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INVOLVEMENT OF THE ETR1 AND ERS1
ETHYLENE RECEPTORS IN REGULATING
SEED DORMANCY IN ARABIDOPSIS

A thesis

presented to

The Faculty of Graduate Studies

of

Carleton University

by

Graham B. Thurston

In partial fulfillment of the requirements

for the degree of

Master of Science

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Abstract

In this thesis, two papers and two appendices are presented. In the first paper, we propose that the ethylene receptors serve non-redundant roles based on phenotypic analysis of the dormancy pattern of developing gain-of-function mutant seeds. We have observed that two receptor mutants, *etr1-1* and *ers1-1* show completely opposite patterns of dormancy with respect to wild-type seeds. The developing *etr1-1* mutant seeds have an increased dormancy while the developing *ers1-1* mutant seeds appear to be less dormant. We support this proposal of non-redundancy with gene expression data of the receptors throughout plant development as well as a phylogenetic analysis of receptor protein sequences across various plant species. The relative levels of four other hormones and their metabolites may be responsible for the observed dormancy pattern of the *ers1-1* and *etr1-1* gain-of-function mutant seeds as we show that these mutations significantly affect the levels of abscisic acid, cytokinins, auxins and gibberellins in developing siliques and seeds. Thus it is possible that these receptors may signal through or converge with different hormone pathways to elicit the observed phenotype. In the second paper, we investigate the paradigm that the ETR1 and ERS1 receptors signal through CTR1, a downstream repressor of the ethylene signal transduction pathway. The non-dormant pattern of developing loss-of-function *ctr1-1* mutant seeds and the previously mentioned dormancy patterns of the developing *ers1-1* and *etr1-1* mutant seeds was interesting as both receptors are proposed to signal through CTR1. The dormancy pattern of these two receptor loss-of-function mutants throughout seed

development revealed a non-dormant phenotype for loss-of-function *etr1* and loss-of-function *ers1* mutants. Possible explanations are presented to justify these results with respect to receptor signalling. Appendix I describes the preliminary data on creating double mutants between gain-of-function ethylene receptor mutants and abscisic acid-deficient mutants. Absciscic acid is an important repressor of seed germination and the single abscisic acid-deficient mutants, *aba1-6*, *aba2-3* and *aba3-1*, revealed an expected non-dormant phenotype throughout seed development. Independent *etr1-1;aba1-6*, *etr1-1;aba2-3*, *etr1-1;aba3-1*, *ers1-1;aba1-6*, *ers1-1;aba2-3* and *ers1-1;aba3-1* double mutants were created for the analysis of the contribution of other hormone pathways, in this case abscisic acid, on the determination of dormancy in the later stages of seed development. Appendix II describes the preliminary work on creating global gene expression profiling for wild-type, *etr1-1* and *ers1-1* silique and seed tissue at two key stages in development. Developing seeds and siliques for stage 2, the stage prior to the entrance into dormancy and stage 5, the stage after dormancy, were used for this analysis as these stages were predicted to show the greatest difference in gene expression between the mutants and wildtype.

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Abbreviations

2iP	isopentenyladenine
7'OH-ABA	7'-hydroxy-ABA
A	Adenine
a.a.	Amino acid
ABA	Abscisic acid
ABA1 or aba1	ABA deficient 1
ABA2 or aba2	ABA deficient 2
ABA3 or aba3	ABA deficient 3
ABA-GE	ABA glucose ester
ABI or abi	Abscisic acid insensitive
ACC	1-aminocyclopropane-1-carboxylic acid
AdoMet	S-adenosylmethionine
AGI	Arabidopsis genome initiative
Asp	Aspartic acid
c.s.	Coding sequence
CIA	Chloroform:isoamyl alcohol (24:1)
Col	Columbia
C-terminal	Carboxy-terminal
CTR1 or ctr1	Constitutive triple response 1
Cys	Cysteine
DAP	Days after pollination
DPA	Dihydrophaseic acid
DW	Dry weight
EIN2 or ein2	Ethylene insensitive 2
EIN3 or ein3	Ethylene insensitive 3
EIN4 or ein4	Ethylene insensitive 4
EMS	Ethyl methanesulfonate
ER	Endoplasmic reticulum
ERA or era	Enhanced response to ABA
EREBP	Ethylene response element binding protein
ERS1 or ers1	Ethylene response sensor 1
ERS2 or ers2	Ethylene response sensor 2
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
ETR1 or etr1	Ethylene response 1
ETR2 or etr2	Ethylene response 2
g	Gram
G	Guanine
GA	Gibberellic acid
GAF	cGMP-specific and -regulated cyclic nucleotide phosphodiesterase, <u>a</u> denylyl cyclase, <u>E</u> h1A
GIN1 or gin1	Glucose insensitive 1
GIN4 or gin4	Glucose insensitive 4

GIN5 or gin5	Glucose insensitive 5
Glc	Glucose
GOF	Gain-of-function
His	Histidine
HPLC	High performance liquid chromatography
Hpt	Histidine-containing phosphotransfer protein
IAA	Indole-3-acetic acid
IAAsp	Indole-3-acetyl-aspartate
IPA	Isopentenyladenosine
KOH	Potassium hydroxide
LOF	Loss-of-function
M&S	Murashige and Skoog
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
Met	Methionine
ng	Nanogram
N-terminal	Amino-terminal
PA	Phaseic acid
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
Ws	Wassilewskija
WT	Wildtype
Z	Zeatin
ZR	Zeatin riboside

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Introduction

Plants are sessile and have therefore evolved very complex response mechanisms to deal with continuously changing of biotic and abiotic factors. There are five classical phytohormones including abscisic acid, gibberellic acid, auxin, cytokinins and ethylene, although many other small molecular weight compounds such as brassinosteroids, polyamines and even some types of sugars are being recognized as major signalling compounds in plants. Phytohormones can act as positive or negative signals for various physiological phenomenon including growth and development as well as many stress responses. For instance, auxins participate in cell expansion associated with phototropic movement; abscisic acid is involved in the plant's response to drought; gibberellic acid has a positive effect on seed germination; ethylene participates in signalling many responses associated with pathogens and cytokinins are involved in senescence of leaf tissue (Taiz and Zeeger, 2002). Our current understanding of phytohormone action has been made possible through investigations using model plant species such as tomato, pea, rice and barley but mostly from studies involving *Arabidopsis thaliana*.

Arabidopsis belongs to the *Brassicaceae* family, which also include agronomically important crop species such as mustard, broccoli, kale, cauliflower, and canola. The genome of *Arabidopsis* has been completely sequenced and annotated (*Arabidopsis* Genome Initiative, 2000). For plants, it has a relatively small genome (approximately twenty five thousand genes), making it well suited for genetic studies. The plethora of resources available in

Arabidopsis has facilitated the discovery and characterization of many genes. Many gene knockouts, gain-of-function and loss-of-function mutants are available through seed stock centres such as the Arabidopsis Biological Resource Centre (ABRC) or the Nottingham Arabidopsis Stock Centre (NASC). Arabidopsis is also amenable to many transformation techniques, allowing for the introduction of reporter-fusion constructs, for tracking gene expression or protein localization, or generation of other types of transgenic plants. Arabidopsis is a prolific seed producer and also has a short life cycle, going from seed to seed in just a few weeks time and it can be easily grown in greenhouses or growth chambers anywhere in the world. These features are among just some of the reasons why Arabidopsis is at the forefront of physiological, biochemical and genetic studies in plant systems.

Ethylene, despite its simple structure, is an important hormone in plants. It is involved in the regulation of many developmental processes including: cell elongation and division, flowering, fruit ripening, seed germination, organ senescence and abscission as well as many other abiotic and biotic stress responses (Abeles *et al.*, 1992; Johnson and Ecker, 1998). Ethylene is unique among the major plant hormones as it is a gas and is freely diffusible throughout the plant. Perhaps then, it is not surprising that ethylene is perceived inside the cell at the endoplasmic reticulum (ER) (Chen *et al.*, 2002) as opposed to the periphery of a cell (plasma membrane anchored receptor).

The hierarchy of the components in the ethylene signal transduction pathway has been delineated largely due to the use of mutant screens of seedlings displaying altered triple response phenotypes. The triple response is a morphological response to high levels of ethylene in etiolated seedlings and is characterized by a shortened root, a shortened and thickened hypocotyl, and an exaggerated apical hook (Figure 1) (Guzman and Ecker, 1990). The various mutants identified to date fall into three distinct categories: constitutive triple-response mutants, ethylene-insensitive mutants and those mutants that affect only a subset of organs (Kieber, 1997; Johnson and Ecker, 1998). Ethylene is perceived by a multigene family of receptors in *Arabidopsis* that share similarity to bacterial two-component regulators; in fact this was the first two-component regulator to be identified in plants (Chang *et al.*, 1993). Two-component systems in bacteria are involved in responding to many environmental stimuli including chemotaxis and osmotic sensing (Parkinson and Kofoed, 1992). Two-component regulators are proteins which modify the activity of a target protein through a phosphotransfer relay. Protein modification by phosphorylation predominantly occur on serine, threonine or tyrosine residues, however two-component regulators phosphorylate on histidine (His) and aspartic acid (Asp) residues (Schaller *et al.*, 2002). A bacterial two-component system is comprised of two proteins, a sensor His kinase protein (receptor) and a response regulator protein (usually a transcription factor). When the receptor is stimulated, the His kinase domain of the sensor protein (receptor) autophosphorylates on a conserved His residue. This catalyzes a phosphotransfer from this His residue to a conserved

Figure 1: The triple response in *Arabidopsis*. This figure illustrates the morphological change of etiolated seedlings (three days) when germinated in the presence of air compared to ethylene. When wild-type seeds are germinated in the dark (etiolated here for three days) in the presence of air (right), the resulting seedlings have elongated hypocotyls. This elongation occurs when a seed is buried deep in the soil and is an attempt to push the cotyledons, and apical meristem, upwards towards the light source. When wild-type seeds are germinated in the dark and in the presence of high levels of ethylene (or its biochemical precursor 1-aminocyclopropane-1-carboxylic acid – ACC)(left), the resulting seedlings display a remarkable change in morphology, coined the triple response. The triple response is characterized by a shortened root, a shortened and thickened hypocotyl, and an exaggerated apical hook (note in figure, curvature of apical is difficult to distinguish as it is curving outwards from the page). It is believed that in situations where the germinating seed encounters a physical obstacle in the soil, ethylene is produced as a stress hormone. The exaggeration of the apical hook is believed to protect the apical meristem from damage as the seedlings attempts to grow around the obstacle. Mutants of various components from the ethylene signal transduction pathway have been isolated from mutagenized seed populations based on altered triple response morphology. For instance, the ethylene receptor ETR1 was isolated in a screen for ethylene insensitivity. The ETR1 mutant exhibited an ethylene insensitive morphology (similar to wild-type seedling morphology in air) when grown in the presence of ethylene. Subsequent characterization of this mutant revealed that the mutant ETR1 receptor was incapable of binding ethylene.

Asp residue in the receiver domain of the response regulator (usually a transcription factor) protein. This phosphorylation modifies the response regulator such that the appropriate transcription response is initiated (Schaller *et al.*, 2002). Plant two-component systems are built upon the same principal, but with some alterations, probably due to difference between bacterial and plant cell architecture. In plant systems, there exists a multi-step phosphorelay to transduce the signal from the receptor to downstream effectors. The sensor protein (receptor) is usually a hybrid histidine kinase protein, in that the receiver domain (found in the bacterial response regulator protein) is fused to the histidine kinase domain of the sensor protein. A second protein, a histidine-containing phosphotransfer (HPt) protein acts as an intermediary between the sensor protein and the response regulator. The phosphotransfer occurs from His to Asp residues of the sensor protein (receptor) to the His residue of the HPt protein and finally to the Asp residue of the response regulator protein which mediates the proper response (Schaller *et al.*, 2002). An HPt protein can allow a signal to be transduced from two distant regions of the cell, such as the plasma membrane and the nucleus. The cytokinin receptor(s) of Arabidopsis follow this hybrid kinase to HPt protein to response regulator protein phosphoryl cascade to regulate cytokinins signalling (Hutchison and Kieber, 2002).

The first ethylene receptor, ETR1, was identified in Arabidopsis (Bleecker *et al.*, 1988; Chang *et al.*, 1993). Based on DNA sequence similarities, four other *ETR1*-like receptors have been isolated and characterized, these include *ERS1*, *ETR2*, *EIN4* and *ERS2* (Hua *et al.*, 1995; Hua *et al.*, 1998; Sakai *et al.*, 1998).

The ethylene receptor family of *Arabidopsis* has been subdivided into two subfamilies based on the presence or absence of certain protein domains (Hua *et al.* 1998). All receptors contain a sensor domain consisting of an N-terminal membrane (ER) spanning domain responsible for ethylene binding, a GAF domain of unknown function and a putative C-terminal His kinase domain (Hua *et al.*, 1998). Subfamily I (ETR1 and ERS1) have three predicted N-terminal membrane spanning domains and also contain all the conserved residues required for functional His kinase activity. Subfamily II (ETR2, ERS2 and EIN4) receptors lack one or more conserved residues needed for His kinase activity, as well, they all contain an extra N-terminal hydrophobic membrane spanning domain. ETR1, ETR2 and EIN4 also have a C-terminal receiver domain (Hua *et al.*, 1998). Thus, ETR1, ETR2 and EIN4 are two-component hybrid His kinase sensor proteins, as they contain a sensor protein with a fused receiver domain, while ERS1 and ERS2 more closely resemble the prokaryotic two-component His kinase sensor protein. The receptors have been isolated as homodimers from membrane protein extracts, and are believed to function as homodimers. However there is some speculation as to whether different receptors can form heterodimers in the ER signalling complex.

Dominant gain-of-function (GOF) mutations in the N-terminal sensor domain of any of the receptor homologues relieves the association with an essential copper co-factor (Rodriguez *et al.*, 1999) and confers ethylene insensitivity which suggests that all receptors participate in sensing ethylene (Hua and Meyerowitz, 1998). Recessive loss-of-function (LOF) mutations in

single receptors, which cause unstable or truncated protein products, do not show appreciable phenotypes with respect to the triple response assay, and it is not until combinatorial receptors are knocked out that constitutive ethylene responses are observed (Hua and Meyerowitz, 1998). Taken together, these data suggested that the receptors perform redundant or overlapping functions and that the ethylene receptors function to negatively regulate ethylene responses.

