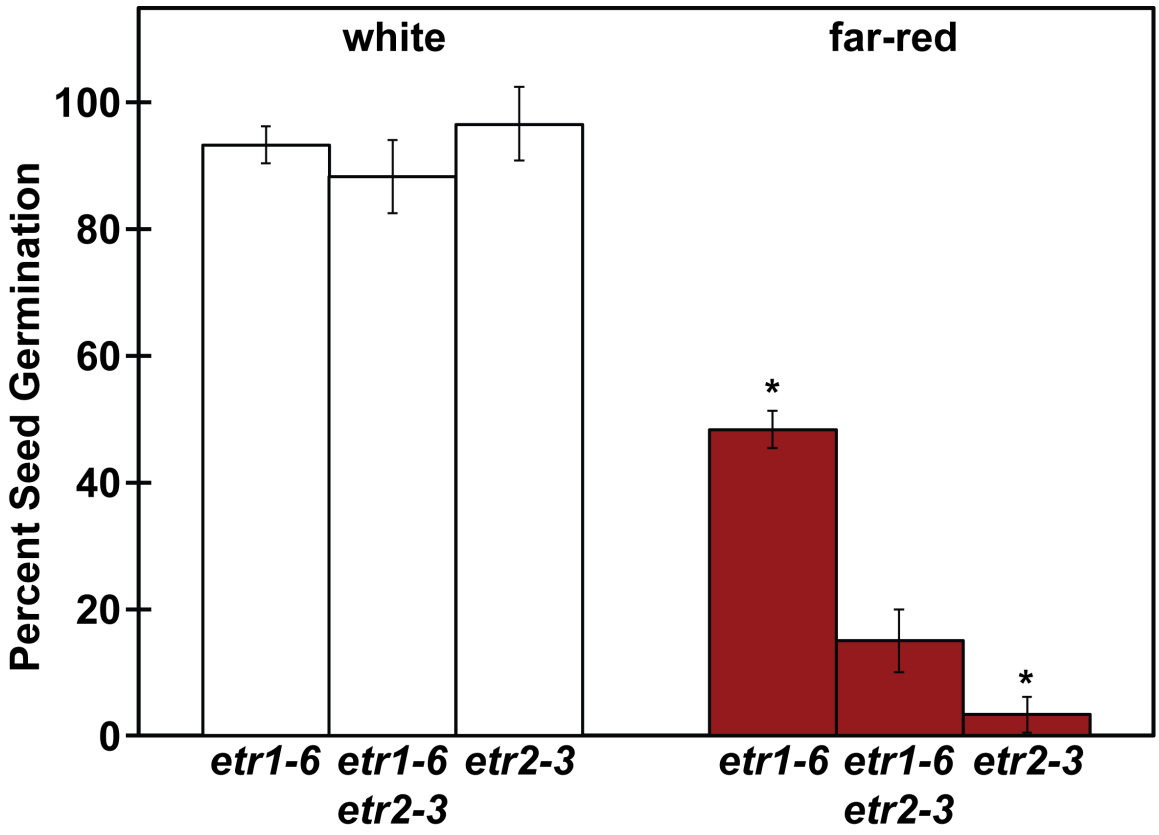


Figure III-3. Effect of far-red light on germination of *etr1-6 etr2-3* double mutant.

Seed germination of ethylene receptor double loss-of-function mutant *etr1-6 etr2-3* is compared to the single *etr1-6* and *etr2-3* mutants. Seeds were treated with 4 h of white light followed by an additional 3 h in white light or 3 h in far-red light. The seeds were then placed in the dark for 7 days after which the number of germinated seeds was determined. The average percent of germinated seeds ± SD from at least three biological replicates is shown. *Denotes a statistical difference from the double mutant in that light treatment where $p < 0.05$ with a t-test.

Ethylene

Ethylene in combination with GA promotes germination in far-red treated lettuce seeds (Burdett and Vidaver, 1971). In order to determine whether differences in ethylene production or sensitivity are responsible for the difference in germination between *etr1-6* and wild-type following far-red treatment, we examined the effect of ethylene and the ethylene biosynthesis inhibitor, AVG, on germination. As discussed in chapter II, if ethylene is involved in the difference in germination following far-red treatment, the *etr1* LOF mutant should either produce more ethylene or be more sensitive to ethylene than wild-type. In these conditions, following white light treatment, *etr2-3* germinated statistically less than wild-type, but not *etr1-6* (Figure III-4). This difference in germination following white light treatment was eliminated by treatment with 0.001 ppm ethylene and treatment with higher concentrations of ethylene hand no further effect on germination (Figure III-4). Following treatment with far-red light, 0.1 and 1 ppm ethylene significantly improved the germination of wild-type and slightly, but statistically significantly improved the germination of *etr2-3* (Figure III-4). Ethylene had no measureable effect on the germination of *etr1-6* following far-red light treatment (Figure III-4).

The *etr1-6* mutant was not more sensitive to ethylene as would be predicted if ethylene was involved in the improved germination of *etr1-6* following far-red light. However, it is possible that *etr1-6* produces more ethylene than wild-type and is already saturated in this response. We were unable to detect any ethylene production prior to radical emergence (data not shown) and instead evaluated the effect of AVG on germination following far-red light. Following white light control treatment, AVG did not have a statistically significant effect on the germination of wild-type, *etr1-6*, or *etr2-3* (Figure III-5). AVG also did not affect wild-type or *etr2-3* germination following far-red treatment, however germination of *etr1-6* was statistically reduced by 20% in this condition (Figure III-5). Although AVG reduced the germination of *etr1-6* following far-red light treatment, it still germinated to a statistically greater percent than wild-type. These data suggest that part, but not all of the difference in germination between wild-type and *etr1-6* may be due to an increase in ethylene production or ethylene sensitivity in this mutant.

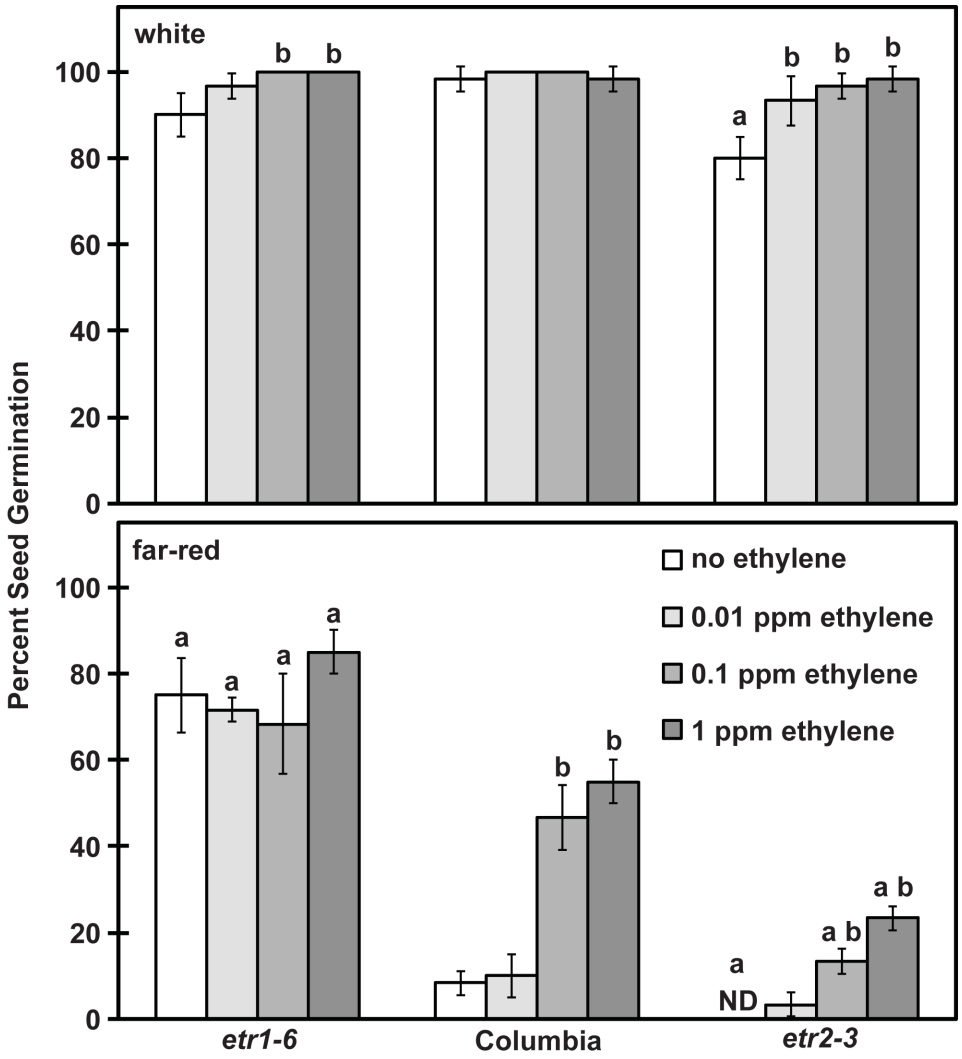


Figure III-4. Effect of ethylene on germination of etr1-6, etr2-3 and Columbia seeds following far-red treatment.

Seed germination of *etr1-6* and *etr2-3* is compared to the Columbia wild-type. Seeds were treated with 4 h of white light followed by an additional 3 h in white light (top) or 3 h in far-red light (bottom). The seeds were then treated with the indicated concentrations of ethylene and placed in the dark for 7 days after which the number of germinated seeds was determined. The average percent of germinated seeds ± SD from at least three biological replicates is shown. [a]Denotes a statistical difference from the wild-type in the same light at the same ethylene concentration and [b]denotes a statistical difference from the no ethylene control in the same light condition for that seed line where $p < 0.05$ with a t-test. ND denotes no germination detected.

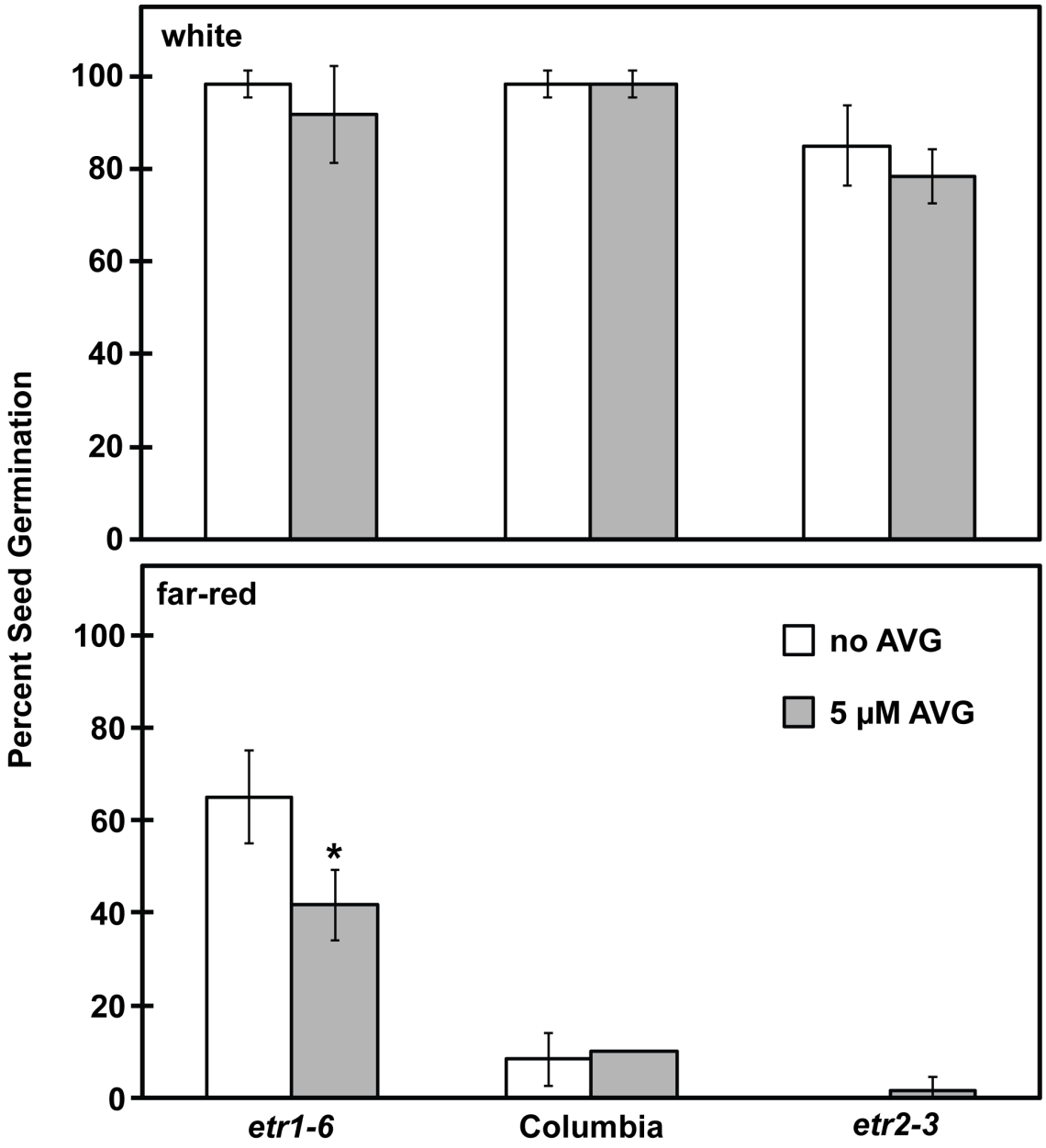


Figure III-5. Effect of AVG on germination of *etr1-6*, *etr2-3* and Columbia following far-red treatment.

Seed germination of *etr1-6* and *etr2-3* is compared to the Columbia wild-type in the presence and absence of 5 µM AVG. Seeds were treated with 4 h of white light followed by an additional 3 h in white light (top) or 3 h in far-red light (bottom). The seeds were then placed in the dark for 7 days after which the number of germinated seeds was determined. The average percent of germinated seeds ± SD from at least three biological replicates is shown. *Denotes a statistical difference from the no AVG control of that seed line where $p < 0.05$ with a t-test.

GA and ABA

Red light promotes seed germination through regulation of GA and ABA metabolism (Oh et al., 2006; Seo et al., 2006). We examined the effect of GA and the ABA biosynthesis inhibitor norflurazon on the seed germination of *etr1-6*, *etr2-3* and Col following far-red light treatment. Following the control white light treatment, there was no difference in germination between *etr1-6*, *etr2-3* and Col (Figure III-6 and Figure III-7 top). Following far-red treatment, 10 μM GA significantly improved the germination of all three seed lines, but was not able to improve germination of Col or *etr2-3* to that of *etr1-6* (Figure III-6). Addition of norflurazon improved the germination of *etr1-6*, but had no effect on Col or *etr2-3* germination following far-red treatment (Figure III-7). These data suggest that differences in GA and ABA sensitivity or production may be responsible for the improved germination of the *etr1-6* mutant following far-red light treatment.

In order to further elucidate any differences in GA and ABA metabolism, we used qRT-PCR to analyze the expression of several genes involved in GA and ABA metabolism. Compared to far-red light, red light is known to promote expression of the GA biosynthesis genes GA3ox1 and GA3ox2 and repress expression of the GA catabolism gene GA2ox2 (Oh et al., 2006). On the other hand, the ABA biosynthesis genes ZEP, NCED6 and NCED9 are decreased in red compared to far-red light while the ABA catabolism gene CYP707A2 is increased by red light (Seo et al., 2006; Seo et al., 2009). The transcript abundance of these genes was evaluated in Col and *etr1-6* during a germination time-course starting with dry seeds. The seeds were imbibed for 4 hours under white light, 3 hours under far-red light and then were transferred to the dark. RNA was isolated immediately following the 4 h white light treatment and 3 h far-red light treatment and then at 6, 12 and 24 h after transfer to the dark. We found that the GA biosynthesis genes, *GA3ox1* and *GA3ox2* increased following the 4 h white light treatment and were more highly expressed in *etr1-6* than Col (Figure III-8). The expression of both genes was reduced following the 3 h far-red treatment, but *etr1-6* maintained a higher expression of these genes than Col after transfer to dark (Figure III-8). Expression of *GA2ox2* increased following far-red light treatment and was greater in Col than *etr1-6* in dry seeds and through the far-red treatment (Figure III-8). Expression of the ABA synthesis genes *ZEP*, *NCED6*, and *NCED9* increased in Col following

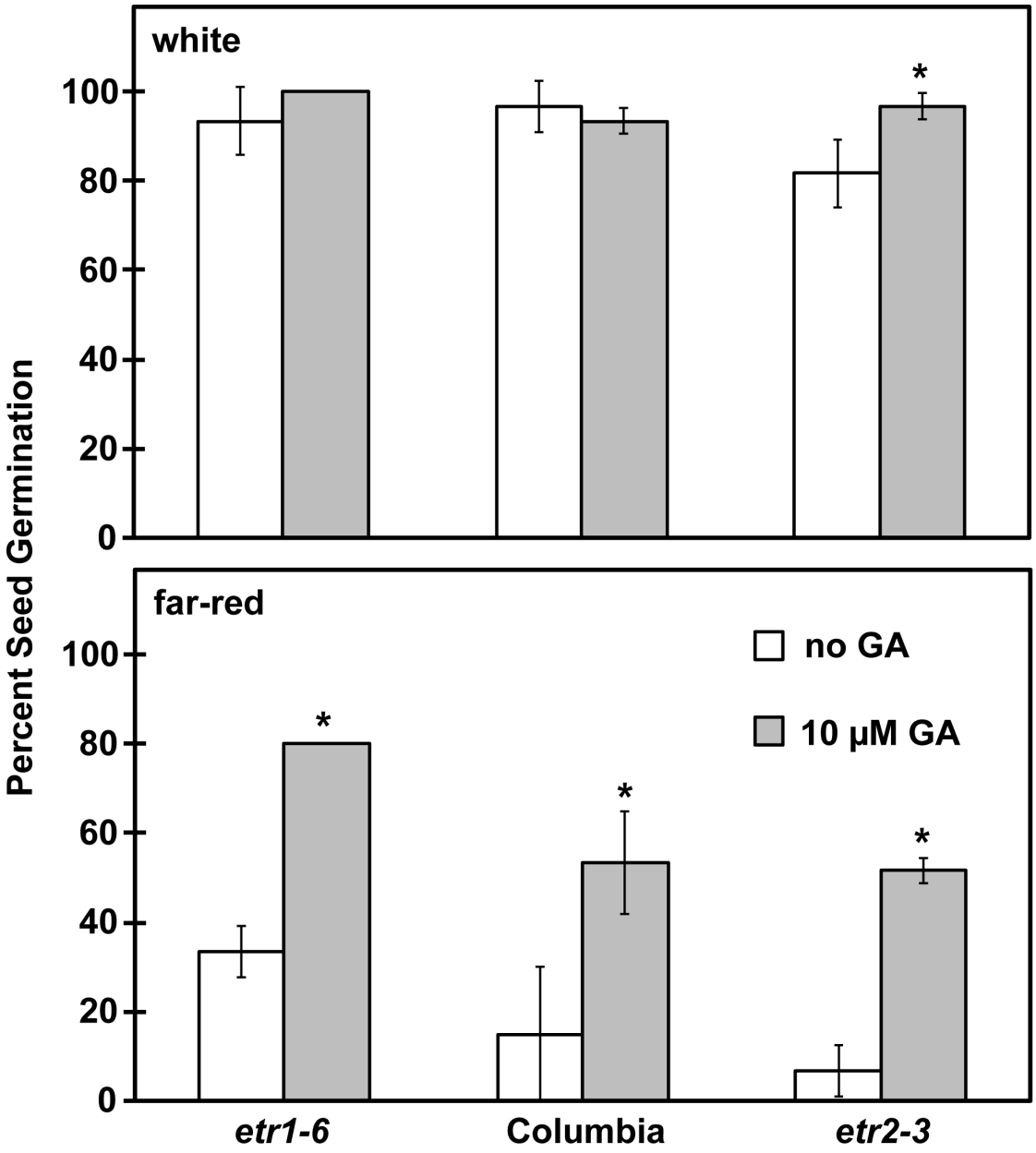


Figure III-6. Effect of GA on germination of *etr1-6*, *etr2-3* and Columbia following far-red treatment.