The current model of ethylene signalling from the receptors involves a protein complex at the ER. CTR1, a Raf-like serine/threonine mitogen activated protein kinase (MAPK) kinase kinase, is a negative regulator of the ethylene signalling pathway, as loss-of-function mutants display constitutive ethylene responses (Kieber *et al.*, 1993). CTR1 has been co-purified with ETR1 from membrane extracts and has been shown to directly interact with the histidine kinase domains of ETR1 and to a lesser extent ERS1 as well as ETR2 *in vitro* (Clark *et al.*, 1998; Gao *et al.*, 2003). The CTR1 protein is composed of a novel N-terminal domain, which is responsible for the interaction with the receptors, and a C-terminal Raf-like kinase domain responsible for signal output (Gao *et al.*, 2003, Huang *et al.*, 2004). Evidence that CTR1 is required as a signalling intermediate comes from double mutant analysis between all GOF receptor mutants with the LOF *ctr1-1* mutant. The introduction of the constitutive ethylene response phenotype (always triple response regardless if grown in air or ethylene) of the *ctr1-1* mutant into the dominant ethylene insensitive (never triple response, regardless if grown in ethylene or air) GOF mutant receptor backgrounds

resulted in double mutant lines showing the constitutive ethylene responses (Hua *et al.*, 1995). Therefore the receptors must signal through CTR1. Further support comes from observations that specific mutations in the N-terminal or C-terminal regions of CTR1 results in constitutive ethylene responses. Mutation of specific conserved residues of the N-terminal domain (*ctr1-8*) alleviate the interaction between the receptors and CTR1, resulting in mis-localization of CTR1 to the cytosol (Huang *et al.*, 2003) abolishing signal transmission from the receptors. Whereas mutations that alleviate CTR1 kinase activity (*ctr1-1*) do result in proper localization of CTR1 to the ER protein complex, however de-repression of ethylene responses are compromised due to the non-functional CTR1 kinase domain (Gao *et al.*, 2003; Huang *et al.*, 2003). Signalling is therefore thought to progress through CTR1 and possibly through a MAPK cascade (Hua and Meyerowitz, 1998), however the placement of a MAPKK and MAPK into this cascade has yet to be confirmed (Ouaked *et al.*, 2003; Ecker, 2004).

The exact domains required for signal transmission from the ethylene receptors to CTR1 is currently unknown. The region of the ethylene receptors shown to be involved in the interaction with CTR1 is found in the C-terminal His kinase domain. However, there are a few lines of evidence that put the hypothesis of CTR1 regulation by His Kinase activity of the receptors into question. 1) The presence of a degenerate His kinase domain in subfamily II receptors and the ability of GOF subfamily II receptors to confer ethylene insensitivity 2) The His kinase domain of ETR1 is required for CTR1 binding, however the activity of this domain is not required for binding (Gao *et al.*, 2003).

3) The recent discovery that all of the receptors except ETR1 autophosphorylate predominantly on serine residues *in vitro* (Moussatche and Klee, 2004). These data taken together may suggest that ethylene signal transduction from receptor to CTR1 may not occur through a His kinase phosphorelay mechanism. However these data do not preclude the possibility of the receptors modifying other, yet unidentified, CTR1-like proteins or other proteins present in the protein complex at the ER involved in regulating CTR1 kinase activity. The presence of hybrid two-component His kinase receptors and a Ser/Thr CTR1 MAPKKK module in the same signalling system raises the possibility that ethylene signalling may be mediated through a novel type of phosphorelay pathway, one that would potentially incorporate elements of the prokaryotic two-component system and the distinctly eukaryotic MAPK system. Regardless of the precise mechanism of signal transmission, it is proposed that the ethylene receptors negatively regulate CTR1. As shown in Figure2, when ethylene is absent, the receptors are 'active' and associate with CTR1. This interaction 'activates' CTR1 and thus represses downstream ethylene responses. In the presence of ethylene, the receptors become 'de-activated' presumably by autophosphorylation, thus causing a change in the ability of the receptor to interact with CTR1, thereby de-repressing CTR1 activity. The 'de-activated' CTR1 de-represses downstream ethylene responses. Downstream components of this pathway include positive regulators such as EIN2, a putative nuclear transporter protein, transcription factors such as EIN3-like proteins and ethylene response binding proteins (EREBP) (reviewed in Chang and Shockey, 1999).

Figure 2: A simplified schematic of the ethylene signal transduction pathway, highlighting early signalling events – receptors and CTR1. Arrows represent positive regulation, while bars represent negative regulation.

A. The ethylene signal transduction pathway is thought to occur through a phosphorylation cascade transmitting the signal from the receptors to the nucleus. The five ethylene receptors are located at the endoplasmic reticulum (ER) and negatively regulate the signalling pathway. All five receptors signal through the mitogen activated protein kinase (MAPK) kinase kinase CTR1, a negative regulator of the ethylene signal transduction pathway. A MAPKK and MAPK are thought to exist in this signalling pathway, however individual proteins have not been identified to date. EIN2, a positive regulator of the ethylene signal transduction pathway is putative nuclear transporter protein and is thought to transduce the signal into the nucleus. EIN3 and EIN3-like (EILs) proteins are involved in activating specific subsets of ethylene regulated genes.

B. The five ethylene receptors ETR1, ETR2, ERS1, ERS2 and EIN4 form homodimers and are comprised of specific protein domains. All receptors have an N-terminal ER membrane spanning domain, however subfamily I receptors (ETR1 and ERS1) have only three membrane spanning regions while subfamily II receptors (ETR2, ERS2 and EIN4) have four membrane spanning regions. All ethylene receptors contain an N-terminal hydrophobic motif for association with a copper co-factor (Cu) required for ethylene binding. All receptors contain a GAF domain (diamond) of unknown function and a C-terminal histidine (His) kinase domain (rectangle). Subfamily I receptors contain all the required residues thought necessary for His kinase activity (black), while Subfamily II receptors lack one or more key residues required for His kinase activity (white). ETR1, ETR2 and EIN4 also contain a C-terminal receiver domain (circle), therefore these receptors resemble the two-component hybrid receptor kinases while ERS1 and ERS2 more closely resemble the prokaryotic two-component receptor kinases. In air, all five receptors are 'active' and negatively regulate CTR1 (dark grey). This interaction 'activates' CTR1 and thus represses downstream ethylene responses.

C. In the presence of ethylene (light grey), the receptors become 'de-activated' presumably by autophosphorylation, thus causing a change in the ability of the receptor to interact with CTR1, thereby de-repressing CTR1 activity (indicated by change in shape). The 'de-activated' CTR1 de-represses downstream ethylene responses.

Because ethylene is a gas and can presumably diffuse throughout the plant, it is reasonable to expect that ethylene production must be tightly regulated. The production of ethylene follows a short linear biosynthetic pathway (Yang and Hoffman, 1984; Wang *et al.*, 2002). Briefly, methionine (Met) is converted via S-Adenosylmethionine (AdoMet) synthetase into AdoMet, which in turn is converted, in the first committed step of synthesis, into 1-aminocyclopropane-1-carboxylic acid (ACC) by a multigene family of ACC synthase proteins. ACC is converted into ethylene in an oxygen dependant reaction by ACC oxidase (Yang and Hoffman, 1984; Wang *et al.*, 2002). Due to the multiple developmental and stress responses that are associated with this hormone, an important biological question that researchers have been addressing is how a plant can regulate ethylene production and ethylene signalling in such a way as to moderate the appropriate developmental or stress response for a given stimuli? The mechanistic understanding of the components that are involved in such process, from the synthesis -> inception of the first signal -> signal processing -> determining the appropriate response, lies at the heart of the genetic, biochemical and molecular approaches to understanding this pathway.

Seed germination is a process known to be regulated by ethylene. Proteomics based studies have identified the regulation of proteins involved in methionine biosynthesis and ethylene biosynthesis during seed germination. Met synthase was up regulated during first day of imbibition of wild-type seeds (Gallardo *et al.*, 2002) while AdoMet synthetase levels were found to increase at

radicle protrusion (Gallardo *et al.*, 2001). ACC oxidase was found to accumulate during germination (Petruzzelli *et al.*, 2000). These data suggest that ethylene synthesis is involved during germination, however there has been little investigation into the role of ethylene during seed development. Our lab has been focusing on the role of ethylene during seed development and specifically seed dormancy using a simple test, seed germination. Our principal approach has taken advantage of ethylene receptor mutants in *Arabidopsis*.

The hypothesis of this thesis states that the subfamily I ethylene receptors have unique functions with respect to seed dormancy. The objective of the first chapter is to provide evidence for non-redundant roles of the ethylene receptor gene family and to propose distinct roles for the subfamily I receptors in regulating seed dormancy. The objective of the second chapter is to provide support for the hypothesis that the ETR1 and ERS1 receptors regulate seed dormancy. The research presented in this thesis is a continuation of the work initiated by Donna Yee and is now being continued by Lisa Koziol, an undergraduate student at Queen's University. My research contributions are as follows: In Chapter 1, I generated half of the metabolite profiles and analysed the receptor gene expression by electronic northern. In Chapter 2, I generated all of the germination profiles except those of CTR1. From the appendices, I have designed the crosses to the other hormone mutants, generated the F₁ and F₂ populations of double mutants and designed the screen to isolate the double mutants. I have trained Lisa Koziol to carry out the final analysis of the double mutants and in teaching Lisa how to stage developing seeds and siliques, I

determined the germination profiles of half of the single ABA-deficient mutants.

As well, I determined the germination characteristics of all single mutants on the various germination media. In appendix II, I collected all tissue, isolated total RNA for microarray analysis that was completed in December 2005 and because it appeared late in my degree, only an outline of the strategy is presented here.

Chapter 1: The Ethylene Receptors Have Non-redundant Roles in Plant Development: Distinct Functions of ERS1 and ETR1 in the Regulation of Seed Dormancy in *Arabidopsis thaliana*.

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Abstract

The five ethylene receptors of Arabidopsis are generally believed to perform redundant functions, partly because ethylene insensitive mutations in any one of these receptors block the triple response. However, we have found several lines of evidence that indicates the receptors have distinct functions during plant development. It would be expected that the expression of the receptors would be co-regulated if they were truly redundant, but detailed examination of gene expression by microarray analysis reveals that they each have distinct and different expression patterns throughout plant development. Phylogenetic analysis of receptor protein sequences across multiple plant species also reveals a distinct pattern of conservation for three groups of receptors, such that ETR1 and ERS1 fall into distinct and separate groups, while ERS2, EIN4 and ETR2 form a third less distinct group. Further evidence that the receptors play distinct roles in development came from the phenotypic analysis of gain-of-function mutants *etr1-1*, *ers1-1*, *etr2-1*, *ein4-1* and *ers2-1*. Several phenotypic differences were identified, but most notable was the difference in primary seed dormancy in *etr1-1* and *ers1-1* compared to wild-type seeds. Developing seeds of *etr1-1* are impaired in their ability to exit dormancy, while *ers1-1* seeds are affected in their ability to enter dormancy. Thus, these two receptors have completely opposite roles in seed development. Additionally, when we analysed the accumulation of all other hormones in these mutants during seed development, we find the mutated receptors differentially affect the accumulation of auxins, cytokinins, gibberellins and abscisic acid. Taken together, these results reveal that the

ethylene receptors have distinct roles in plant development, and more specifically ETR1 and ERS1 play critical roles in seed dormancy that is potentially manifested by an alteration in the accumulation of other plant hormones.

Introduction

The ethylene receptors of *Arabidopsis* are thought to be redundant in function. Individual GOF mutants are all ethylene insensitive and individual LOF mutants do not have any discernible phenotype, at least with respect to the triple response assay. If the receptors participated in independent functions, then the loss of any given receptor should result in a discernible phenotype. Thus far, no significant phenotypes have been reported for single LOF mutants, suggesting that the receptors have overlapping functions and the other four receptors can act to replace the missing receptor. A conceivable explanation for the functional overlap between receptors comes from analysing the sequence similarity of the five proteins. The two subfamilies show between 57-65% sequence similarity, while members of the same subfamily show even higher levels of similarity (Hua *et al.*, 1998). ERS1 and ETR1 are 79% similar while ETR2, EIN4 and ERS2 show between 68-74% similarity (Hua *et al.*, 1998). Hence, due to some shared domains and the high level of sequence similarity, it is possible that these receptors function redundantly. Support for the redundancy hypothesis also comes from the previous expression patterns of the five receptors in different tissues. Similar levels of RNA transcripts for each receptor have been identified in such tissue as etiolated seedlings, leaves, stems, floral organs, ovules and embryos, suggesting similar functions in these tissues (Hua *et al.*, 1998).

Despite the fact that the five receptors showed similar expression patterns in most tissues, it was observed that certain receptors had distinct expression patterns in specific organs and that some receptors were induced by ethylene treatment while others were not (Hua *et al.*, 1998). However, although the expression levels are similar, this doesn't rule out the possibility that the receptors have distinct functions in these tissues. A more complete analysis of ethylene receptors gene expression across several developmental stages might provide more insight. Additionally, we have recently shown that at least one LOF ethylene receptor mutant displays a distinct phenotype. The LOF *etr2-3* mutant is affected in its ability to produce branched trichomes. The trichomes of this mutant are unbranched, unlike the trichomes of wildtype which contain 2, 3 or even 4 branches (Plett *et al.*, 2005). Further support of specific roles for the receptors comes from recent studies where an *etr1* LOF mutant was shown to have an ethylene related phenotype. The LOF *etr1-7* mutant was shown to have an exaggerated response to ethylene which resulted in a smaller plant due to an overall decrease in cell size (Cancel and Larsen, 2002). The fact that the overall protein sequences show distinct differences (lack of the receiver domain in some receptors, lack of conserved His kinase residues) also suggests that they contain distinct functions. Therefore it could be possible that the receptors do perform redundant roles in certain tissues or at certain stages of development but also have distinct tissue specific or developmental specific roles.

Seed development can be divided into two major phases, embryo development and embryo maturation (Bentsink and Koornneef, 2002). Embryogenesis begins after pollen fertilizes an ovule. Two main organs are generated during embryo development; the axis, which gives rise to the root and shoot meristem and the cotyledons, which are differentially terminal and provide a source of nutrient reserves for the germination of young photosynthetically inactive seedlings (Goldberg *et al.*, 1994). During embryo maturation, these organs grow to fill the seed sac and storage compounds begin to accumulate and desiccation and cold tolerance are acquired (Taiz and Zeiger, 2002; Goldberg *et al.*, 1994). Seed embryogenesis terminates with primary seed dormancy. Seed dormancy is defined as the temporary inability of an intact viable seed to germinate under favourable conditions and is a plant adaptive trait that ensures the survival of the next generation (Bewley, 1997). Generally, a seed that has 'passed' primary dormancy is ready to germinate given the appropriate conditions. A non-dormant seed that does not germinate because of the absence of one or more environmental factors is said to be in a state of quiescence, or metabolic inactivity, and can remain so until the appropriate condition is met. Non-dormant seeds that remain in a state of quiescence can re-enter into dormancy, or secondary dormancy constituting a dormancy cycle (Baskin and Baskin, 1985, 2004). Seed dormancy is a very complex trait due to the many genetic, physiological and environmental factors that may influence its development (Bentsink and Koornneef, 2002). Aside from the environmental factors (light/dark, temperature, water etc.), endogenous cues play pivotal roles

in the regulation of dormancy and/or the transition to germination. Of these cues, phytohormones have been identified as primary components. Abscissic acid (ABA) and gibberellins (GA) are believed to play the most important regulatory roles. Mutants deficient in, or insensitive to, ABA show marked reduction of seed dormancy and germinate readily, whereas mutants deficient or insensitive to GA or those treated with GA inhibitors have low germination levels. However, germination can be rescued with exogenous GA application (Koornneef and van der Veen, 1980; Koornneef *et al.*, 1982; Karssens *et al.*, 1983; Koornneef *et al.*, 1984; Nambara *et al.*, 1991). These results have given support to hormone-balance model that proposes antagonistic roles between ABA and GA in regulating seed dormancy and germination. Such that the relative presence of ABA versus GA at any given time controls the response, namely dormancy versus germination (Wareing and Saunders, 1971). Besides ABA and GA, other molecules such as ethylene (promotive), sugar (inhibitory), brassinosteroids (promotive) (Steber and McCourt, 2001), and polyamines (promotive) have been implicated in seed germination (Bentsink and Koornneef, 2002). It is becoming clear that these compounds do not act independently to mediate a response, rather there is significant overlap or cross-talk involved (Gazzarrini and McCourt, 2003). For instance, the ABA, ethylene and sugar signalling pathways interact to regulate various aspects of development including germination and early seedling development (Gazzarrini and McCourt, 2001). Recently, many components of the ABA and ethylene biosynthesis or signalling pathways have been found to be allelic to sugar insensitive mutants (ex. *aba2* (ABA-deficient),

aba3, *abi4* (ABA-insensitive), *ctr1* allelic to *gin1* (glucose-insensitive), *gin5*, *sis5* (sucrose-insensitive), *gin4*, respectively (summarized in Leon and Sheen, 2003). This cross-talk between different hormone signalling cascade may occur through common signalling elements. For instance, the *era3* (enhanced response to ABA) mutant, originally classified as hypersensitive to ABA (and was subsequently found to over-accumulate ABA) was found to be allelic to *ein2*, the putative nuclear transporter involved in ethylene signalling (Ghassemian *et al.*, 2002). Such identifications may lead to the construction of signalling hierarchies between various hormone signalling and/or biosynthesis pathways. The resolving paradigm of hormone signalling describes a very complex network of pathway interactions, where multiple cross-talk events exist to regulate various aspects of development. A recent study using the relatively dormant, ethylene insensitive *etr1-2* GOF mutant revealed differential hormone and hormone metabolite accumulation during the early stages of seed germination compared to wild-type seeds (Chiwocha *et al.*, 2005). Hormone levels of ABA, auxins, cytokinins and GA were altered in the mutant during the early stages of germination, suggesting that the ethylene pathway interacts directly or indirectly with these hormone pathways which might contribute to the increased dormancy state of this mutant (Chiwocha *et al.*, 2005).

In this paper we show distinct dormancy phenotypes for developing GOF *etr1* and *ers1* mutant seeds. Based on the ability of these developing seeds to germinate, we present a rationale for the involvement of ETR1 and ERS1 in regulating the entrance and exit of primary seed dormancy, respectively. In line

with the notion that individual receptors have distinct or independent roles, we present a phylogenetic analysis of the ethylene receptor gene family. Here we show that the ETR1 and ERS1 receptors have been strongly selected for throughout evolution as that there is very little variation between these receptors across various plant species suggesting distinct functions. Furthermore, electronically generated gene expression profiles, show the differential expression of these receptors throughout development in various tissue types and also reveal that their expression is not co-regulated. We show here that GOF mutations in the ethylene receptors ETR1 and ERS1 directly or indirectly affect the accumulation of four other hormones and their metabolites during seed and silique development. These data taken together suggest that the ethylene receptors serve non-redundant roles throughout development and that there is a specific role for the subfamily I receptors in regulating primary seed dormancy.

Materials and Methods

Plant material

All plant types used were of the Columbia (Col-0) ecotype. Wildtype, *ers1-1* and *etr1-1* seeds were obtained from the Arabidopsis Biological Resource Center (ABRC).

Plant growth conditions

Seeds were surface-sterilized with 70% (v/v) ethanol for 2 min, followed by 30% bleach/0.02% Triton X-100 (v/v) solution for 8 min, and rinsed ten times with

sterile distilled water. To stratify seeds, they were incubated at 4°C for 4-7 days in the dark. All plants were grown to maturity in Pro-Mix soil in Enconair AC60 Arabidopsis growth chambers at 22°C. The plants were grown under a long day photoperiod (16h light / 8h dark). White fluorescent lighting provided a light intensity of 130-190 $\mu\text{E}/\text{m}^2/\text{s}$ at the rosette level.

For the GOF ethylene receptor mutants, a triple response assay was performed to screen for ethylene-insensitivity. Surface-sterilized mutant seeds were sown on Petri plates with growth medium consisting of Murashige and Skoog (M&S) basal salt mixture (Sigma) buffered to pH 5.7-5.8 with 1 M KOH, solidified with 0.8% (w/v) agar, and supplemented with 50 μM 1-aminocyclopropane-1-carboxylic acid (ACC-HCl; Sigma). Stratified seeds were germinated in the dark for 4 days at room temperature (25°C). Etiolated mutant seedlings with long hypocotyls and roots (compared to wild-type) confirmed ethylene-insensitivity and were selected for continued growth.

Silique and seed staging

Six distinct stages were identified based on silique and seed morphology and are described below from youngest (stage 1) to oldest (stage 6) with appropriate days after pollination (DAP) for wild-type plants grown under long-day: *Stage 1* – (2.8 ± 0.1 DAP) – silique was flaccid, not fully elongated, and pericarp had a pale green colouration; floral organs usually had not senesced; seeds were small, white and soft; *Stage 2* – (4.4 ± 0.2 DAP) – silique was plump, fully elongated, and pericarp had a bright green colouration; seeds had reached

full size and ranged from a greenish white testa with a visible green embryo to a completely bright green coloured seed coat; *Stage 3* – (12.5 ± 0.1 DAP) – siliques were in the process of losing the green colouration with signs of yellowing evident near the style; seeds had also lost their bright green colour and were showing signs of browning; *Stage 4* – (13.6 ± 0.1) – silique pericarp had a dull yellow colouration; seeds had a tinge of green colour remaining as they continued to brown; *Stage 5* – (14.6 ± 0.1 DAP) – siliques were brown in colour with no signs of desiccation visible; seeds were completely brown with no signs of desiccation visible; *Stage 6* – (15.7 ± 0.2 DAP) – siliques were brown coloured and showed signs of desiccation; seeds were dark brown and hardened due to seed desiccation. At this final stage, seeds were completely mature and ready for dispersal.

Germination potential of a developing seed

To identify stages of seed development and dormancy, the germination potential of seeds from each stage was assessed for WT and mutant plants. Seeds were carefully separated from their siliques and seeds from each stage were visually confirmed prior to being plated (without sterilization) on each of two replicate square Petri dishes water buffered pH 5.7-5.8 0.8% (w/v) agar. Six siliques per stage were used and eight seeds from each silique were plated onto replicate plates (48 seeds/stage). Individual replicates were either subjected to stratification as previously described or not stratified before being incubated in continuous light at room temperature (25°C) in a humidified polystyrene mini-desiccator cabinet (Sanplatec Corp., Osaka, Japan). After 14 days, the number

of seeds that had germinated was recorded for each silique/stage and the average percentages of seed germination $\pm$ standard error were calculated. A seed was considered germinated when the radicle had penetrated the seed coat.

Electronic Northern

Recently, the use of bioinformatics resources has allowed researchers to organize and interpret relevant sets of data from the vast amounts of genomic data being generated. We have determined that the ethylene receptor genes are differentially regulated throughout plant development using the Botany Array Resource (BAR) (<http://bbc.botany.utoronto.ca/>) (Toufighi *et al.*, 2005). The expression patterns of the ethylene receptors throughout development were generated electronically using the Expression Browser tool with the AtGenExpress - Tissue Series dataset on the Botany Array Resource database. All research areas, tissue types and developmental stages were chosen for the analysis. The average of replicate treatments relative to average of appropriate control was selected as the output option to view the log transformed clustered data while the average of replicate treatments was selected as the output option to view the absolute expression levels for these genes. The following AGI codes were used At2g40940, At1g66340, At1g04310, At3g23150 and At3g04580 representing ERS1, ETR1, ERS2, ETR2 and EIN4 respectively. Clustered data was selected to view graphical representation of the gene expressions.

Phylogenetic Analysis

Phylogenetic trees were built using the PHYLIP packages version 3.6a2.1 (Felsenstein, 1993) and TREE-PUZZLE version 5.0 (Strimmer and von Haeseler, 1996). The distance matrices used in neighbour-joining analyses of protein sequences were calculated with the PROTDIST program using the JTT model of sequence evolution and gamma distributed rates. These neighbour-joining analyses were performed using the NEIGHBOR program. The coefficient of variation of substitution rates among sites was calculated using the α values of the gamma distribution measured by TREE-PUZZLE and bootstrap analyses were performed using the SEQBOOT program with 100 replicates. TREE-PUZZLE maximum likelihood trees were built using the JTT model of substitution and a model of rate heterogeneity composed of one invariable and eight gamma rates estimated from the data set.

Amino Acid Substitutions

Average amino acid substitution rates were measured using the MEGA version 2.1 program (Kumar *et al.*, 2001). Within group averages were measured using the gamma model with eight gamma parameters. Standard errors were calculated by the bootstrap method using 500 replications. Rates were compared using *t* tests for unequal variances (Zar, 1999). Site specific amino acid substitutions analyses were performed using the PAML program (Yang, 1997). The likelihood model 8 was used to analyse the sequences of the *etr1*, *ers1* and *etr2* families. Note that, for these analyses, the *Arabidopsis ers2*

sequence was not used because it is much shorter than that of other subfamily II family members.

Low molecular weight profiling sample collection

WT, *ers1-1* and *etr1-1* mutant plants were grown under long-day conditions and siliques were harvested and sorted according to development stage (table 1). To ensure proper staging, siliques were dissected to reveal the appropriate seed stage. Siliques were frozen in liquid nitrogen and stored at -80°C until use. A minimum of 50 mg of dry weight material was required per replicate. Three biological replicates were processed, therefore a minimum of 150 mg of dry weight was required. After lyophilization, water loss ranged from 20% to 80% depending on the developmental stage and therefore a fresh weight of approximately 0.75 g was collected for each stage.

Extraction and purification of samples

When all material had been collected and frozen, samples were lyophilized (FreeZone Freeze Dry System, Labconco, Kansas City, Missouri) for 48 hrs in the dark. The dried samples were then ground into a coarse powder (FastPrep BIO101, Thermo Savant, Milford, Massachusetts) and a triplicate of ~ 50 mg (exact weight recorded) of ground sample from each stage was used for extraction. Each replicate was suspended in 4 ml of extraction buffer consisting of 99:1 isopropanol: glacial acetic acid (v/v) containing 5 ng/ml of each deuterium-labelled internal standard. Extraction occurred overnight on an orbital shaker at 250 rpm at 4°C in the dark. Each replicate sample was centrifuged at

2000 rpm for 10 min and the initial supernatant was collected and pooled with a subsequent supernatant after the pellet was re-suspended in 500 μ l of extraction buffer and centrifuged at 2000 rpm for 5 min. The supernatant extract was then passed through a solid phase extraction column to separate the compounds of interest from interfering compounds. A Sep-Pak C18 chromatography column (Waters Associates, Mississauga, Ontario) was used after conditioning with 5 ml of 100% methanol followed by equilibration with 4 ml of extraction buffer. The loaded column was rinsed with 500 μ l of 80% (v/v) methanol acidified with 1% (v/v) glacial acetic acid and the eluted purified extract was collected. A vacuum apparatus (Supelco Preppy; Sigma, Oakville, Ontario) was used to facilitate column elution. To evaporate solvents from the column eluate, the purified extract was dried in a Speed-Vac (CentriVac Concentration System, Labconco, Kansas City, Missouri). The dried purified extract was reconstituted in 200 μ l of 100% methanol and sonicated for 10 min prior to centrifugation at 13,000 rpm for 10 min. The supernatant of centrifuged reconstituted purified extract was transferred (without including any residual oils) to 200 μ l HPLC vials.