Seed germination of *etr1-6* and *etr2-3* is compared to the Columbia wild-type in the presence and absence of 10 µM GA. Seeds were treated with 4 h of white light followed by an additional 3 h in white light (top) or 3 h in far-red light (bottom). The seeds were then placed in the dark for 7 days after which the number of germinated seeds was determined. The average percent of germinated seeds ± SD from at least three biological replicates is shown. *Denotes a statistical difference from the no GA control of that seed line where $p < 0.05$ with a t-test.

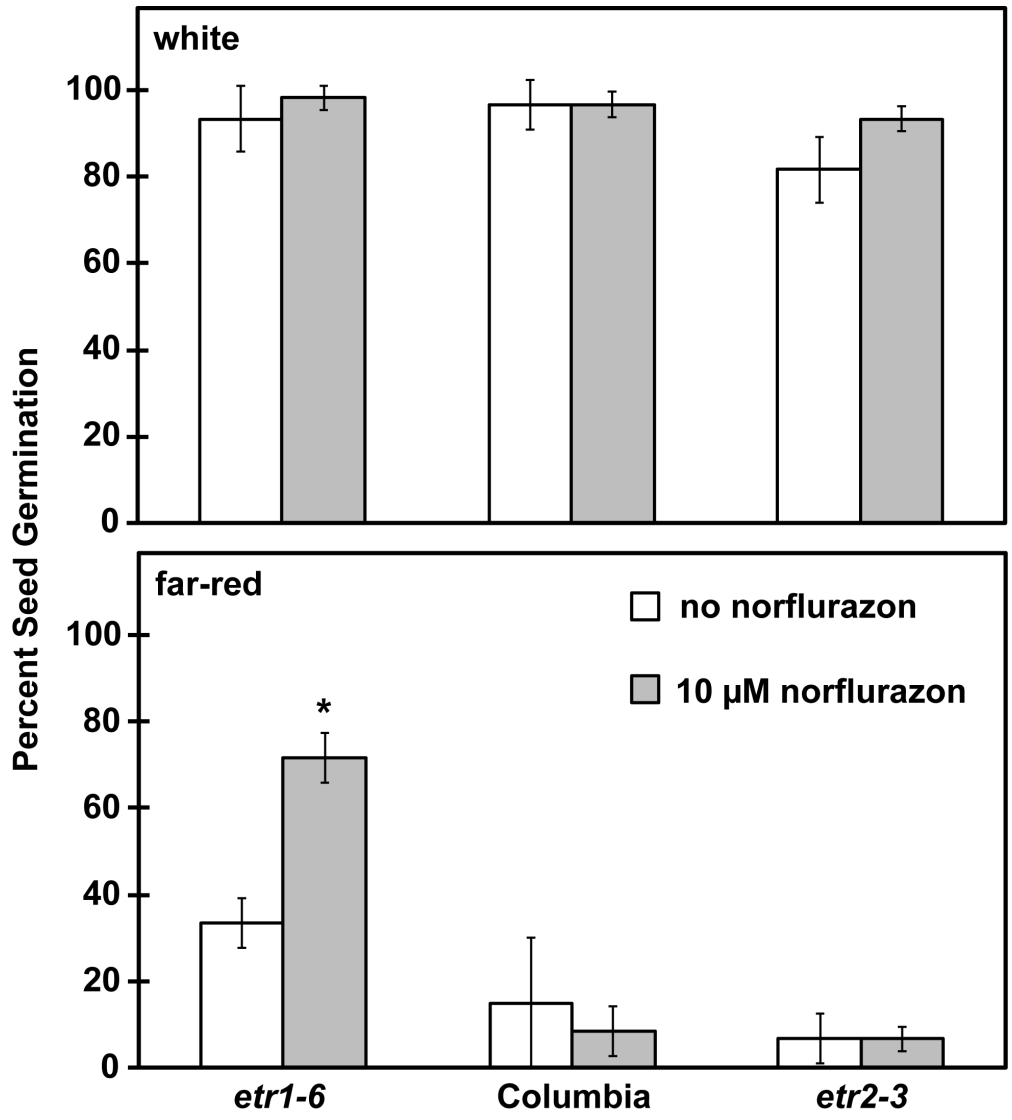


Figure III-7. Effect of norflurazon on germination of *etr1-6*, *etr2-3* and Columbia following far-red treatment.

Seed germination of *etr1-6* and *etr2-3* is compared to the Columbia wild-type in the presence and absence of 10 µM norflurazon. Seeds were treated with 4 h of white light followed by an additional 3 h in white light (top) or 3 h in far-red light (bottom). The seeds were then placed in the dark for 7 days after which the number of germinated seeds was determined. The average percent of germinated seeds ± SD from at least three biological replicates is shown. *Denotes a statistical difference from the no norflurazon control of that seed line where $p < 0.05$ with a t-test.

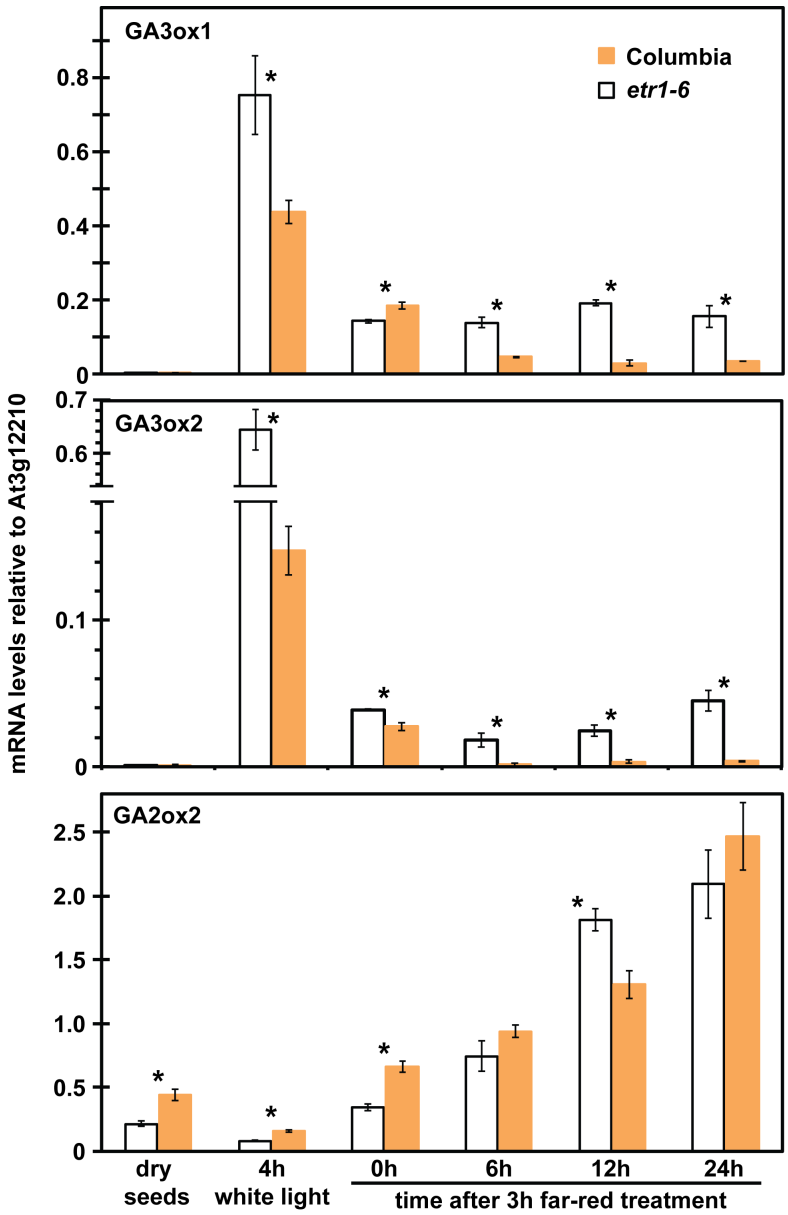


Figure III-8. Effect of far-red light on transcript levels of select GA metabolic genes during *etr1-6* and Columbia germination.

The transcript levels of the GA biosynthesis genes GA3ox1 and GA3ox2 and the GA catabolic gene GA2ox2 in *etr1-6* compared to the Columbia wild-type at various times during germination were determined by qRT-PCR. Data are normalized to the level of At3g12210 in each seed line at each time point. *Denotes a statistical difference between etr1-6 and wild-type at that time point where $p < 0.05$ with a t-test.

the far-red treatment, but were less affected in *etr1-6* (Figure III-9). These genes were expressed to significantly greater levels in Col than *etr1-6* following the far-red light treatment (Figure III-9). Little difference in the expression of the ABA catabolism gene *CYP707A2* was observed between Col and *etr1-6*, although its expression was statistically greater in Columbia than *etr1-6* at 0 and 24 h following the far-red light treatment (Figure III-9).

PhyA and PhyB

The phytochromes are photoreversible switches that promote seed germination in red light and inhibit germination in far-red light (Borthwick et al., 1952). Of the five phytochromes in Arabidopsis, PhyA and PhyB play the major in seed germination (Reed et al., 1994; Poppe and Schafer, 1997; Strasser et al., 2010). In order to determine if germination of the *etr1-6* mutant is photoreversible, a characteristic of functional phytochrome signaling, we treated seeds with a series of five minute pulses of red and far-red light and then allowed the seeds to germinate in darkness. We found that there was no difference in germination between wild-type and *etr1-6* when the series of pulses ended in red light (Figure III-10). In contrast, when the series of pulses ended in far-red light, Col failed to germinate, but about 20% of *etr1-6* seeds germinated (Figure III-10). Even though the *etr1-6* mutant germinates better than wild-type in far-red light, the photoreversibility of *etr1-6* suggests that the phytochromes are still functional in this mutant.

In order to examine the genetic interaction of *etr1-6* with the phytochromes, we generated *etr1-6 phyA* and *etr1-6 phyB* double mutants. Following the control white light treatment, germination of the *etr1-6 phyA* double mutant and its respective single mutants was indistinguishable from wild-type (Figure III-11). However, consistent with previous reports (Shinomura et al., 1994), the *phyB* mutant showed very poor germination after being transferred from white light to the dark (Figure III-11). The *etr1-6 phyB* double mutant germinated to a slightly, but statistically greater percent than the *phyB* single mutant (Figure III-11). Following far-red light treatment, *etr1-6* germinated statistically better than wild-type, while *phyB* germinated statistically worse than wild-type and *phyA* failed to germinate (Figure III-11). In contrast to the single mutants, the *etr1-6 phyA* and *etr1-6 phyB* double mutants germinate similar to wild-type (Figure III-11). These data suggest that ETR1 and PHYA /PHYB act in parallel to inhibit and promote germination respectively.

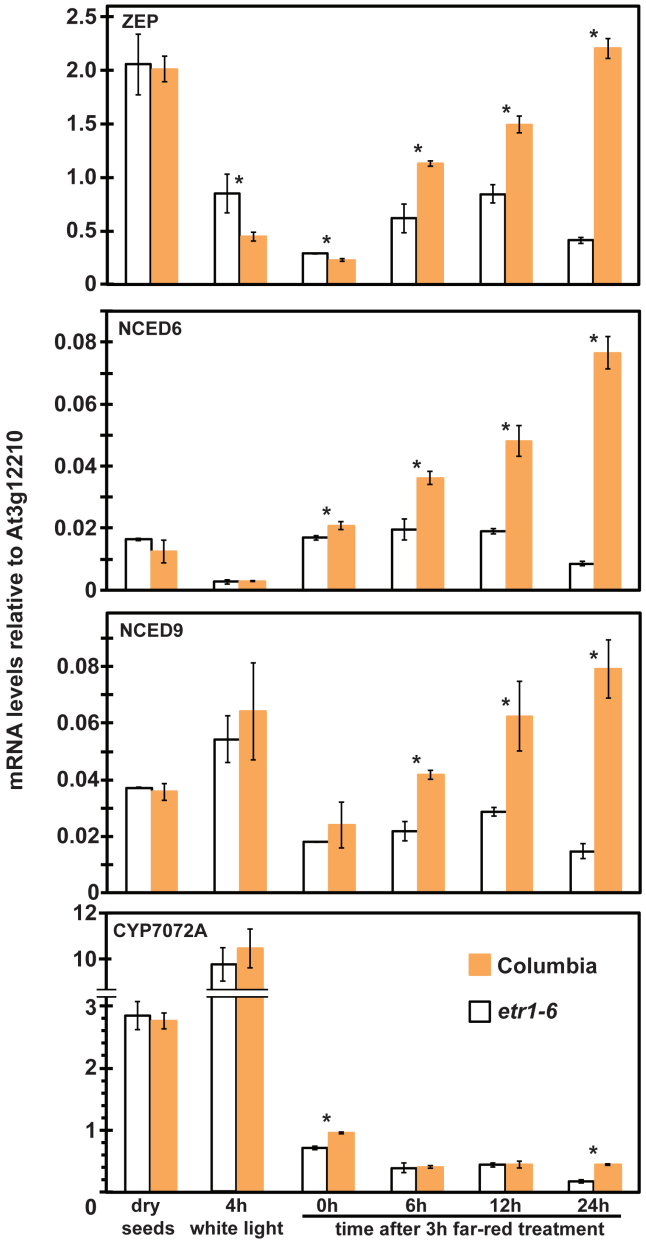
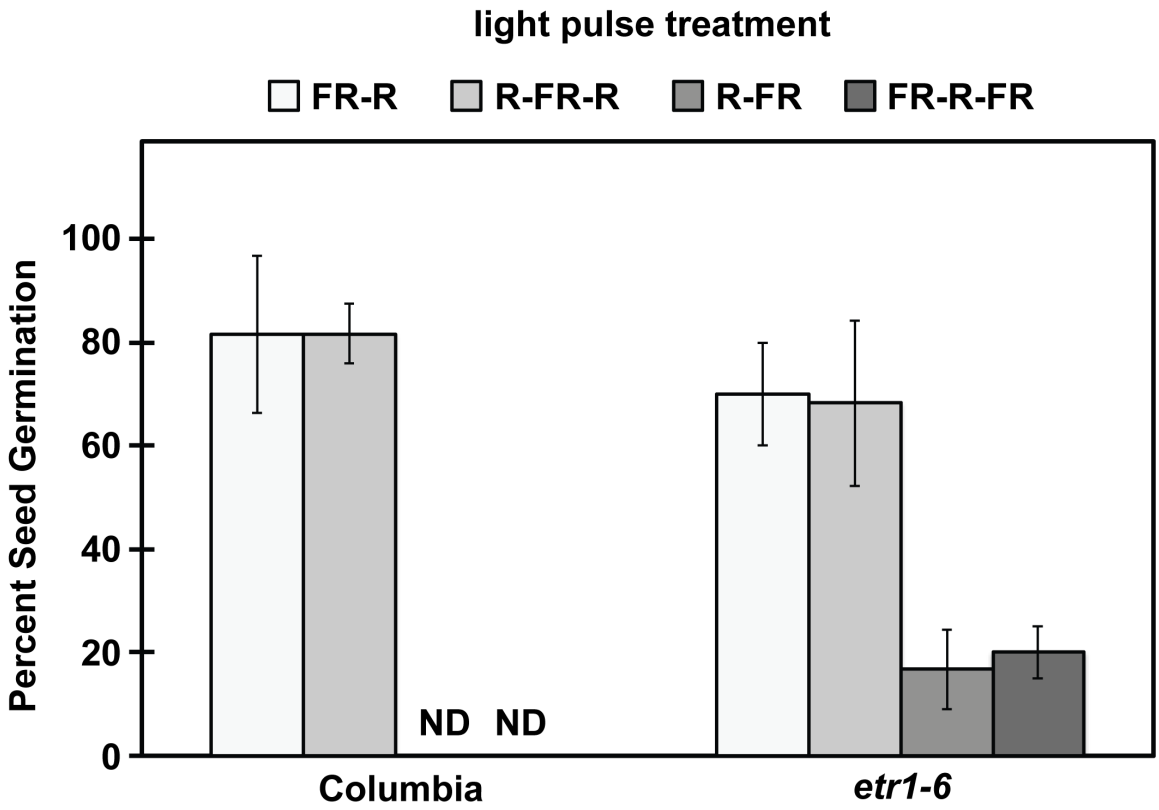


Figure III-9. Effect of far-red light on transcript levels of ABA metabolic genes during *etr1-6* and Columbia germination.

The transcript levels of the ABA biosynthesis genes ZEP, NCED6 and NCED9 and the ABA catabolic gene CYP707A2 in *etr1-6* compared to the Columbia wild-type at various times during germination were determined by qRT-PCR. Data are normalized to the level of At3g12210 in each seed line at each time point. *Denotes a statistical difference between etr1-6 and wild-type at that time point where $p < 0.05$ with a t-test.



In order to further examine the interaction of ETR1 with PHYA and PHYB, we also examined the hypocotyl lengths of the *etr1-6 phyA* and *etr1-6 phyB* double mutants and their respective

Figure III-10. Effect of far-red and red light pulses on *etr1-6* and Columbia germination.

Columbia and *etr1-6* seeds were treated with white light for 3 h followed by the indicated series of 5 min far-red (FR) and red (R) light pulses. The seeds were then placed in the dark for 7 days after which the number of germinated seeds was determined. The average percent of germinated seeds ± SD from at least three biological replicates is shown. ND denotes no germination detected.