HPLC/ESI-MS/MS Analysis

For each sample, 10 μ l was auto-injected into a high-performance liquid chromatography (HPLC) system (Alliance 2695 separation module, Waters Associates, Mississauga, Ontario) coupled to an electrospray ionization source (ESI) linked to a quadrupole tandem mass spectrometer (MS/MS) (Quattro Ultima, Micromass, Manchester). Multiple reaction monitoring (MRM) was used to monitor ions during their elution and ion peak data (including area and

response) were recorded and analyzed with spectrometer software (MassLynx v. 3.5, Micromass, Manchester). The deuterium-labelled compounds were used as internal standards (IS) for each compound and calibration curves were created by running varying concentrations of unlabeled compounds with known IS concentrations and plotting response (= unlabelled product ion peak area x IS concentration / IS product ion peak area) against the known unlabelled concentration (Chiwocha *et al.*, 2003). By relating the response calculated between a known internal standard and its relevant unlabeled plant hormone/metabolite to generated calibration curves, compound quantification was achieved. All HPLC conditions, MS conditions, calibration curve generation, and deuterium-labelled compounds production was carried out exactly as detailed by Chiwocha *et al.* (2003).

Results

Silique and Seed Developmental Stages

We have identified six distinct stages of silique and seed development that reveal the timing of seed dormancy. These stages are based on the number of days after pollination, the length, colour and turgidity of the silique as well as seed size and colour (Table 1). Boyes and colleagues have developed an extensive phenotypic analysis process for the description of growth and developmental stages throughout the entire life cycle of an Arabidopsis plant (Boyes *et al.*, 2001). The growth stage-based analysis of the developing siliques

Table 1: Six stages of seed development in wildtype. WT Col-0 silique and seed identification chart with developmental times points with respect to days after pollination (DAP – long day) and approximated corresponding life growth stages.

Stage	DAP	Silique Characteristics	Seed Characteristics	Apprx. life stages*	Silique**	Silique and seeds†	seed‡
1	2.8 +/- 0.1	dull green pericarp, flaccid and not fully elongated	white/translucent, small - not fully developed, soft seed coat	6.10 to 6.90
2	4.4 +/- 0.2	bright green pericarp, turgid and fully elongated	greenish white testa with a visible green embryo to a completely green coloured seed coat	6.10 < x < 6.30 to 6.90
3	12.5 +/- 0.1	green with some yellowing of the pericarp near the tip (style/stigma), still turgid	losing bright green colour and showing signs of browning	~ 6.50 to 6.90
4	13.6 +/- 0.1	yellow pericarp, still turgid	mostly brown with a tinge of green remaining	6.50 to 6.90
5	14.6 +/- 0.1	brown pericarp with no signs of desiccation, still turgid	brown with no signs of desiccation	6.50 to 6.90
6	15.7 +/- 0.2	brown pericarp with signs of desiccation	dark brown with signs of desiccation, hardened seed coat	~ 6.90 to 8.00

* as reported by Boyes et al., 2001, ** apprx. 4X magnification, † apprx. 8X magnification, ‡ apprx. 16X magnification

can only be assigned after flowering is complete, because it is based on percentages and requires knowledge of the total number of flowers produced. Therefore, the corresponding 'Boyes' developmental stages indicated in Table 1 are only roughly comparable and were approximated using our DAP to the number of days after the initiation of principle growth stage 6 (flower production) (Boyes *et al.*, 2001). It is important to note that differences in growth regimes exist between the two studies, which would invariably affect the developmental stage of the tissue.

Developmental stages have also been characterized during morphogenesis of the embryo based on certain hallmarks of embryo shape, including globular, heart, torpedo, walking stick, curled cotyledon and green cotyledon shaped (Bowman, 1994). Although we did not dissect the embryos from the developing seeds, our DAP data indicates that our stage 1 is analogous to the heart through walking stick stages of embryo development and our stage 2 is analogous to the curled cotyledon to green cotyledon stage of embryo development.

Phases of late seed development based on germination

Seeds of the six developmental stages were isolated from siliques and were either stratified or not stratified to test the ability of a developing seed to germinate. The typical seed germination profile for wildtype is shown in Figure 1 and Figure 2A. Stage 1 seeds failed to germinate after stratification or without stratification. This indicates that the seeds at this stage have not undergone sufficient development to acquire the ability to germinate. Stage 2 seeds

Figure 1: Germination plates showing wild-type seed germination at various stages of development. Seeds at the appropriate developmental stages were excised from their siliques and transferred to germination media (water and agar media) to test the ability of a seed to germinate. **A.** After seeds had been plated, the plates were wrapped in foil (dark) and placed at 4°C for seven days (stratification) before being removed to long day growth conditions for 14 days, photographed on day 14. Stage 1 immature seeds do not germinate; stage 2 seeds show precocious germination but germinate with less vigour than non-stratified stage 2 seeds; stage 3 and stage 4 seeds show very low levels of germination and are considered to be dormant; mature stage 5 and stage 6 seeds germinate. **B.** After seeds were plated, plates were placed in long day growth conditions for 14 days, photographed on day 14. Each square represents eight seeds from one silique at the appropriate stage. . Stage 1 immature seeds do not germinate; stage 2 seeds show precocious germination; stage 3 and stage 4 seeds show very low levels of germination and are considered to be dormant; Note the lack of germination in stage 5 and stage 6 seeds (see figure 2 and discussion). Note the missing seeds from stage 1 A and B. This was due to the lack of enough siliques at this stage at the time of harvesting. Also note the contamination present in A Stage 4 column. Seeds were plated directly from siliques without any surface sterilization, making this a rather punctilious task.

A **B**

Figure 2: Graphical representation of developing seed germination patterns of wildtype and gain-of-function ethylene receptor mutants. Staged seeds were harvested from their corresponding siliques and plated on germination media. Germination plates were either stratified or not stratified and grown under long day conditions for 14 days. Seed germination counts were scored on the fourteenth day. Germination was scored by radicle protrusion. Black bars represent non-stratified seeds, grey bars represent stratified seeds.

A. wildtype (Col-0). Four distinct phases based on seed germination can be observed in wild-type developing seeds. Stage 1: Non-viable seeds unable to germinate; Stage 2: Precocious germination; Stage 3 and Stage 4: Dormancy; Stage 5 and Stage 6: Mature seed germination. Note that the non-stratified stage 5 and stage 6 seeds were unable to germinate and only after 23 days of after-ripening were non-stratified stage 6 seeds able to germinate (see figure 3).

B. *ers1-1*. The four phases observed for wild-type seeds were not observed in the *ers1-1* mutant. Immature stage 1 seeds did not germinate; stage 2 seeds showed precocious germination, however the ability of both stratified and non-stratified seeds from this stage showed a reduced ability to germinate when compared to wild-type stage 2 seeds. Unlike wild-type stage 3 and stage 4 seeds, the *ers1-1* stage 3 and stage 4 non-stratified and stratified seeds were able to germinate and appear to be non-dormant. Both stratified and non-stratified stage 5 and stage 6 seeds are able to germinate.

C. *etr1-1*. The four phases observed for wild-type seeds were not observed in the *etr1-1* mutant. Immature stage 1 seeds did not germinate; stage 2 seeds showed precocious germination, however the ability of both stratified and non-stratified seeds from this stage showed a reduced ability to germinate when compared to wild-type stage 2 seeds. Stage 3 and stage 4 seeds were unable to germinate. Unlike wild-type seeds, stage 5 and stage 6 *etr1-1* seeds showed a reduction in the ability to germinate and appear to have augmented seed dormancy.

A. **B.** **C.**

exhibited an ability to germinate with or without stratification, however seed germination was much more vigorous in non-stratified seeds and is characteristic of precocious germination. It seems that these seeds have developed to the point of being capable to germinate however are not fully cold tolerant. Stage 3 and stage 4 seeds displayed minimal germination regardless of being stratified or not. These seeds have entered primary dormancy and cannot be broken by stratification. Stage 5 and stage 6 seeds showed germination primarily in stratified seeds. These seeds have exited seed dormancy. The ability of seeds to germinate in non-stratified conditions increased with time (after 23 days), suggesting the seeds have undergone after-ripening to become receptive to germination (summarized in Figure 3). Therefore, four phases of seed development are observed when plating these six stages of seed development, immature non viable seeds, precocious germination, dormancy, and mature seeds whose dormancy can be broken by stratification.

The subfamily I ethylene receptor mutants show aberrant germination profiles across seed development

The same germination tests were performed on the ethylene insensitive GOF ethylene receptor mutants. The subfamily II GOF *etr2-1*, *ers2-1* and *ein4-1* mutant germination profiles resembled (with some minor variations) those of wildtype as the major phases were seen (precocious germination, dormancy and germination of mature seeds) (data not shown). The subfamily I GOF *etr1-1* and *ers1-1* mutants, however revealed dramatically different germination profiles.

Figure 3: Sequential steps of Arabidopsis seed development including the wildtype germinate profile. Integrating the wild-type germination profiles across seed development (Figures 1 and 2) into the current model of seed developmental events (Harada 1997, Raz *et al.*, 2001) generates the following figure. Dashed diagonal lines represent an approximation of the start/end of each developmental event. Initial broken bars of the rectangles (post seed dispersal) are meant to represent that time is required for certain developmental (after-ripening) events to proceed before a seed becomes receptive to germination conditions as was inferred from wild type stage 6 seeds requiring at least 23 days of 'maturation time' to germinate (Figure 2).

For the *etr1-1* mutant, the stage 1 seeds failed to germinate (Figure 2C). Precocious germination was observed in stage 2 *etr1-1* seeds however there was a notable reduction in the percentage of non-stratified seeds that germinated. Seeds at stage 3 and stage 4 did not germinate and had presumably entered into primary seed dormancy. However by stage 5 and stage 6, neither stratified nor non-stratified seeds were able to germinate at wildtype-like levels. The *etr1-1* seeds appear to remain in primary seed dormancy.

The germination profile of the *ers1-1* mutant is in sharp contrast to the *etr1-1* mutant germination profile. Stage 1 *ers1-1* seeds failed to germinate (Figure 2B). Stage 2 *ers1-1* seeds demonstrated precocious germination, however unlike wildtype, the percentage of seed germination in non-stratified seeds was similar to stratified seeds at this stage. *ers1-1* seeds maintained the ability to germinate throughout stage 3 and stage 4 compared to wildtype. In stage 5 and stage 6 seeds are still able to germinate with or without stratification. Interestingly the proportion of non-stratified seeds able to germinate is much higher in the *ers1-1* seeds. The *ers1-1* seeds appeared to not enter into a dormant state in the latter stages of seed development.

Differential receptor expression patterns

The electronically generated gene expression profiles in Figure 4 reveal the differential expression of the ethylene receptors throughout development in various tissue types. For instance, *ERS2* is down-regulated between 1.9 to 2.3 fold during young rosette development but is up-regulated 0.6 to 0.9 fold in the

Figure 4: An analysis of ethylene receptor expression throughout plant development using averages of the AtGenExpress Tissue Data Set (Weigel *et al.*, unpublished results) available via the Botany Affymetrix Resource Expression Browser tool at <http://bbc.botany.utoronto.ca>.

A. From left to right, this figure provides an analysis of the relative changes occurring in the expression levels of the five ethylene receptors throughout the lifespan of Arabidopsis. Tissue types are abbreviated in this figure: first node (1stNd), cauline leaf (CaulLf), cotyledons (Cotyl), rosette after flowering (FlwRos), flower (Flw), carpel (Crp), petal (Ptl), sepal (Sep), stamen (Stm), hypocotyls (HypTl), leaf (Lf), mature pollen (Mat.Poll) rosette leaf (RosLf), green parts of seedling (SdIng.grn), senescing leaf (ScnLf), Shoot apex (ShoApx), vegetative rosette (VegRos) Numbers associated with the tissue abbreviation correspond to the developmental stage of the tissue as reported by Boyes *et al.*, 2000. Expression levels relative to median value for each developmental time point (Res. Area) on a per gene basis. Values have been $\log_2$ -transformed, and levels 4-fold or more above the median are indicated in red, while levels 4-fold or more below the median are indicated in blue. A comprehensive summary of all the features from this tool are presented in a recent publication (Toufighi *et al.*, 2005).

B. From left to right, this figure provides an analysis of the absolute expression levels for the five ethylene receptors throughout the lifespan of Arabidopsis. Tissue types are identical to A. A strong red colour indicates more than 1000 expression units of expression in the indicated tissue. The average-linkage hierarchical trees at the right indicates the degree of co-regulation between the genes, with the metric used for determining this the Pearson correlation coefficient. No ethylene receptor exhibits more than 50% co-regulation.

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shoot apex/inflorescence tissue and 1.5 to 2.6 fold in various flower organs. Interestingly, *ETR2* and *EIN4* were also found to be up-regulated 1.5 to 2.3 fold in the shoot apex /inflorescence tissue, respectively. *ETR2* was also seen to be up-regulated 2.3 fold in the carpels of the flowers. The *ERS1* and *ERS2* were differentially regulated in young seed and silique tissues. *ERS1* was down-regulated 2.0 to 3.0 fold in young seed tissue (approximately corresponding to the walking-stick to early curled cotyledons stage through to green cotyledons stage, respectively) as defined by Bowman in (Bowman, 1994) (the later stage is approximate to our stage 2). *ERS2* was down-regulated 2.3 to 7.5 fold in very young seed and silique tissue. Intriguingly, the levels of *ETR1* were not seen to change significantly across development for any of the available tissues. Although, *ERS2*, *ETR2* and *EIN4* were up-regulated in the shoot apex/inflorescence tissues, no ethylene receptor exhibits more than 50% co-regulation.

Independent Conservation of the subfamily I protein sequences

The neighbour-joining phylogenetic tree shown in Figure 5 was built using the first 650 amino acids of our 860 amino acid alignment. This region was chosen because it is well conserved and common between ethylene receptor proteins. The three groups shown in Figure 5 are also present when the tree was built using the maximum-likelihood method implemented in TREE-PUZZLE (results not shown). The maximum-likelihood phylogenetic trees based only on aligned amino acid position 48 to 168 (ethylene binding domain), 169 to 386 (GAF domain), 387 to 668 (histidine kinase domain), or 669 to 860 (receiver

Figure 5: Phylogenetic analysis of ethylene receptor protein sequences across various plant species. Ethylene receptor protein sequences from various plant species were used to generate this phylogenetic tree. A strong trend towards the independent conservation of ETR1 and ERS1 sequences is observed, while there is less conservation of the other ethylene receptors.

domain) all had the same three groups shown in Figure 5 (results not shown). These partitions correspond to the domains defined by Hua and colleagues (Hua *et al.*, 1998).