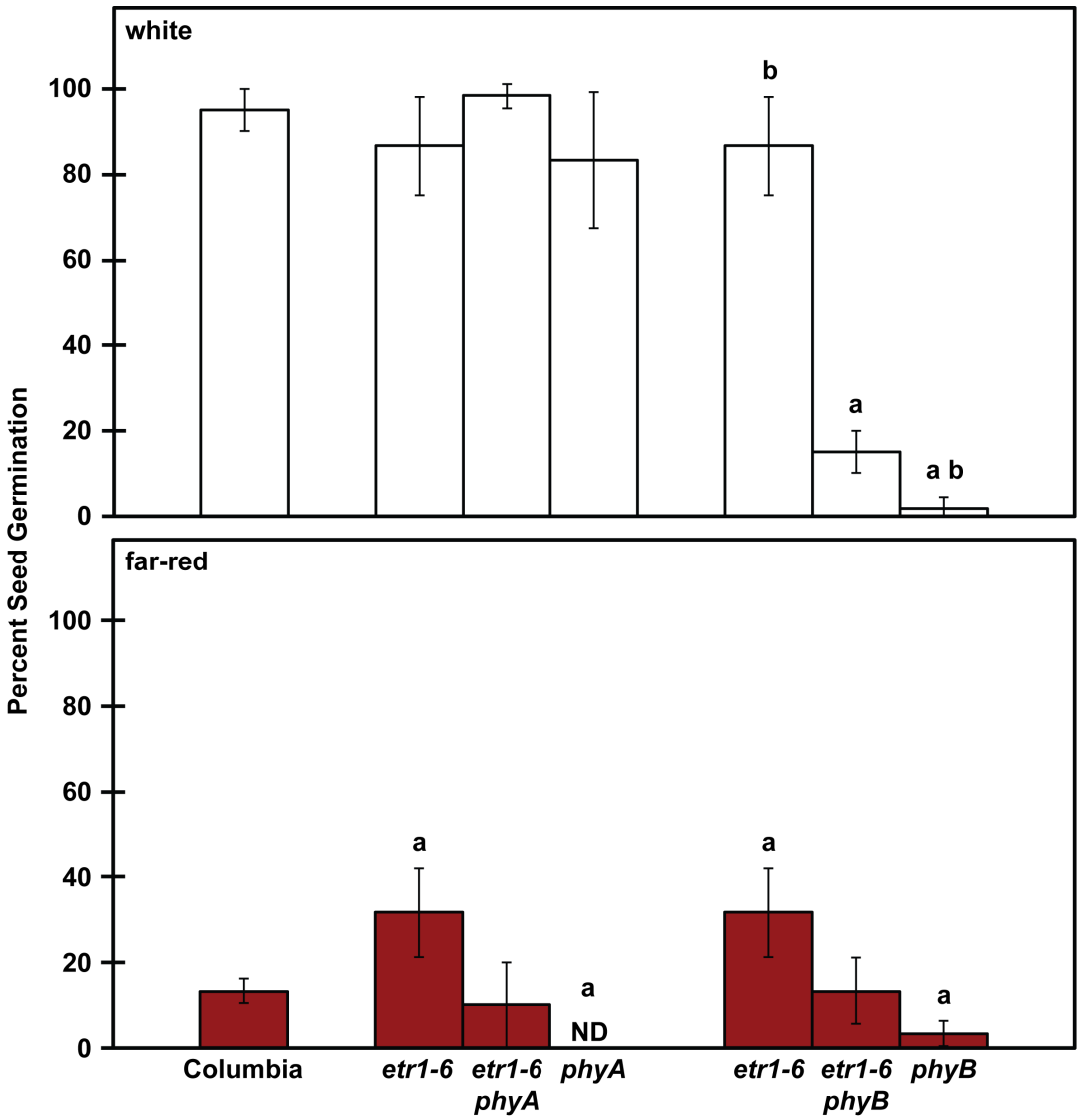


Figure III-11. Epistasis analysis of *etr1-6* and *phyA* or *phyB* germination following far-red treatment.

Seed germination of etr1-6 phyA or etr1-6 phyB double mutants compared to their single mutants and wild-type. Seeds were treated with 4 h of white light followed by an additional 3 h in white light (top) or 3 h in far-red light (bottom). The seeds were then placed in the dark for 7 days after which the number of germinated seeds was determined. The average percent of germinated seeds ± SD from at least three biological replicates is shown. [a]Denotes a statistical difference from wild-type in that light condition and [b]denotes a statistical difference from the double mutant in that light treatment where $p < 0.05$ with a t-test.

single mutants. This is a trait known to be affected by phytochromes. Consistent with previous research, the *phyA* and *phyB* mutants grew taller in far-red and red light respectively compared to wild-type (Figure III-12) (Reed et al., 1994). Growth of the *etr1-6* hypocotyl is similar to wild-type in both red and far-red light. In red light, the single *etr1-6* and *phyA* mutants are taller than the double *etr1-6 phyA* mutant (Figure III-12 top). In far-red light, *etr1-6* is shorter and *phyA* is taller than their double mutant (Figure III-12 bottom). Similarly, in red light, *etr1-6* is shorter and *phyB* is taller than their double mutant (Figure III-12 top) and in far-red there is no difference between the single and double *etr1-6 phyB* mutants (Figure III-12 bottom). These data suggest that ETR1 may genetically interact with PHYA and PHYB.

Discussion

The ethylene receptors have been shown to have unique and sometimes opposite functions to one another (Shakeel et al., 2013). Previously we showed that ETR1 and ETR2 function oppositely to inhibit and promote seed germination respectively during salt stress (Wilson et al., 2014). In this study we examined the role of the individual ethylene receptors in seed germination following far-red light treatment, another condition known to inhibit seed germination (Borthwick et al., 1952). We found that ETR1 inhibits germination following far-red light treatment. Although it was not evident in the single LOF mutant, epistasis analysis between *etr1* and *etr2* LOF mutants suggests that ETR2 may promote seed germination following far-red light as it does during salt stress.

It is known that *etr1* LOF mutants are slightly more sensitive and responsive to ethylene (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002) and that ethylene promotes seed germination in lettuce following far-red light treatment in conjunction with GA (Burdett and Vidaver, 1971). Therefore, it is possible that the improved germination of *etr1-6* could be due to an increase in ethylene production and/or sensitivity to ethylene by this mutant. However, we found that ethylene had no effect on *etr1-6* germination. This suggests that *etr1-6* is not more sensitive to ethylene for this trait, however we can not rule out the possibility that *etr1-6* produces a saturating amount of ethylene. In support of this, although we were unable to detect any ethylene

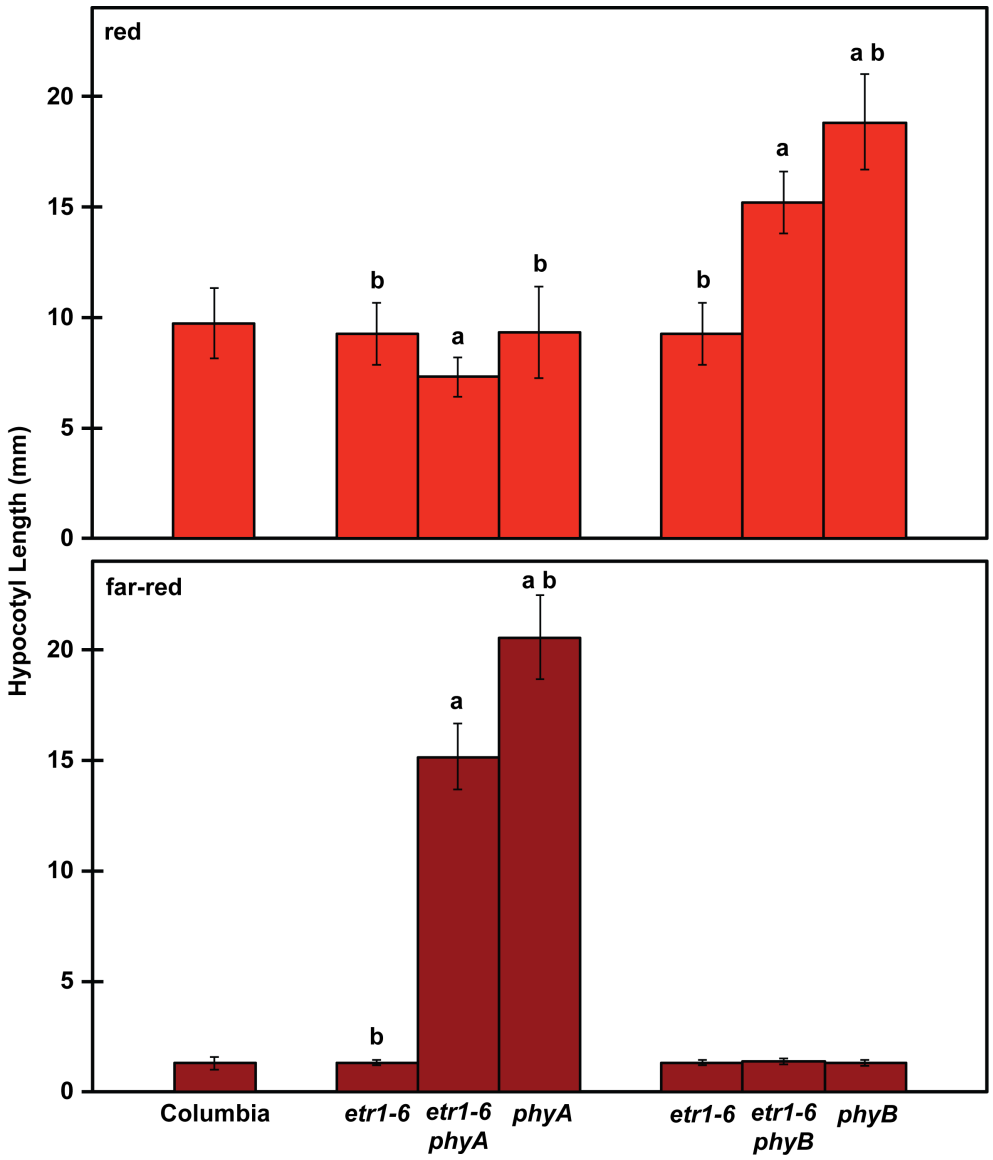


Figure III-12. Epistasis analysis of *etr1-6* and *phyA* or *phyB* hypocotyl growth following red and far-red treatment.

Hypocotyl lengths of etr1-6 phyA or etr1-6 phyB double mutants compared to their single mutants and wild-type. Seeds were treated with 24 h of white light followed by 6 days of continuous red light (top) or far-red light (bottom). The hypocotyl lengths were then measured. The average hypocotyl length ± SD of at least 10 seedlings is shown. [a]Denotes a statistical difference from wild-type in that light condition and [b]denotes a statistical difference from the double mutant in that light treatment where $p < 0.05$ with a t-test.

production prior to seed germination, addition of AVG to inhibit ethylene production reduced the germination of *etr1-6* seeds following far-red treatment by 20% while having no effect on the germination of Col or *etr2-3* seeds. This did not reduce germination of *etr1-6* seeds to that of Col, but suggests that contrary to during salt stress, ethylene is playing a role in the enhanced germination of *etr1-6* following far-red light treatment.

Differences in ABA production or sensitivity were likely the cause for the opposite effects of *etr1-6* and *etr2-3* on germination during salt stress. Surprisingly, however, addition of the ABA biosynthesis inhibitor norflurazon failed to improve the germination of Col or *etr2-3* following far-red light, but significantly improved the already enhanced germination of *etr1-6*. GA, on the other hand, significantly improved the germination of all three seed lines. Similar to our results, previous studies show that inhibition of ABA biosynthesis causes a very slight, but significant improvement of wild-type germination following far-red treatment (Seo et al., 2006), whereas addition of GA improves germination of far-red treated wild-type seeds dramatically (Oh et al., 2006). Analysis of the GA biosynthesis genes GA3ox1 and GA3ox2 suggests they were expressed at a low, but steady state level in *etr1-6* following far-red treatment while they were almost undetectable in wild-type. Additionally, analysis of ABA biosynthesis genes suggests that *etr1-6* produces less ABA than wild-type. This suggests that *etr1-6* produces more GA and less ABA than wild-type following far-red treatment and explains the improved germination of *etr1-6* in these conditions. It may also explain the enhanced germination of *etr1-6* under norflurazon treatment.