Regardless of the method or the region of the protein chosen, this analysis presents evidence that the ETR1 and ERS1 receptors have been strongly selected for throughout evolution. This conservation was not seen for members of the subfamily II receptors, those being ERS2, ETR2 and EIN4.

The rates of amino acid substitutions (+/- standard error) are 0.124 +/- 0.009, 0.292 +/- 0.014 and 0.606 +/- 0.025 substitution/site for the *etr1*, *ers1* and *etr2* families respectively. These substitution rates are all statistically different. The ERS1 family evolves faster than the ETR1 family ($t = 6.06$, d.f. = 39, $p < 0.001$) and the ETR2 family evolves faster than the ERS1 family ($t = 10.97$, d.f. = 11, $p < 0.001$).

Figure 6 shows the posterior probability for site classes with different selective pressures (as measured by the w ratio) obtained with model 8 of the PAML program. The w ratio corresponds to the ratio of non-synonymous to synonymous substitutions at each site. The probabilities shown in Figure 6 indicate whether a site is likely to be highly conserved (low w values), conserved (intermediate w values) or subject to weaker selective constraints (higher w values). This figure shows that variable sites are not distributed equally along the sequences of the ERS1, ETR1 and ETR2 protein families. However, the overall distribution of variable sites for these three families show the same general pattern. The first and second third of these sequences are composed of

Figure 6: Likelihood of amino acid substitutions in the ethylene receptor protein sequences. Areas indicated by low w values correspond to sites which are likely to be highly conserved, whereas areas indicated by high w values correspond to sites which are likely to be less conserved. The ETR1 and ERS1 sequences show areas of high conservation while the ETR2 sequence is much less conserved.

more conserved regions separated by a more variable region and the third third, of these proteins, is more variable.

Hormone and hormone metabolite accumulation across silique and seed development

Recent advances in quantification, identification, and chemical analysis have now made it possible to measure low-level molecules, such as plant hormones, with unprecedented accuracy. The liquid chromatography-electrospray ionization tandem mass spectrometry method has already been successfully used to follow the hormone and metabolite levels associated with seed dormancy and germination in lettuce and *Arabidopsis* (Chiwocha *et al.*, 2003; Chiwocha *et al.*, 2005). The relative accumulation of four major plant hormones were monitored across seed and silique development, the major changes in hormone profiles between the receptor mutants and wild-type are reported in Figure 7. It is important to note here that the hormone profiles are representative of the developing seed and silique tissues. It is probable that both the embryo/seed and maternal tissue hormone levels contribute to the observed levels described below.

ABA profiles

The ABA content of very young seeds and siliques was found to be 321.5 ng/g DW in wild-type stage 1 tissue. The levels of ABA were slightly lower in *ers1-1* at 297.9 ng/g DW and much lower in *etr1-1* at 154.8 mg/g DW (Figure 7-

Figure 7: Hormone and hormone metabolite profiles across wild-type (A), *ers1-1* (B) and *etr1-1* (C) silique and seed development. Hormone profiles across silique and seed development were generated using a LC-ESI-MS/MS based method. Four major hormone groups were monitored including ABAs (1), cytokinins (2), auxins (3) and GAs (4). Selected ABAs included abscissic acid (ABA), phaseic acid (PA), dihydrophaseic acid (DPA), 7'-hydroxy-ABA (7'OH-ABA) and ABA-glucose ester (ABA-GE). Selected cytokinins included zeatin (Z), zeatin riboside (ZR), isopentenyladenine (2iP) and isopentenyladenosine (IPA). Selected auxins included indole-3-acetic acid (IAA) and indole-3-acetyl aspartate (IAAsp). Selected gibberellins included GA₁, GA₃, GA₄ and GA₇. Briefly, differences were seen not only in the relative levels of certain hormones but also in the relative accumulation of metabolites between *ers1-1*, *etr1-1* and wild-type seeds and siliques. The *ers1-1* seeds and siliques differentially accumulated ABA and ABA metabolites as well as cytokinins compared to wild-type seeds and siliques whereas *etr1-1* seeds and siliques had comparable levels of these hormones to wild-type seeds and siliques. The *etr1-1* seeds and siliques differentially accumulated auxins and GAs compared to wild-type seeds and siliques whereas *ers1-1* had comparable levels of these hormones to wild-type seeds and siliques. Surprisingly, of the four GAs monitored, none were observed in any stages of development in wild-type seeds and siliques.

1.) ABA dropped steadily as the maturation of the wild-type seeds and siliques occurred, at stage 4 the concentration reached 40.4 ng/g DW, rose slightly to 45.2 ng/g DW at stage 5 and declined further to 29.6 ng/g DW in stage 6 seeds and siliques. However, in the *ers1-1* and *etr1-1* mutants, ABA levels increased from stage 1 to 347.8 ng/g DW and 170.6 ng/g DW at stage 2, respectively. As with wildtype, the levels of ABA have decreased by stage 3 and at stage 4. ABA is present at 45.9 ng/g DW in the *ers1-1* and at 28.3ng/g DW in the *etr1-1* seeds and siliques. Unlike wildtype, however, the levels of ABA rise slightly towards the final stages of development in the mutant seeds and siliques and by stage 6, ABA is present at 53.1ng/g DW in the *ers1-1* mutant and at 46.4 ng/g DW in the *etr1-1* mutant. Phaseic acid (PA), metabolite of ABA, was only detected in Stage 1 seeds and siliques at 0.48ng/g DW in wild-type seeds and siliques. PA was present in all stages of *ers1-1* but most abundantly in stage1 and stage 2 at 133.4 and 116.9 ng/g DW before dropping to approximately 20ng/g DW in the latter stages. PA is only present in the first three stages of *etr1-1* seeds and siliques and most abundantly in stage 2 seeds and siliques at 60ng/g DW. Dihydro-PA (DPA), a metabolite of PA, levels gradually decreased across wild-type and *etr1-1* seed and silique development from 156.6ng/g DW and 110.4ng/g DW in Stage 1 to 24.1ng/g DW and 21.6 ng/g DW in stage 6, respectively. The major difference noticed in the ABA profiles revealed that the *ers1-1* seeds and siliques accumulate high levels of DPA in early development (574.6ng/g DW in stage 1), however these levels regained wildtype-like levels in the later stages of development. 7'OH-ABA, an unstable derivative of ABA, levels remained low

throughout development in wild-type and *etr1-1* seeds and siliques approximately 10-15 ng/g DW and where slightly higher in *ers1-1* seeds and siliques at approximately 45ng/g DW. ABA-GE, a conjugated form of ABA, levels was different across development in every plant type but tended to decrease gradually towards the later stages of development. In relation to the germination profiles generated for these plant types, it is interesting to note that there is no direct correlation between ABA abundance and maintenance of dormancy. In fact, in the *ers1-1* mutant that germinates in stage 3 and stage 4 (wild-type seeds remain dormant during these stages), increased ABA levels in stage 1 and stage 2 were observed. Interestingly, in the *etr1-1* mutant seeds that do not germinate in the later stages of seed development (wild-type stage 5 and stage 6 seeds are able to germinate at these stages) there were no appreciable increase in ABA or ABA metabolites in the seed and silique tissue compared to wildtype. These results reveal a remarkable re-organization of ABA metabolism throughout seed and silique development that is regulated directly or indirectly by the ERS1 but not the ETR1 receptor.

Cytokinins profiles

Generally, zeatin (Z) was not present in abundance in any of the silique and seed stages. The conjugated form of Z, zeatin riboside (ZR) was the most abundant cytokinin across wild-type and *etr1-1* seed and silique development (Figure7-2), being the most abundant during stage 1 at 98.1ng/g DW and 115.4ng/g DW, respectively. ZR levels were detected in only the first two stages

of development in the *ers1-1* mutant. The levels of 2iP, a bioactive cytokinin, remained constant throughout wild-type and *etr1-1* seed and silique development at approximately 12 ng/g DW but were not detected in any stage in the *ers1-1* seeds and siliques. The major difference noticed in the cytokinin profiles revealed that the *ers1-1* seeds and siliques accumulate high levels of isopentenyladenosine (IPA), a conjugated form of 2iP, (86.4 ng/g DW in stage 1 and dropping to approximately 50ng/g DW from stage 3 through stage 6). This was the most abundant cytokinin metabolite across all stages of development in this mutant. Whereas in the WT and *etr1-1* seeds and siliques, IPA was present in relative abundance in the early stages of development at 44.3 ng/g DW and 17.8ng/g DW, respectively before dropping to very low levels from stage 3 through stage 6. In summary, it appears that the ERS1 receptor is important in regulating the metabolism of cytokinins across seed and silique development as an ethylene insensitive ERS1-1 protein dramatically changes the accumulation of cytokinins across seed and silique development.

Auxins profiles

Relative to all other hormones monitored, indole-3-acetic acid (IAA), a bioactive auxin, was the most abundant compound detected throughout wild-type and *ers1-1* seed and silique development (Figure 7-3). The trend in the accumulation of IAA was similar throughout wild-type and *ers1-1* seed and silique development. Stage 1 seeds and siliques accumulated 834.5 ng/g DW and 1258.2ng/g DW in wildtype and *ers1-1*, rising to 1201.2 ng/g DW and 1477.3

ng/g DW at stage 2, respectively. IAA levels remained high from stage 3 through stage 5 at approximately 600 ng/g DW and 750 ng/g DW in wild-type and *ers1-1* seeds and siliques, before dropping to 150.05 ng/g DW and 239.3 ng/g DW, respectively. The trends for the abundance of Indole-3-aspartate (IAAsp), a conjugated form of IAA, were also somewhat comparable between wild-type and *ers1-1* seeds and siliques. However, the relative levels of IAAsp were much lower in the *ers1-1* mutant by approximately 5 fold. The major difference noticed in the auxin profiles were observed in the *etr1-1* mutant. The levels of IAA were considerably lower (203.7 ng/g DW in stage 1 and declining to 61.8 ng/g DW in stage 6) than those of wild-type or *ers1-1* seeds and siliques. The levels of IAAsp in the *etr1-1* mutant were similar to those found in the *ers1-1* mutant across seed and silique development. IAA appears to be required at high concentrations across seed and silique development and the ETR1 receptor is directly or indirectly required to maintain proper IAA levels in these tissues.

Gibberellin profiles

Surprisingly, none of the GAs being monitored (GA₁, GA₃, GA₄ or GA₇) were observed in any stage of seed and silique development in wildtype (Figure 7-4). Very low levels of GAs were observed across seed and silique development in the *ers1-1* mutant. GA₄ and GA₇ were detected at approximately 3.5 ng/g DW and approximately 0.5 ng/g DW in all stages of development. GA₁ was detected at 2.2 ng/g DW only in stage 4 in the *ers1-1* mutant. The major difference noticed in the GA profiles was surprisingly in the *etr1-1* mutant with GA₄ and GA₇ being highly abundant across seed and silique development, especially in stage 1 at

984.8 ng/g DW and 896.7 ng/g DW, respectively. These GAs were the most abundant compound detected throughout *etr1-1* seed and silique development. Apparently, a functional ETR1 protein is required to mediate GA signalling in these tissues.

Discussion

Ethylene Receptor Redundancy

The previous assumption that the ethylene receptors serve redundant functions is founded largely on the triple response assay as an indicator of ethylene response. The fact that a GOF mutation in any one of the five receptors conferred ethylene insensitivity (and therefore a loss of the triple response) as well as the fact that a single LOF mutation in any one of the receptors had no appreciable phenotype led significantly to the conclusion for this redundancy. The triple response assay has proven itself a great asset for the isolation of multiple mutants impaired in ethylene related events, primarily because it is a simple assay and large populations of seeds can be screened with ease. However, perhaps conceptually at least, seedlings are not the most practical developmental stage from which to base such conclusions, especially when one considers the myriad of responses associated with ethylene, both developmental and stress related throughout the life cycle of the plant. Visual (qualitative) observation throughout the life cycle of GOF and LOF ethylene receptor mutants revealed differences in leaf size and shape, trichome form and patterning, anthocyanin accumulation and flowering time (data not shown). Furthermore,

testing responses of these mutants to various 'stimuli' also revealed differential phenotypes, in this case, plating developing GOF mutant seeds revealed differential dormancy patterns compared to wildtype. In light of these sets of data, the possibility that the ethylene receptors serve non-redundant roles in specific developmental or response programs but share redundant functions with respect to early developmental responses such as the triple response is much more probable.

Evolutionary Conservation of Subfamily I Receptors

A required gene function is subject to pressure throughout evolution and will have minimal variation from generation to generation as mutations in that gene will often produce a negative effect to the required gene function, putting the progeny at a competitive disadvantage. Therefore genes required for certain key developmental decisions should show high conservation across various plant species while those genes with non-essential or redundant function are less restricted by the pressures of evolution and are 'more free' to accepting changes. A phylogenetic analysis of the specific ethylene receptor protein sequences across 57 different plant species revealed the conservation of the ETR1 and ERS1 receptors but not for the EIN4, ETR2 and ERS2 receptors (Figure 5). This conservation is apparent in eudicots and monocots indicating the presence, if not the necessity of these receptors before the divide between these two angiosperm groups. Thus, it is apparent from this analyses, and in conjunction with our biological observations, that the ETR1 and ERS1 receptors are under selective pressure(s) to remain conserved as they are required for specific response

programs (ex. development, stress, etc.) while it appears that the EIN4, ETR2 or ERS2 receptors are not under such pressure(s). This data supports the observation that the *etr1;ers1* double loss-of-function mutants exhibit severe constitutive ethylene response phenotypes, implicating a required role for subfamily I receptors across development (Wang *et al.*, 2003; Hall and Bleecker, 2003). Analysis of the probabilities for conservation of certain gene regions, Figure 6 reveals the areas of the gene families showing the highest levels of conservation. It appears that there are pockets or islands within the sequence that are essential (won't accept mutations - highly conserved across these plant species) in all receptors. For instance, consider the first third, there appears to be an island in all three groups which correlates to the ethylene binding region. Mutations in this region have been shown to abolish ethylene binding (Rodriguez *et al.*, 1999) and thus conservation of such sequences would remain high throughout evolution. The third region contains possible substitution sites in all three conserved receptor groups suggesting that this region is highly variable and is not highly conserved.