PIL5 is a negative regulator of seed germination that has been shown to enhance ABA synthesis while reducing sensitivity to and production of GA. The phytochromes have been shown to promote seed germination by negatively regulating PIL5. They do this by translocating into the nucleus when in their active Pfr form where they phosphorylate PIL5 ultimately leading to its degradation via the 26S proteosome (Oh et al., 2004; Oh et al., 2006; Oh et al., 2007). It is unlikely that the phytochromes would negatively regulate ETR1 which is located in the endoplasmic reticulum in the same manner as they regulate PIL5. Instead, epistasis analysis of *etr1-6 phyA etr1-6 phyB* suggests that ETR1 and PHYA/PHYB act in parallel to inhibit and promote germination respectively (Figure III-13). In support of this model, the EIN3/EIL1

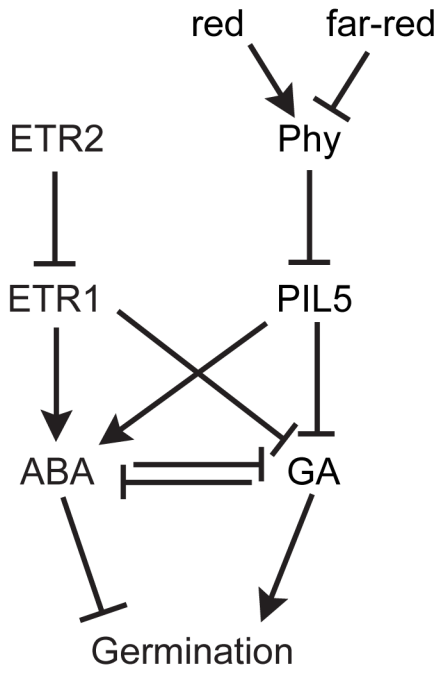


Figure III-13. Genetic model of ETR1 and ETR2 in germination following exposure to far-red light.

The phytochromes are known to promote seed germination by promoting degradation of PIL5, a negative regulator of seed germination. PIL5 inhibits seed germination by promoting ABA biosynthesis while inhibiting GA biosynthesis (Oh et al., 2004; Oh et al., 2006; Oh et al., 2007). In this model, in the presence of far-red light, ETR1 functions in parallel with the phytochromes and inhibits seed germination by promoting ABA biosynthesis while inhibiting GA biosynthesis. ETR2 inhibits the function of ETR1.

transcription factors were found to function in parallel with PIL5 to promote seedling greening (Zhong et al., 2009). Nevertheless, we cannot rule out the possibility that one of the other phytochromes promote germination through inhibition of ETR1.

Materials and Methods

Chemicals

The plant hormones gibberellic acid (GA) and abscisic acid (ABA) were obtained from ACROS Organics (Belgium). Norflurazon (NF), an inhibitor of ABA biosynthesis, was obtained from Fluka (Switzerland). Aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis was a gift from Rohm Haas (Philadelphia).

Plants

All of the plants used in this chapter were described previously (Hua and Meyerowitz, 1998; Qu and Schaller, 2004; Rösler et al., 2007; Sung et al., 2007; Kim et al., 2011). The *etr1-6*, *etr1-7*, *etr2-3*, *ein4-4*, *phya-t* and *phyb-9* mutants are in the Columbia (Col) background and the *etr1-9*, *ers1-3*, and *ers2-3* mutants are in the Wassilewskija (WS) background.

Germination Assays

In order to minimize biological variation, each experiment used a batch of age matched seeds between 250 and 300 μm in size from plants that were grown together in long day conditions as previously described (Hensel et al., 1993). Prior to use, the seeds were allowed to after-ripen in a room temperature desiccator for at least three weeks and were then sorted by size using sieves (Elwell et al., 2011). The seeds were surface sterilized in 70% ethanol for 30 seconds and allowed to dry on filter paper prior to imbibition on 0.8% (w/v) agar plates containing half-strength Murashige and Skoog (MS) basal medium with Gamborg's vitamins (Sigma, St. Louis, MO) at pH 5.7 with no added sugar. Where specified, GA, ABA, and NF were prepared as 10,000x stocks in ethanol, filter sterilized and added to the media at the indicated concentrations after autoclaving (the solvent control plates contained 0.01% ethanol). AVG was prepared in water, filter sterilized and added to the media after autoclaving. The seeds were exposed to

ethylene by placing the plates in a sealed container and injecting ethylene into the container to yield the indicated concentration. Twenty seeds of one genotype were placed on the agar plates in two rows of ten seeds with 5 mm space between the seeds. The plates were sealed with micropore surgical tape (3M, St. Paul, MN) to allow for gas exchange and prevent the accumulation of ethylene while also preventing water loss (Buer et al., 2003). The seeds were not stratified. Unless otherwise noted, after sowing, the seeds were treated with 45-55 μmol m^{-2} s^{-1} white light for 4 h followed by a 3 h treatment with 12 μmol m^{-2} s^{-1} blue (λ_{max} = 470 nm), green (λ_{max} = 525 nm), red (λ_{max} = 672 nm), or far-red light (λ_{max} = 732 nm). The monochromatic light was provided by LED arrays (Quantum Devices, Inc., Barneveld WI). As a positive control, seeds were treated with an additional 3 h of 45-55 μmol m^{-2} s^{-1} white light instead of the monochromatic light. Following these light treatments, the seeds were allowed to germinate for 7 days in the dark after which germination was scored. In some experiments, seeds were exposed to continuous light and germination was scored every 12-24 h. Germination of continuous monochromatic light treatment was scored in dim green light (about 0.02 μmol m^{-2} s^{-1}). After each experiment, plates containing seeds that did not germinate were transferred to white light and germination was evaluated after 7 days. In all cases, seeds germinated to at least 95% indicating that the seeds were viable. In all experiments, germination was scored as the visible rupture of the testa (seed coat).

Ethylene Measurements

The concentration of ethylene produced by 22 mg (dry weight) of seeds was measured every 6h using an ETD-300 photoacoustic ethylene detector (Sensor Sense, The Netherlands). Prior to the first measurement, the seeds were placed in a 6 mL glass vial on 1 mL half strength MS media prepared as described above, sealed with a rubber septum and treated with 4 h white light followed by 3 h white or far-red light as described above for the germination experiments.

Hypocotyl Growth Assay

For the hypocotyl growth assay, seeds were placed on agar plates and treated with 45-55 μmol m^{-2} s^{-1} white light for 24 h. The plates were then placed vertically under continuous 12 μmol m^{-2}

s^{-1} red or far-red light for 6 days. The plates were then scanned with a flat-bed scanner and the seedling length was measured using ImageJ (version 1.43u).

RNA Isolation and qRT-PCR

Total RNA was isolated from 25 mg dry seeds or 25 mg dry seeds imbibed in half strength MS with no added sugar and light treated as specified for the indicated times. RNA was isolated according to Meng and Feldman (2010), but instead of resuspending the pellet in Trizol, the RNA was further purified using the Spectrum Plant Total RNA Kit (Sigma, St. Louis, MO). Total RNA was treated with DNase I (Invitrogen) and 800 mg of the RNA was used for cDNA synthesis with the ImProm-II Reverse Transcription System (Promega) according to the manufacturer's instructions. Each qPCR reaction consisted of 5 μL of SsoFast EvaGreen Supermix (Bio-Rad), 0.5 μL each of the forward and reverse primers (10 μM) and 4 μL of cDNA diluted 1:8. The qPCR reactions were run on a BioRad iQ5 Real-Time PCR Detection System (Bio-Rad) with the following conditions: an initial denaturation step of 95 °C for 1 min followed by 45 cycles of 15 sec at 95 °C, 30 sec at 58 °C and 10 sec at 72 °C. Transcript data was normalized to At3g12210, which was validated as a stably expressed reference gene in Arabidopsis seeds by Dekkers et al. (2011), using the method of Livak and Schmittgen (2001) for each seed line at each condition to obtain the relative amount of transcript. The primers used for *GA3ox2* are 5'-GTTCTTTAATAAGAAGATGTGGTCCG-3' (forward) and 5'CATCAACTTGGCTGCCAACTTT-3' (reverse). The primers for GA3ox1, GA2ox2, ZEP, NCED6, NCED9, and CYP707A2 were described previously (Seo et al., 2004; Kim et al., 2012; Shu et al., 2013).

Chapter IV
Conclusions and Future Directions

The contribution of individual ethylene receptors to seed germination was examined during salt stress or following treatment with far-red light (Table IV-1). Both of these treatments are known to inhibit seed germination. NaCl stress is known to delay seed germination due to ion toxicity, by preventing imbibition, and through the action of the phytohormone ABA (Saleki et al., 1993). Far-red light reversibly inhibits seed germination through a phytochrome controlled increase in ABA biosynthesis and simultaneous decrease in GA biosynthesis and sensitivity (Oh et al., 2004; Oh et al., 2006; Oh et al., 2007). In both of these conditions, ETR1 was found to inhibit and ETR2 to promote seed germination. During seed germination under salt stress, high concentrations of exogenously applied ethylene were found to eliminate the difference in germination between *etr1-6* and *etr2-3*, but there was no evidence that physiologically relevant concentrations of ethylene or differences in sensitivity to ethylene were playing a role in the opposite effects of ETR1 and ETR2 on germination. On the other hand, differences in sensitivity or biosynthesis of ABA were found to play a large role in the opposite effects of ETR1 and ETR2 on germination. Therefore, it is possible that the ethylene receptors affect seed germination though both ethylene perception dependent and independent mechanisms. Following far-red treatment, treatment with the ethylene biosynthesis inhibitor AVG reduced the germination of *etr1*-6 suggesting that ethylene is playing a larger role in this condition than during salt stress. However, analysis of the transcript levels of ABA and GA metabolic genes, suggest that differences in ABA and GA synthesis are likely to underlie the difference in germination between wild-type and *etr1-6*. Although ETR1 inhibits germination during both NaCl stress and following far-red light, analysis of these responses suggest that it may function in slightly different ways in these conditions (Figure IV-1).

It is surprising that two ethylene receptors thought to signal through the same downstream components would have opposite roles in seed germination, however opposite roles for the ethylene receptors have been observed in other ethylene related phenotypes (Binder et al., 2006; Plett et al., 2009a; Kim et al., 2011). The next major step in this research is to identify the mechanism by which the ethylene receptors differentially control ethylene mediated responses.

Table IV-1 Unique Functions of the Ethylene Receptors

Trait	ETR1	ERS1	ETR2	EIN4	ERS2
Growth	++	++/-	+	+	+
Growth Recovery	+		+	+	
Nutations	+	-	-	-	-
Inhibition by Silver	++	+		+	+
Fumonisin B_1 Resistance	-			+	
Trichome Development			+		
Germination during salt stress	--		+	-	
Germination in far-red light	--		+		

A plus sign (+) indicates that the relative degree to which the receptor promotes the response. A minus sign (-) that indicates that the receptor inhibits the response. No sign indicates that the receptor was not observed to effect the response. This table is modified from Shakeel et al. (2013).

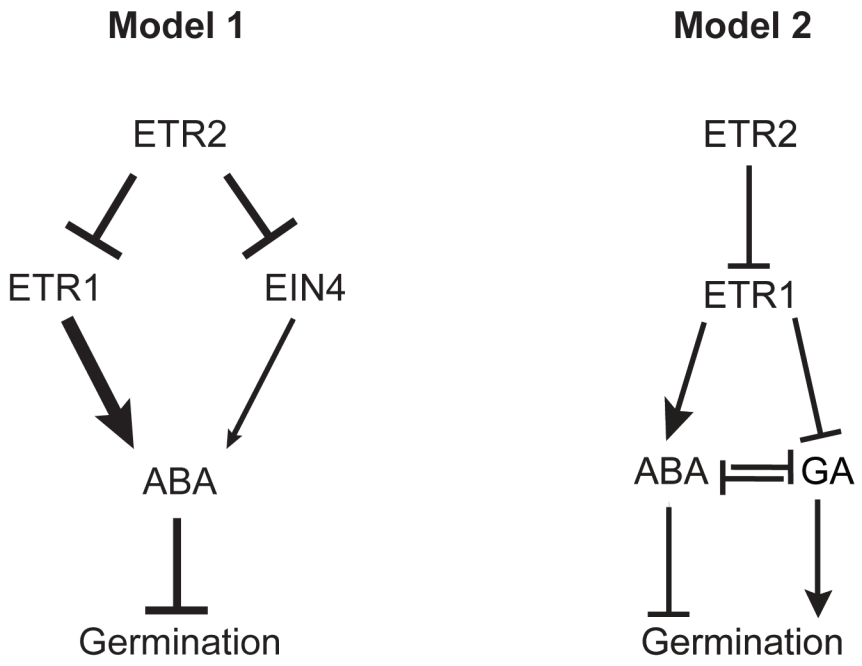


Figure IV-1. Genetic models for the role of ETR1, EIN4 and ETR2 in germination during salt stress and following exposure to far-red light.

During salt stress (Model 1), ETR1 and EIN4 are thought to inhibit seed germination by increasing ABA biosynthesis or sensitivity and ETR2 is thought to inhibit ETR1 and EIN4 function. Following far-red light treatment (Model 2), ETR1 is thought to inhibit seed germination by increasing ABA biosynthesis and decreasing GA biosynthesis.

Although time consuming, genetic epistasis analysis is an inexpensive method that can help elucidate the genetic interactions and possible differences in interaction of *etr1-6* and *etr2-3* with components of hormone signaling pathways.

Although ethylene is known to improve seed germination (Bleecker et al., 1988) and ethylene signaling has been shown to promote germination during salt stress (Wang et al., 2007; Wang et al., 2008; Lin et al., 2012), our data suggests that *etr1-6* and *etr2-3* may be affecting germination independently of ethylene perception. Nevertheless, *etr2-3* does have the same phenotype as *ein2* and the triple LOF mutant *etr1-6 etr2-3 ein4-4* has the same phenotype as *ctr1* mutants suggesting that they may function through ethylene signaling. Crosses between *etr1-6* or *etr2-3* and *ctr1* or *ein2* would help to determine if the receptors are functioning through the canonical ethylene signaling pathway. Because ETR1 and ETR2 are known to function upstream of CTR1 and EIN2, if ETR1 or ETR2 are functioning through these proteins, we would expect to see the *ctr1* or *ein2* phenotype in their respective crosses. If the *etr1 ein2* or *etr2 ctr1* double mutants yield an intermediate or wild-type phenotype or the *etr1 ctr1* or *etr2 ein2* mutants yield an additive phenotype (germinate better or worse than both single mutants), then this would suggest that ETR1 or ETR2 are signaling independently of the canonical ethylene signaling pathway.

The ETR2 loss-of-function mutant is hypersensitive to both NaCl and ABA during germination. Examination of germination of *etr2-3* crosses with ABA signaling components may help determine whether ETR2 functions to reduce sensitivity to ABA during germination under salt stress. If enhanced sensitivity to ABA is responsible for the poor germination of *etr2* during salt stress then mutations that reduce sensitivity to ABA should have improved germination similar to the ABA insensitive mutant. Beaudoin et al. (2000) showed that *ein2* reduces sensitivity to ABA by crossing it to the *abi3* LOF mutant that has greater than 1000 fold reduction in ABA sensitivity at germination. An *etr2 abi3* cross should yield the same insight into the function of ETR2.

During salt stress, GA only had a small effect on *etr2-3* seed germination and was not able to improve the germination of *etr2-3* seeds to that of *etr1-6*. However, we found that *etr1-6* was able to germinate to a higher percent than *etr2-3* and wild-type in the presence of the GA

biosynthesis inhibitor PAC. Although PAC affected seed germination differently than NaCl by predominantly reducing the percent of seeds that germinated rather than affecting the onset of germination, this result is still interesting. It suggests that even in the absence of salt stress, *etr1-6* produces more GA than wild-type, is more sensitive to GA than wild-type and/or is able to germinate independently of GA biosynthesis. We found that *etr1-6* seeds had higher transcript levels of the GA biosynthesis genes GA3ox1 and GA3ox2 four hours after the start of imbibition in white light and that these transcript levels remained higher in *etr1*-6 up to 24 hours after treatment with far-red light suggesting that *etr1-6* produces more GA than wild-type in both of these light conditions. In order to more thoroughly examine the effect of ETR1 on GA during seed germination, *etr1-6* could be crossed to a GA deficient mutant such as *ga1*. The *ga1* mutant cannot germinate in any light condition without exogenous application of GA, however, the *ga1 aba1* double mutant deficient in both GA and ABA biosynthesis is able to germinate (Koornneef et al., 1982). If the *etr1-6 ga1* double mutant, like the *ga1* mutant fails to germinate without exogenous GA application, this would suggest that *etr1-6* requires GA for germination and that ETR1 regulates seed germination through GA. On the other hand if, like the *ga1 aba1* double mutant, the *etr1-6 ga1* double mutant is able to germinate this would suggest that *etr1-6* functions predominantly by decreasing ABA biosynthesis or sensitivity and that the effects of GA are secondary to this.

Interestingly, loss of the cytokinin receptors, Arabidopsis Histidine Kinase2 (AHK2), AHK3, and AHK4, which like ETR1 resemble two-component histidine kinases, also leads to improved germination following far-red light treatment (Riefler et al., 2006). Unlike the ethylene receptors, the cytokinin receptors actually signal through a His-to-Asp phosphorelay that includes the Arabidopsis Histidine Phosphotransfer (AHP) proteins and the Arabidopsis Response Regulator (ARR) proteins (Hwang et al., 2012). ETR1 was shown to interact with AHP1, AHP2 and AHP3 in a yeast-two hybrid assay suggesting that ETR1 may directly interact with cytokinin signaling (Urao et al., 2000). AHP2 is known to interact with ARR4 (Imamura et al., 1999). ARR4 is hypersensitive to red light with respect to hypocotyl elongation (the *arr4* LOF has a shorter hypocotyl than wt) and has been shown to stabilize the Pfr form of PhyB. Additionally, the quadruple *arr3,4,5,6* mutant is hypersensitive to ABA (Wang et al., 2011). ARR4, ARR5 and ARR6 were all shown to interact with the ABI5 transcription factor and they are thought to

negatively regulate ABA signaling through this interaction (Wang et al., 2011). It would be interesting to determine whether ETR1 cross-talks with cytokinin signaling to affect seed germination and whether this could account for the opposite function of ETR1 and ETR2 in seed germination during salt stress or following far-red light treatment.

In addition to epistasis analysis, a more thorough method may be useful to shed light on the mechanism of ETR1 inhibition of seed germination during salt stress and following far-red light treatment and the opposing role of ETR2 in these conditions. A good place to start may be with analysis of the transcriptome and proteome of *etr1-6, etr2-3* and Col at multiple time points prior to seed germination in the presence and absence of salt stress and/or following far-red light treatment. One would expect to find opposite effects on the transcript, protein and metabolites between *etr1-6* and *etr2-3* compared to Col during the stress and less or no difference in the absence of the stress. Because the *etr1-6* mutant is in the *etr1-1* background and because these two mutants have opposite phenotypes on seed germination during salt stress, it may be helpful to include *etr1-1* either as a control to help eliminate false positives or possibly in place of *etr2-3* in these experiments. RNA sequencing and two-dimensional difference gel electrophoresis (2D DIGE) have been successfully used to identify transcript and protein differences respectively in Arabidopsis seeds (Chen et al., 2009a; Wang et al., 2013). It would also be insightful to examine the metabolome of these seeds or to look at specific hormones known or expected to be involved in these conditions. The successful profiling of specific hormones during germination of the *etr1-2* gain-of-function mutant could serve as a starting point for these particular experiments (Chiwocha et al., 2005). Transcript of the GA biosynthesis genes GA3ox1 and GA3ox2 were up regulated in *etr1-6* compared to wild-type after a four hour white light treatment. Although differences in GA production or sensitivity were not thought to play a role in germination during salt stress, it is possible that there is an increase in GA biosynthesis in *etr1-6* during germination on salt stress. The expression of these same genes could easily be evaluated during germination on salt stress.