Developmental expression patterns

Differential expression of the ethylene receptors has been reported in *Arabidopsis* (Hua *et al.*, 1998), tomato (Klee and Tieman, 2002; Payton, *et al.*, 1996) and muskmelon (Sato-Nara *et al.*, 1999). Specifically in the later case, the ETR1 and ERS1 melon homologues were shown to be differentially expressed during fruit development (Sato-Nara *et al.*, 1999). The five ethylene receptors

were seen to be expressed ubiquitously in all tissues in Arabidopsis, however each receptor was observed to have distinct expression patterns in specific tissues (Hua *et al.*, 1998). The Botany Array Resource's 'Expression Browser' tool (Toufighi *et al.*, 2005) was used with the AtGenExpress tissue series dataset (see methods) to probe the expression of the ethylene receptor family throughout development. In this analysis, it was also observed that the receptors have their own unique expression pattern in specific tissues. For instance, *ers2* and *ers1* were observed to be down-regulated during the early stages of silique and seed development (Figure 4). Theoretically, the sensitivity of a tissue to a given elicitor can be augmented by increasing the levels of the elicitor and/or decreasing the levels of the receptor in that specific tissue. If each receptor served distinct functions, and assuming a linear relationship between gene expression and protein product, the fact that *ers1* and *ers2* are down-regulated may imply a requirement for sensitivity or signalling from those receptors during early silique and seed development. Interestingly, the ERS1 receptor was observed to be down-regulated at approximately stage 2 of our silique and seed development stages. If we assume that at stage 2 there is a required signal from the ERS1 receptor then the reduction in the expression of this receptor could imply that the ERS1 receptor is required at Stage 2. Considering the increased germination of GOF *ers1-1* seeds post stage 2, we could then predict that the signal required from this receptor is involved in initiating primary seed dormancy. Unfortunately, data does not currently exist in the AtGenExpress dataset used here in order to monitor the expression level of *etr1* across our later stages of

seed development. Quantitative PCR data will be invaluable in supporting these aforementioned predictions as well as determining the expression levels of receptors across all of our stages of seed and silique development. The lack of co-regulation of any receptors indicates that they are expressed independently from each other in specific tissues and are likely regulated by different developmental cues.

Effect of the GOF mutations on dormancy

The late stages of wild-type seed development exhibit four distinct phases based on the ability of the seed to germinate (Figure 3). Using stratified and non-stratified seeds of the various stages simplifies the delineation of these phases. The phases are: immature non-germination or previable phase corresponding to stage 1; precocious germination phase corresponding to stage 2; dormancy phase corresponding to stage 3 and stage 4; mature, dormancy breakable phase corresponding to stage 5 and stage 6. Stage 1 seeds have presumably not developed enough to allow germination of the embryo under our conditions. Stage 2 seeds have reached a developmental state at which the embryo is viable and able to germinate. These immature seeds are full sized, completely green and have yet to acquire cold tolerance, as stratified seeds exhibited an overall percentage of germination lower than that of the non-stratified seeds. Stage 3 and stage 4 seeds are dormant and germination is not observed even after stratification. Stage 5 and stage 6 non-stratified seeds remain dormant but are able to break dormancy as stratified seeds show complete germination. Mature,

non-stratified seeds do become receptive to germination under these conditions as after-ripened stage 6 seeds (left in siliques on the maternal plant for another 23 days past stage 6) germinate. The presence of the dominant GOF mutation of the *ers1-1* or *etr1-1* gene products has a dramatic effect on these phases of seed development. Two opposite dormancy phenotypes were observed for these mutants. *etr1-1* developing seeds appear to have an increased dormancy as mature stage 5 and stage 6 seeds have a lower overall percentage of germination than the corresponding wild-type seeds at the same stage (Figure 2). Whereas *ers1-1* developing seeds do not enter into dormancy and have a greater overall percentage of germination compared to wild-type seeds at stage 3 and stage 4 (Figure 2). These two phenotypes offer an interesting hint that accumulation of other hormones might be changing since the relative levels of GA versus ABA are responsible for germination versus dormancy, respectively. If this were the case, we might expect that the ABA levels in the *etr1-1* seeds would be greater when compared to wildtype, and that the GA levels in the *ers1-1* seeds would be greater when compared to wildtype. However, the opposite situation was observed (Figure 7). The *etr1-1* developing seeds and siliques accumulated higher than wildtype levels of GA and the *ers1-1* developing seeds and siliques accumulated higher than wildtype levels of ABA.

A possible hormone contribution

ABA plays an integral role in the dormancy status of a seed. Mutants deficient (*aba1*, *aba2*, *aba3*) or insensitive to ABA (*abi1* to *abi5*) have reduced levels of dormancy and germinate with a higher percentage than wild type seeds

(reviewed in Bentsink and Koornneef, 2002). Conversely, mutants which have an exaggerated response to ABA, for example *era* (Cutler *et al.*, 1996, Ghassemian *et al.*, 2002) have a super-sensitivity to ABA and have low germination percentages. Immature developing seeds rarely germinate and seeds that germinate precociously are often associated with ABA deficiency or insensitivity (Bewley, 1997). In a study to monitor the induction of vivipary; seeds enclosed in siliques, non-dried ovules, or dried ovules at various developmental stages were sown onto water-agar media and analysed for their ability to germinate (Raghavan, 2002). Our results agree well with this study as young seeds exhibit a precocious germination and enter into a dormant state later in development (around the time when the seeds begin to turn from green to brown). Contrary to our findings, non-dried green seeds (comparable to our stage 2 seeds) showed no germination after stratification, however germination percentages were taken after only four rather than fourteen days (Table 3 Raghavan, 2002). Typically, ABA levels during seed development are low in the early stages, rising through mid-development during seed reserve accumulation and decline as the seed undergoes maturation and drying (Bewley, 1997). Our wildtype ABA profiles (they represent seed and silique tissue) demonstrate this typical ABA profile across seed development, although the very early stages are not represented here as those stages of embryogenesis are not represented in our developmental stages. The developing seed and silique hormone profiles of the ethylene receptor mutants contrasted significantly from wildtype profiles. It is important to note here that these hormone profiles represent only a snapshot of

targeted metabolites and do not represent a global representation of all hormone levels. That being said, they still provided a novel opportunity to propose hypothesis for the interactions between ethylene and other hormone pathways. It is also important to note that these profiles are more amenable to deducing the signals required for the inception of primary dormancy as the transition to germination undoubtedly requires re-organization at the metabolic level.

Most surprising, was the relative accumulation of ABA in the *ers1-1* mutant and GA in the *etr1-1* mutant compared to wildtype (Figure 7-1). The *ers1-1* profiles show higher than wildtype accumulation of ABA in the early stages of development which is somewhat paradoxical as these seeds do not exhibit the induction of primary dormancy seen in stages 3 and 4 of wildtype seeds (Figure 2B). Not only where the levels of ABA different in this mutant but also the levels of ABA metabolites compared to wildtype and the *etr1-1* mutant suggesting that ethylene may somehow regulate the biosynthesis of ABA through action of the ERS1 receptor. Possible explanations for the lack of dormancy could be, that due to the GOF mutation, the *ers1-1* seeds are less sensitive to ABA during seed development at stage 3 and stage 4 as they have comparable ABA levels to those in wild-type seeds of the same stage. It could also be possible that the *ers1-1* seeds are more sensitive to the relative levels of GAs at these stages of seed development. Another possible explanation for this result could be that these seeds are accumulating ABA to stimulate the transition into primary dormancy of these non-dormant seeds. There is evidence that components of the ethylene and ABA signalling pathways interact and that the

ethylene acts upstream of the ABA biosynthesis pathway with respect to seed germination (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000). The ethylene insensitive *etr1-1* mutant was shown to have increased sensitivity to ABA (Ghassemian *et al.*, 2000) and our results support this finding as not only mature, but developing *etr1-1* seeds have lower than wild-type germination and do not demonstrate an increase in ABA accumulation across seed development. The levels of ABA in a similar ethylene insensitive allele, *etr1-2*, are markedly different in mature seeds from those reported here as they represent the levels of ABA metabolism in response to signals promotive to germination (imbibition) and not during development (as reported here) (Chiwocha *et al.*, 2005). Inexplicably, the *etr1-1* mutant has a dramatic increase in GA content compared to wildtype (Figure 7-4). This observation was surprising because of the known promotive role GA plays in seed germination and that the seeds of this mutants exhibit a deeper dormancy than wild-type seeds across seed development. GA₁, GA₃, GA₄ or GA₇ are apparently not sequestered throughout wild-type silique and seed development, however the relative accumulation of at least GA₄ and GA₇ across seed development does not cause and increase in the ability of a seed to germinate with an ethylene insensitive ETR1-1 receptor. It is apparent that 'normal' signalling from the ETR1 receptor is directly or indirectly required to mediate 'normal' GA accumulation across seed and silique development. Conversely, the lack of GA accumulation in seeds and siliques with an ethylene insensitive ERS1-1 receptor do not appear to be correlated with the reduced primary seed dormancy state of this mutant. It is possible that the *etr1-1*

ethylene insensitive seeds and siliques are also less sensitive to endogenous GAs and perhaps this accumulation is an over-compensatory effect as it is known that ethylene and GAs play synergistic roles with respect to seed germination (Karssen *et al.*, 1983). The fact that these seeds over accumulate GAs to such a large degree during development but not in mature seeds is also quite perplexing. The *ers1-1* mutant had comparable GA levels to wild type seeds and siliques for all the GAs measured. The lack of any detectable GAs in stage 1 and stage 2 wild-type seeds and siliques and low levels found in the *ers1-1* mutant was rather surprising, as these tissues are still growing and expanding.

Because the *ers1-1* mutant had increased accumulation of ABA but lacked an increase in GA, it is conceivable that these seeds germinate during the inception of primary dormancy with a higher than wildtype percentage because of an increased sensitivity to GAs and/or a reduced sensitivity to ABA

It is likely that many hormone signalling pathways interact together to regulate various aspects of development (Gazzarrini and McCourt, 2003). *ein2*, first identified as a downstream component of the ethylene signal transduction pathway, has been isolated in screen for mutants impaired in various other hormones pathways including auxin transport inhibitors, ABA, cytokinin and in screens for mutants with delayed senescence (Alonso *et al.*, 1999). To this end, it is also interesting that these two ethylene receptor mutants showed dramatic changes in the levels of auxins and cytokinins throughout seed and silique development (Figures 7-3 and 7-4). The roles of auxin and cytokinin

have been less studied with respect to seed dormancy compared to ABA and GAs. In a study to find genes differentially regulated by exogenous GA application during seed germination, several auxin biosynthetic genes were found to be up-regulated suggesting a role for auxins in germination (Ogawa *et al.*, 2003). The lower levels of the conjugated auxin, IAAsp, in both mutants compared to wildtype and the reduced levels of IAA in the *etr1-1* mutant reveal an interaction between the auxin and ethylene during seed and silique development. In the cytokinin profiles, the most notable variation was the predominance of ZR in wildtype and *etr1-1* mutant as the primary cytokinin metabolite and IPA in the *ers1-1* mutant. Both ZR and IPA are low bioactive conjugates of more bioactive Z and 2iP, respectively. It is interesting to note that a loss of ethylene signal through the ERS1 receptor shifts the cytokinin metabolism towards IPA. The possibility of both auxins and cytokinins involved in seed dormancy is interesting, but will require further investigation to understand their potential roles and how they interact with ABA, GA and ethylene. We have used developing seeds as a tool to dissect the individual roles of the receptors. The two ethylene receptors, ETR1 and ERS1 have contrasting roles with respect to many aspects of plant development including primary dormancy. They may mediate this control, through hormone regulation as dominant mutations in these receptors vary the relative levels of hormones and hormone metabolites in other major hormone pathways throughout the development of seeds and siliques. The hormone data reveal unexpected interactions (auxin or cytokinin) and also provide new insight into the interaction

with ABA and GAs. It is presumed that these interactions are in part responsible for the dormancy differences observed between developing mutant and wild-type seeds. Phylogenetic and expression data show the conservation of the subfamily I receptors across many plant species and show that they are differentially regulated across development. Taken together these data indicate that the receptors have distinct functions for plant development.

Chapter 2: Ethylene Receptor Signalling: New Insights from Germination of Developing Mutant *etr1*, *ers1* and *ctr1* Seeds.

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Abstract

In *Arabidopsis*, ethylene signalling is mediated in part by a multi-gene receptor family. Five receptors have been identified and characterized based on sequence similarities. Similarities in protein domains as well as supporting genetic data suggest that these receptors function redundantly to negatively regulate ethylene responses. We have previously shown that the gain-of-function *etr1-1* and *ers1-1* mutants display an altered dormancy phenotype during seed development. The *etr1-1* seeds appear to exhibit a deeper dormancy while the *ers1-1* seeds appeared to have reduced dormancy. Here we further investigate the role of these receptors in seed dormancy with the analysis of loss-of-function *etr1* and *ers1* mutants. Furthermore, loss-of-function *ctr1-1* developing seeds were used to investigate the paradigm that all receptors signal through CTR1 to mediate an ethylene response. We have found that the ETR1 receptor may signal through CTR1 to signal the termination of seed dormancy. However, based on our data, it remains to be determined whether the ERS1 receptor also signals through CTR1.

Introduction

The five ethylene receptors of *Arabidopsis* are proposed to signal through CTR1, a downstream negative regulator of the ethylene signal transduction pathway (Figure #####). The exact method of signal transmission from receptors to CTR1 has been studied in detail and the role of the receptor kinase domain is the obvious candidate. However, the difference in domain architecture between the various receptors questions the notion that these receptors truly function redundantly. The mechanisms of signal transmission from the receptors to downstream components remains the focus of many studies and it has been proposed that the N-terminal domain, the histidine kinase domain and the receiver domain may all have distinct signalling responsibilities (Qu and Schaller, 2004).

Previous studies have shown that the histidine kinase activity of ETR1 may not be needed in signalling (Gamble *et al.*, 2002; Wang *et al.* 2003). Recently, it was shown that the histidine kinase activity of the ETR1 protein may in fact mediate ethylene signalling (Qu and Schaller, 2004). As previously mentioned, combinatorial LOF mutants lead to constitutive ethylene responses. The *etr1,etr2,ein4* triple LOF mutant exhibit constitutive ethylene responses in etiolated seedlings and was used as a genetic background to elucidate which receptor domains of ETR1 were involved in signal transmission (Qu and Schaller, 2004). Introduction of successively longer ETR1 deletion constructs into this genetic background while monitoring for the recovery of seedling growth determined that the histidine kinase activity of ETR1 was required for signal

transmission (Qu and Schaller, 2004). While these data support a role for ETR1 histidine kinase in receptor signalling, the genetic background used here contained a functional ERS1 protein (like ETR1, ERS1 contains all the appropriate residues for histidine kinase activity) and until these results are followed up using a background incorporating a true *ers1* LOF allele, the role of the histidine kinase domain in regulating receptor signalling will remain the topic of much discussion (Qu and Schaller, 2004).

Intriguingly, it was shown in a recent study that all receptor homologues were able to autophosphorylate *in vitro*, but unlike ETR1, the remaining four homologues were able to phosphorylate on serine residues (Moussatche and Klee, 2004). ERS1 was found to be capable of phosphorylating on histidine and serine residues, however the authors assume that histidine phosphorylation would not occur *in vivo* due to the relative cellular levels of di-cation metals (Moussatche and Klee, 2004). This may provide an opportunity for the receptors to regulate ethylene responses by two independent signalling methods, histidine dependant and/or serine dependant receptor phosphorylation.

Much of the data used to construct the current model of the ethylene signal transduction pathway comes from monitoring the triple response assay of etiolated seedlings. At such a young developmental stage, if the receptors function in specific independent signalling pathways, it is difficult to dissect the individual roles of each receptor since the two modalities in this assay are triple response (or the degree of triple response) or lack of triple response. Since ethylene regulates many other developmental and stress responses, it may be

possible to delineate specific roles of the receptors by monitoring various receptor mutants at later stages of development. One such developmental process that may be used to investigate receptor signalling is based on the ability of a developing seed to germinate. We have previously observed a general pattern of dormancy during the development for wild-type seeds dissected out of their siliques based on the ability of the seed to germinate (Thurston *et al.*, Chapter 1). In wildtype, there are four distinct phases of seed development based on germination; non-viable immature seeds (Stage 1), precocious germination (viable immature seeds – stage 2), dormancy (stage 3 and stage 4) and viable mature seeds (stage 5 and stage 6). Previously, we have proposed that two receptors, ETR1 and ERS1, have non-redundant functions based on phylogenetic, gene expression, and metabolomic evidence (Thurston *et al.*, Chapter 1). Analysis of the divergence of all the ethylene receptor protein sequences across a variety of plant species demonstrated a strong trend towards the independent groupings of ETR1 group, an ERS1 group and a lesser defined ETR2 group consisting of the ETR2, ERS2 and EIN4 receptors. The strong independent grouping of the ETR1 and ERS1 receptors across a broad range of plant species indicated that the ETR1 and ERS1 proteins serve important function(s) in general plant growth and/or development. Expression analysis of all five receptor homologues revealed differential gene expression patterns throughout development, a characteristic that would not be expected if all receptors were truly redundant. Strong evidence that the ETR1 and ERS1 receptors serve non-redundant and opposite functions with respect to

determining the fate of seed dormancy is based on the ability of GOF mutant seeds to germinate throughout the previously mentioned stages of seed development. Compared to wild-type seeds, developing *etr1-1* seeds do not show the same phases of seed development, rather they appear to be much more dormant across all stages of seed development - a characteristic that has been previously observed for mature *etr1-1* seeds (Bleecker *et al.*, 1988) and is apparently attributed to a super-sensitivity to endogenous ABA (Beaudoin *et al.*, 2000, Ghassemian *et al.*, 2000). Developing *ers1-1* seeds do not show the same phases of seed development as wild-type seeds and appear to be less dormant as they are able to germinate during the dormancy phase of wild-type seeds (stage 3 and stage 4) even after non-stratification conditions. Due to the ethylene insensitivity of the GOF mutations, it appears that ethylene signalling through the ETR1 receptor is responsible for seed germination or the release of dormancy, while ethylene signalling through the ERS1 receptor is responsible for entrance into seed dormancy. It is possible that these two receptors regulate seed dormancy/germination by modifying the accumulation of other hormones since the *ers1-1* seeds have altered abscisic acid and cytokinin levels whereas *etr1-1* seeds have altered auxin and gibberellin levels (Thurston *et al.*, Chapter 1)

Using the transition into and out of dormancy to follow ERS1 and ETR1 receptor function individually, we can now address whether both receptors signal through CTR1 during this developmental process. Here we present the dormancy pattern of developing LOF *etr1*, *ers1* and *ctr1* developing seeds and discuss these results in context of receptor signalling.

Materials and Methods

Plant material

All plants used in these experiments were of the Columbia (Col-0) or Wassilewskija (Ws-2) ecotypes. The following mutants were of the Columbia (Col-0) ecotype: *ctr1-1*, *etr1-5*, *etr1-6* and *etr1-7*. The loss-of-function *ers1-3* mutant was of the Wassilewskija (Ws-2) ecotype. Isogenic wild type plants were used as controls for the developing seed germination assay. All seeds were obtained from the Arabidopsis Biological Resource Center (Columbus, OH).

Plant growth conditions

Seedlings and plants were handled and grown as stated in (Thurston *et al.*, Chapter 1).

Silique and seed staging for germination throughout seed development

Siliques and seeds were staged as previously reported (table 1, Thurston *et al.*, Chapter 1). Briefly, siliques corresponding to the appropriate stage were dissected and seeds corresponding to the appropriate stage were transferred to germination media consisting of water and agar (0.8% w/v) media pH 5.7-5.8. Six siliques per stage were used and eight seeds from each silique were plated onto replicate plates (48 seeds/stage). Plates were wrapped in parafilm and one replicate plate was used for analysis of germination after stratifying conditions (7 days incubation at 4°C in the dark) the other was used for analysis of germination under non-stratifying conditions. Germination was scored by radicle protrusion

after 14d incubation at 22°C under constant light (*ctr1-1*) or long-day (16hlight/8hdark) (*etr1-7*, *etr1-6*, *etr1-5*, *ers1-3*, Ws wild type) growth conditions.

Results

Dormancy pattern of LOF *etr1* developing seeds

The *etr1-1* mutant was created by the mutagen EMS and is the result of a G to A transition at nucleotide 194 of the coding sequence (c.s.) and results in a single amino acid substitution (Cys to Tyr) in the N-terminal domain of the receptor (Hua and Meyerowitz, 1998). This mutation abolishes the localization of a copper cofactor (Rodriguez *et al.*, 1999) which has been shown to be necessary for ethylene binding (Woeste and Kieber, 2000), and thus the *etr1-1* mutant is insensitive to ethylene. In the presence of ethylene, the mutated receptor is not de-activated and therefore maintains the repression of CTR1. Since CTR1 is not de-repressed then repression of ethylene related responses is maintained. Many loss-of-function mutation mutants have been isolated from the *etr1-1* genetic background, as intragenic suppressors of the gain-of-function phenotype (Hua and Meyerowitz, 1998). The resulting proteins are presumably truncated or unstable as they have not been detected from protein extracts (Hua and Meyerowitz, 1998; Chen *et al.*, 2002; Zhao *et al.*, 2002). The three LOF *etr1* mutants used in this study are *etr1-7* (G to A transition at nucleotide 222 c.s.), *etr1-6* (G to A transition at nucleotide 907 c.s.) and *etr1-5* (G to A transition at nucleotide 1689 c.s.) and if translated into a stable protein would result in a 73

a.a. (~9.9% of *etr1-1* protein and would contain incomplete (two of the three) N-terminal membrane spanning domains), 306 a.a (~41.5% of *etr1-1* protein and would contain complete membrane spanning domain and GAF domain) and 562 a.a (~76.1% of *etr1-1* protein and would contain complete membrane spanning domain, GAF domain and histidine kinase domain – but no receiver domain) proteins respectively (Hua and Meyerowitz, 1998; Zhao *et al.*, 2002).

Developing LOF *etr1* mutant seeds were assayed on their ability to germinate across the six stages of seed development. Since developing GOF *etr1-1* seeds were more dormant throughout the four phases of seed development when compared to wild-type seeds (Figures 1A and 1C from chapter 1), the LOF mutants may have an opposite phenotype. There was a remarkable difference in the ability of the LOF *etr1* seeds to germinate depending on the proximity of the LOF mutation to the translational start site of the predicted protein. *etr1-7* developing seeds would theoretically have the shortest truncated *etr1-1* protein product and these seeds were able to germinate with approximately 100% germination throughout stages 2-6 of seed development and displayed no dormancy (Figure 1C). Similarly, *etr1-6* developing seeds are also able to germinate throughout seed stages 2-6 (Figure 1B), however the percent germination was slightly lower than that of *etr1-7* mutant. The *etr1-5* mutant would theoretically produce 76.1% of the *etr1-1* protein product, and the seeds in this mutant displayed a wildtype-like germination pattern such that the four phases of seed development could be observed (Figure 1A). Thus, the three LOF *etr1* mutants used here display distinct phenotypes with respect to

Figure 1: Graphical representation of developing seed germination patterns of loss-of-function *etr1* mutants. Staged seeds were harvested from their corresponding siliques and plated on germination media. Germination plates were either stratified or not stratified and grown under long day conditions for 14 days. Seed germination counts were scored on the fourteenth day. Germination was scored by radicle protrusion. All loss-of-function alleles used here were able to rescue germination from the dormant phenotype of the gain-of-function *etr1-1* seeds. Black bars represent non-stratified seeds, grey bars represent stratified seeds

A. *etr1-5*. The four phases observed in wild-type seeds are also observed in the *etr1-5* mutant. Immature stage 1 seeds do not germinate. Seeds germinate precociously at stage 2 but with more vigour than wild-type stage 2 seeds. Stage 3 and stage 4 seeds have a reduced ability to germinate. Stage 5 and stage 6 stratified seeds are able to germinate. It was not determined whether an after-ripening period is required for non-stratified seeds to germinate.

B. *etr1-6*. The four phases observed for wild-type seeds were not observed in the *etr1-6* mutant. Immature stage 1 seeds did not germinate; stage 2 seeds showed precocious germination but with a higher vigour than stage 2 wild-type seeds. Unlike wild-type stage 3 and stage 4 seeds, the *etr1-6* stage 3 and stage 4 non-stratified and stratified seeds were able to germinate and appear to be non-dormant. Both stratified and non-stratified stage 5 and stage 6 seeds are able to germinate. Note that germination levels are much higher at all stages in this mutant.

C. *etr1-7*. Similar to the *etr1-6* mutant, the four phases observed in wild-type seeds were not observed in the *etr1-7* mutant. Immature stage 1 seeds did not germinate; stage 2 seeds showed precocious germination but with a higher vigour than stage 2 wild-type seeds. Unlike wild-type stage 3 and stage 4 seeds, the *etr1-6* stage 3 and stage 4 non-stratified and stratified seeds were able to germinate and appear to be non-dormant. Both stratified and non-stratified stage 5 and stage 6 seeds are able to germinate. Note that germination levels are much higher at all stages in this mutant.

A.

B.

C.

seed dormancy. All three LOF mutants are able to restore germination in the *etr1-1* background, *etr1-5* to wildtype -like levels, while *etr1-6* and *etr1-7* seeds show a strong LOF phenotype and do not become dormant.

Dormancy pattern of LOF *ers1* developing seeds

The *ers1-1* mutation is functionally equivalent to the substitution of the *etr1-1* mutation and was created using a forward genetics approach (by site-directed mutagenesis of the wild-type ERS1 protein). This construct was transformed into wild-type plants resulting in the ethylene insensitive *ers1-1* mutant (Hua *et al.*, 1995). Because of the difference in generating the *etr1* and *ers1* gain-of-function mutants, it is not possible to generate intragenic suppressors of the *ers1-1* mutant as the native ERS1 protein is also present in this genetic background. The LOF *ers1* mutants available to date were created by T-DNA knockout strategy. Therefore, a potentially important difference between LOF *etr1* and LOF *ers1* mutants is that if the resulting LOF *ers1* proteins are expressed and fold properly, they may retain their ethylene binding capacity, and depending on which domains are responsible for signal transmission, may be able to transmit signals to downstream components. In summary, the LOF *etr1* mutants are true null lines (no input and no output capabilities) while the *ers1* LOF lines may be capable of signal input and thus, potentially output. The *ers1-2* mutant, a putative LOF allele, is the result of a T-DNA insertion into the 5' untranslated region of the *ers1* gene (Wang *et al.*, 2003). This mutant has recently been suggested a possible hypomorphic allele rather than a true LOF

mutant. This insertion presents several ATG sites into the 5' untranslated region of the gene however, whereas the wild type *ers1* gene also contains two ATG sites in the 5' untranslated region. Therefore this T-DNA mutant may still allow for protein translation of the ERS1 protein (Gamble *et al.*, 2002; Wang *et al.*, 2003; Qu and Schaller 2004). Due to the complexity of this mutant, we did not use this mutant for analysis of seed dormancy patterns for a LOF *ers1* mutant. Another T-DNA insertional LOF *ers1* mutant has recently been isolated by the Schaller lab and is the result of insertion into the *ers1* coding sequence. *ers1-3* is the result of a T-DNA insertion at nucleotide 471 c.s. and would result, if translated, in a truncated protein of 168 a.a which would be approximately 27% of the native ERS1 protein (consisting of a membrane spanning domain just up to the GAF domain of ERS1) (ABRC, 2005). The *ers1-3* mutant was isolated from a T-DNA knockout population of seeds in the Wassilewskija (Ws) ecotype. For an isogenic control, wild-type Ws seeds were assayed on their ability to germinate across the six stages of seed development. The germination pattern of Ws wildtype and Col wildtype were different (compare Figure 2A presented in this chapter with Figure 2A from chapter 1). Similar to wild-type Col seeds, wild-type Ws stage 1 seeds were unable to germinate and stage 2 seed showed precocious germination. However the subsequent stages of wild-type Ws seeds including stage 3 and stage 4 were able to germinate whereas germination was only seen in the wild-type Col stage 5 and stage 6 seeds. The wild-type Ws seeds appear to be less sensitive to cold during the dormancy stages seen in the Col ecotype (stage 3 and stage 4) as the Ws wild-type stratified seeds were able

Figure 2: Graphical representation of developing seed germination patterns of loss-of-function *ers1* receptor mutant and Ws wildtype. Staged seeds were harvested from their corresponding siliques and plated on germination media. Germination plates were either stratified or not stratified and grown under long day conditions for 14 days. Seed germination counts were scored on the fourteenth day. Germination was scored by radicle protrusion. Black bars represent non-stratified seeds, grey bars represent stratified seeds

A. wildtype (Ws). The germination pattern of wild-type Ws developing seeds varies from the germination pattern on wild-type Col developing seeds. Immature stage 1 seeds do not germinate. Non-stratified stage 2 seeds germinate with a higher percentage than stratified stage 2 seeds. Stage 3 have a reduced ability to germinate but show higher germination levels compared to wild-type (Col) stage 3 seeds. Unlike wild-type (Col), wild-type (Ws) stage 4 seed show a high level of germinating stratified seed. Stage 5 and stage 6 seeds are able to germinate. Stratified seeds are able to germinate throughout seed development.