Finally, because salt stress is biologically similar to drought and cold stress (Mahajan and Tuteja, 2005), it would be interesting to examine the role of the individual ethylene receptors (particularly ETR1 and ETR2) during these conditions.

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Appendix

Site-directed mutagenesis of ETR1 receiver domain

Introduction

Nutations are ethylene dependent nodding motions made by both the root and hypocotyl that are thought to facilitate their penetration through the soil (Binder et al., 2006). ETR1 has a unique role amongst the ethylene receptors in ethylene stimulated nutations. It is the only ethylene receptor that is required and sufficient for nutations (Binder et al., 2006; Kim et al., 2011). Interestingly, the ETR1 receiver domain has been shown to be required for nutations, but neither histidine kinase activity nor phosphotransfer through the receiver domain are required for nutations (Binder, 2006; Kim et al., 2011). Furthermore, a chimeric ETR1 protein that contains the EIN4 receiver domain in place of its own receiver domain fails to rescue nutations. This suggests a unique function for the receiver domain of ETR1.

In order to examine the unique function of the ETR1 receiver domain in various traits including ethylene stimulated nutations, thirteen amino acid residues in the ETR1 receiver domain were targeted for site-directed mutagenesis (Figure A-1). Because replacing the ETR1 receiver domain with the EIN4 receiver domain and expressing it under the control of the ETR1 promoter fails to rescue nutations, we chose residues for site-directed mutagenesis that are differ between ETR1 and EIN4. To further narrow down the residues that may be important, we took advantage of the crystal structure available for the receiver domain of ETR1 (Müller-Dieckmann et al., 1999) and chose residues that may result in the functional difference of ETR1. The gamma loop of ETR1 is interesting because its orientation differs from that of the bacterial receiver domains. Four divergent residues in the gamma loop of ETR1 (Gly664, Val665, Glu666, and Asn667) will be the targets of alanine scanning site-directed mutagenesis. Gly664 is particularly interesting because the crystal structure shows that it forms a hydrogen bond with Lys702 and may contribute to the unusual orientation of the gamma loop in ETR1. Glu617, which is part of the active site, and the nearby residues Asn618 and Cys661 will also be investigated. The remaining residues that we will target (Gln681, Arg682, Gln684, Glu730, Leu734, and Tyr735) are all thought to be important for dimerization of the receiver domain. Glu730 and Leu734 are particularly interesting because a homology model of EIN4 shows this region to be in a drastically different orientation than that of ETR1.

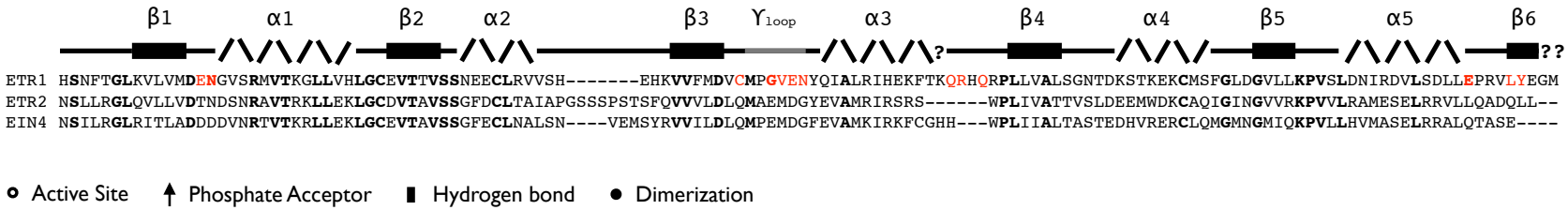


Figure A-1. Arabidopsis ETR1 receiver domain amino acid residues 604-738.

The residues highlighted in red were be targeted for alanine scanning site-directed mutagenesis. The Secondary structure is adapted from Müller-Dieckmann et al. (1999)

Results

Constructs containing the mutated *getr1* or a wild-type *gETR1* were transformed into Arabidopsis lacking the three receiver domain containing ethylene receptors (ETR1, ETR2, EIN4) and 2-3 independent homozygous lines were identified for twelve of these constructs and wild-type *gETR1* (Table A-1). Initially, etiolated seedlings of these plants will be evaluated for rescue of growth in air, growth recovery after ethylene removal and nutations, but these lines may also be of use in identifying residues important for other unique ETR1 functions. Because the receiver domain is not required to rescue growth in air and growth recovery after removal of ethylene (Kim et al., 2011), we expect all of the constructs to rescue these phenotypes indicating that a functional protein is made. Therefore, any mutations that rescue growth in air or growth recovery, but fail to rescue nutations will indicate that wild-type amino acid is required for nutations. These mutant lines are now being analyzed.

Materials and Methods

Plasmid Construction

Construction of the plasmids pBluescript II SK- gETR1 and pPZP211-gETR1 were described previously (Wang et al., 2003; Binder et al., 2004b). Several silent mutations were incorporated into pBluescript II SK- gETR1 leading to a novel avrII restriction site in ETR1 that can be used for genotyping. These mutations, along with the following ones, were made using LaTaq polymerase (TaKaRa) according to the manufacturer's instructions with the primers listed in Table A-2. This plasmid was then used as the template to create the following point mutations in gETR1: E617A, N618A, C661A, G664A, V665A, E666A, N667A, Q681A, R682A, Q684A, E730A, L734A and Y735A. The wild-type and mutant gETR1 fragments containing the silent mutations were then cloned into the pPZP211-gETR1 plasmid with the restriction enzymes AflII and KpnI. All plasmids constructs and point mutations were confirmed by sequencing.

Generation of Transgenic Lines

All of the gETR1 constructs created above were transformed into *Agrobacterium tumefaciens* strain GV3101 pMP90 and then transformed into Arabidopsis *etr1-6 etr2-3 ein4-4* plants with

Table A-1. Homozygous lines and progress of site-directed mutagenesis of ETR1 receiver domain.

Independent homozygous lines for each mutation and wild-type containing silent mutations for genotyping in *etr1-6 etr2-3 ein4-4* were identified by analyzing both segregation of kanamycin resistance and complementation of dark grown seedling hypocotyl length. The line numbers are given.

Construct	Homozygous lines
gETR1	L3 (4-2), L4 (6-2), L7 (3-6)
E617A	L6 (1-2), L7 (2-3), L10 (8-1)
N618A	L4 (5-3), L6 (3-1)
C661A	L4 (5-2), L5 (5-4)
G664A	no homozygous lines identified in T3 seeds
V665A	L2 (6-2), L8 (6-3), L9 (3-2)
E666A	L2 (8-2), L6 (1-3), L7 (1-4)
N667A	L5 (3-4), L6 (8-2), L13 (2-8)
Q681A	L3 (3-3), L4 (4-2), L6 (3-12)
R682A	L3 (4-2), L9 (1-1)
Q684A	L4 (3-2), L7 (1-6), L10 (8-2)
E730A	L4 (1-A), L7 (3-5)
L734A	L3 (2-3), L6 (3-1), L10 (7-5)
Y735A	L2 (4-4), L7 (1-2)

Table A-2. Primers used for site-directed mutagenesis

The primers for the point mutations were designed by HeeJung Kim.

Primer Name	Primer Sequence 5' to 3'
etr1-silent-F	CAGCCATTCCtaGgCAcagtAATTTCACTGGACTTAAGG
etr1-silent-R	CAGTGAAATTACTGTGCCTAGGAATGGCTGGAACTTTCG
E617A F	CATGGATgctAACGGGTTAGTATAAGC
E617A R	ACCCGTTagcATCCATGACAAGAACCT
New 618A F	atgaggctgggttagtataagctt
New 618A R	aacccagcctcatccatgacaa
661A F	catggacgtgGCTatgcccggggtcgaaaac
661A R	cgggcatAGCcacgtccatgaagaccac
G664A F	CATGCCCgctGTCGAAAACTACCAAATCG
G664A R	GTTTTCGACagcGGGCATGCACACGTCCATG
V665A F	GCCCGGGgctGAAAACTACCAAATCGCTCT
V665A R	AGTTTTCagcCCCGGGCATGCACACGTCCATG
E666A F	CGGGGTCgctAACTACCAAATCGCTCTCCGTAT
E666A R	TGGTAGTTagcGACCCCGGGCATGCACACG
N667A F	GTCGAAgctTACCAAATCGCTCTCCGTAT
N667A R	ATTTGGTAagcTTCGACCCCGGGCATGC
Q681A F	TCACAAAAgctCGCCACCAACGGCCACTAC
Q681A R	TGGTGGCGagcTTTTGTGAATTTCTCGTGAAT
R682A F	CAAAACAAgctCACCAACGGCCACTAC
R682A R	CGTTGGTGagcTTGTTTTGTGAATTTCTCG
Q684A F	CGCCACCAAgctCCACTACTTGTGGCA
Q684A R	GTAGTGGCCGagcGTGGCGTTGTTTTGTG
New 730A F	ttctcgctccccgggtactgt
New 730A R	tacccggggagcgagaagat
L734A F	CCGGGTAgctTACGAGGGCATGTAAAG
L734A R	TGCCCTCGTAagcTACCCGGGGCTCGAGAA
Y735A F	GTACTGgctGAGGGCATGTAAAGGC
Y735A R	ATGCCCTCagcCAGTACCCGGGGCT

the floral dip method. T_1 seeds were plated on 0.8% (w/v) agar plates containing half-strength Murashige and Skoog (MS) basal medium with Gamborg's vitamins (Sigma, St. Louis, MO) at pH 5.7 with no added sugar and 50 μg/mL kanamycin and resistant seedlings were transferred to soil. Two to three homozygous lines were identified for wild-type gETR1 and each mutation by analyzing both segregation of kanamycin resistance and complementation of dark grown seedling hypocotyl length.

Vita

Rebecca Wilson was born in Bradenton, FL. She graduated from Messiah College with a Bachelor of Science degree in biochemistry. In 2014, she received a Ph.D. from the University of Tennessee.