B. *ers1-3*. The four phases observed in wild-type (Col) were not observed in the *ers1-3* mutant. Stage 1 seeds are unable to germinate. Both stratified and non-stratified stage 2 seeds germinate precociously, however stage 2 stratified seeds germinate with less vigour than the wild-type (Ws) stage 2 stratified seeds. Stage 3 to stage 6 stratified seeds display a trend of increasing seed germination. Note that the trends in the germination profile of *ers1-3* mutant seeds resembles that of wildtype (Ws).

A.

B.

to germinate. Developing LOF *ers1-3* mutant seeds were assayed on their ability to germinate across our six stages of seed development. The germination pattern of the *ers1-3* seeds paralleled those of the isogenic wild-type seeds (Figures 2A and 2B). Wild-type Ws and *ers1-3* non-stratified seeds showed precocious germination in stage 2 and a reduction of germination in stage 3, stage 4 and stage 5 followed by an increase in germination at stage 6, however the number of wild-type Ws seeds that germinated were roughly twice that of the *ers1-3* seeds for this stage. The stratified seeds in both wild-type Ws and *ers1-3* germinated precociously at stage 2 but had increased germination in stages 3, stage 4, stage 5 and stage 6. Unlike the *etr1* LOF mutants, the *ers1* LOF mutant did not show a strong LOF phenotype.

Dormancy pattern of LOF *ctr1* developing seeds

Previous studies have shown that the *ctr1-1* mutant displays constitutive ethylene responses and is the result of a mutation in the C-terminal kinase domain of the CTR1 protein (Kieber *et al.*, 1993; Gao *et al.*, 2003). The *ctr1-1* mutation alleviates the kinase activity of CTR1 and thus, regardless of the signalling state of the receptors, a *ctr1-1* mutant is unable to repress downstream ethylene responses. Therefore, as both the ETR1 and ERS1 receptors have been shown to directly interact with, and are directly downstream to CTR1 we sought to determine the phenotype for the germination of developing loss-of-function *ctr1-1* mutant seeds. The developing *ctr1-1* seeds do not show the same phases of seed development as wild-type seeds based on their ability to

germinate (compare Figure 3 from this chapter and Figure 2A from chapter 1). The *ctr1-1* seeds are non-viable at stage 1 but they do germinate precociously at stage 2. However, similar to *ers1-1* developing seeds, *ctr1-1* developing seeds appear to be less dormant as they are also able to germinate during the normal dormancy phase of wild-type seeds (stage 3 and stage 4). Also similar to *ers1-1* developing seeds, the *ctr1-1* developing seeds exhibited a greater than wild-type ability to germinate without stratification throughout the latter five stages of seed development

Discussion

In the current model of the ethylene signal transduction pathway, five receptors perceive ethylene and previous genetic and biochemical evidence indicate that they transmit this signal through CTR1, a downstream MAPKKK negative regulator of this pathway. We have previously shown that two ethylene receptor mutants do not follow the same phases of seed dormancy compared to wild-type developing seeds, in that *ers1-1* seeds do not enter the dormancy phase of seed development and *etr1-1* seeds exhibit a deeper dormancy throughout all developmental stages (Figure 2, chapter 1). Since the GOF mutations are dominant, the mutated receptor is activating CTR1 independent of the presence of ethylene to repress downstream ethylene responses. Due to the nature of these dominant mutations in the receptors, if the *etr1-1* developing seeds have an exaggerated seed dormancy throughout the phases of seed development, and this is due to the constant activation of CTR1 by the *etr1-1*

Figure 3: Graphical representation of developing seed germination patterns of a loss-of-function *ctr1* mutant. Staged seeds were harvested from their corresponding siliques and plated on germination media. Germination plates were either stratified or not stratified and grown under constant light conditions for 14 days. Seed germination counts were scored on the fourteenth day. Germination was scored by radicle protrusion. Black bars represent non-stratified seeds, grey bars represent stratified seeds. The four phases observed for wild-type seeds were not observed in the *ctr1-1* mutant. Immature stage 1 seeds did not germinate; stage 2 seeds showed precocious germination. Unlike wild-type stage 3 and stage 4 seeds, the *ers1-1* stage 3 and stage 4 non-stratified and stratified seeds were able to germinate and appear to be non-dormant. Both stratified and non-stratified stage 5 and stage 6 seeds are able to germinate.

protein, then the wild-type ETR1 receptor is likely involved in regulating seed germination (or the release from dormancy). Similarly, the wild-type ERS1 receptor is likely involved in initiating seed dormancy as GOF *ers1-1* seeds do not enter into the dormancy phase of seed development. Analysis of the LOF mutants of these receptors could therefore substantiate the idea that the ETR1 and ERS1 receptors serve specific and independent signalling functions with respect to seed dormancy. The opposite dormancy patterns observed for the GOF *etr1* and *ers1* mutant seeds with respect to wild-type seeds present a unique opportunity to examine the roles of these two receptors with respect to regulating seed dormancy.

The LOF alleles result in truncated or unstable proteins as these mutations introduce premature stop codons or interfere with intron splice sequences. The protein products of LOF *etr1* mutants have not been detected *in vivo* using specific antibodies raised against different portions of the wild-type protein receptors, suggesting these LOF mutants do not produce stable truncated proteins (Hua and Meyerowitz, 1998; Zhao *et al.*, 2002). Unlike the GOF mutants in which the mutated receptor constantly activates CTR1, it is presumed that in a LOF background, CTR1 regulation by the LOF protein is lost as no receptor is being synthesized. Therefore, if we assume that these receptors do serve specific and independent signalling functions, the LOF mutants should reveal phenotypes in which specific activation of CTR1 by that receptor is lost. Furthermore, LOF phenotypes may be opposite in phenotype to the GOF mutants. With respect to seed dormancy and in the case of ETR1,

which potentially signals through CTR1 to stimulate germination (or release of dormancy), the loss of regulation of CTR1 by the ETR1 protein (a LOF *etr1* mutant), should result in the loss of regulation of seed germination (or release of dormancy) by ethylene. Likewise the loss of regulation of CTR1 by the ERS1 protein (LOF *ers1* mutant) should result in the loss of initiation of seed dormancy by ethylene.

Analysis of the four phases of seed development in the three LOF *etr1* mutants used in this study revealed that, the *etr1-5*, *etr1-6* and *etr1-7* mutations were able to restore germination in the *etr1-1* background. The *etr1-7* mutant showed the strongest LOF phenotype as close to 100% seed germination was observed in the latter five stages of seed development (Figure 1C). This phenotype is opposite to that displayed by the GOF *etr1-1* mutant, in which seeds in the latter five stages of seed development exhibited greater than wildtype dormancy (Figure 2C in chapter 1). The *etr1-6* mutant also showed an *etr1-7*-like lack of seed dormancy across seed development, except for a roughly 40% reduction in germination of stage 3 seeds (Figure 1B). However, the *etr1-5* mutant showed wildtype-like germination patterns across the four phases of seed development. Stage 1 seeds did not germinate, stage 2 seeds germinated precociously but to a higher percentage than wild-type stage 2 seeds (compare Figure 1A from this chapter with Figure 2A from chapter 1). Seed germination was greatly reduced during stage 3 and stage 4, however not to the extent of wild-type seeds at stage 3 and stage 4, and finally complete germination was observed in mature stage 5 and stage 6 seeds after stratifying conditions. The

analysis of both LOF and GOF *etr1* seeds taken together suggest that the ETR1 protein is needed for the regulation of seed germination (or release from dormancy) as GOF mutations prevent germination and extreme LOF mutations activate germination throughout seed development. But how could the LOF *etr1-5* allele result in a wildtype-like phenotype if this mutant doesn't produce a protein product? We would expect that all LOF *etr1* alleles would result in a similar phenotype if all alleles do not produce a protein product. If we assume that these LOF alleles do produce protein products, then it is possible that the ETR1 protein interacts with downstream components to mediate germination (or the exit of dormancy) as the corresponding truncated versions of *etr1-1* protein product (ETR1-7 and ETR1-6) are not sufficient to maintain the repression of ethylene responses. Perhaps the *etr1-5* protein product, having 76.1% of the *etr1-1* protein product is able to weakly interact with these downstream components and results in a slight LOF phenotype resembling wild-type germination levels. Alternatively, certain protein signalling domains (or the lack of certain protein signalling domains) could be responsible for the observed phenotypes. The only difference between the putative *etr1-5* and *etr1-1* protein products would be the absence of the C-terminal receiver domain in *etr1-5*. Perhaps the C-terminal receiver domain is involved in a yet to be identified two-component phosphorelay signalling pathway to regulate seed germination (or release from seed dormancy). However such an assumption would have to take into account the strong LOF phenotypes of the *etr1-7* and *etr1-6* mutants as their putative protein products would also lack the C-terminal receiver domain as well as other *etr1*

protein domains (the *etr1-6* putative protein would be comprised of approximately 41.5% of the *etr1-1* protein product and would be lacking the putative histidine kinase domain and receiver domain. The *etr1-7* protein product would contain only 9.9% of the *etr1-1* protein product and would lack all domains of this protein except 2 membrane spanning motifs of the N-terminal domain). Perhaps then, it is the simultaneous action of multiple protein domains present on the receptor that determines the appropriate response. Removing certain domains (as in the *etr1-7* and *etr1-6* putative protein products) would modulate the signalling capability of the receptor. Identification of the truncated protein products from these LOF mutants would be necessary to corroborate this proposal. As well, further biochemical analysis of the ETR1 receptor domains in the context of seed germination will be required to support such assumptions. However, these results do not preclude the possibility that the receptors could be forming heterodimer complexes. If the LOF *etr1* mutants do produce a truncated protein product and these truncated proteins heterodimerize with other native full length receptors, especially with ERS1 all the necessary residues for His Kinase activity are conserved in ERS1, then perhaps ethylene signalling from the truncated LOF ETR1 protein could be modulated through such heterodimer complexes.

Interestingly, the protein product of *etr1-5* would contain all the functional domains present in the GOF *ers1-1* protein, since the difference between the ETR1 and ERS1 receptor is the presence of the fused receiver-domain in ETR1. Therefore, it is interesting to note that ethylene insensitive GOF *ers1-1* mutant showed higher than wildtype levels of germination through seed development

(Figure 2B from chapter 1), while the ethylene insensitive LOF *etr1-5* mutant showed wildtype-like germination levels (Figure 1A).

Developing GOF *ers1-1* mutant seeds are less dormant than wild-type seeds when they are tested for their ability to germinate (Figure 2B, chapter 1). Based on these results, if we assume that the ERS1 protein is involved in the initiation of seed dormancy, then we would expect the LOF *ers1* developing seeds to have an increased dormancy. However, the LOF *ers1-3* developing seeds have wildtype-like germination across our six stages of seed development (Figure 2B). Interestingly the dormancy phase of seed development (stage 3 and stage 4) is seen in the *ers1-3* seeds under non-stratifying conditions, however this dormancy phase is lost after seeds are stratified, a trend that was also observed in the isogenic control. The fact that the LOF *ers1-3* mutant seeds do not result in the expected dormant phenotype could be due to several reasons. This *ers1-3* mutant may not be a LOF mutant as there is currently no published literature about this mutant. In this case, the germination pattern seen in the 'LOF *ers1-3*' mutant would be similar to wildtype, as was observed. If the *ers1-3* mutant is a true LOF *ers1* allele, then perhaps the expected results are being masked because of the differences in the germination pattern between the Ws wildtype and Col wildtype throughout seed development. The fact that this mutant is in another ecotype as the GOF mutant used in previous studies (Wassilewskija rather than Columbia) may be responsible for the unexpected LOF phenotype. Alternatively, if the ERS1 protein is not involved in the entrance into dormancy, then the LOF mutants would not be expected to have an ethylene

response phenotype. Perhaps the GOF *ers1-1* mutant gained the ability to interact with factors that regulate seed dormancy. However, the previously reported gene expression patterns of ERS1 during the early stages of seed development, as well as the previously generated hormone profiles tend to suggest that the ERS1 protein is involved in seed dormancy (Thurston *et al.* Chapter 1).

In our previous study, we found that the GOF *etr1* and GOF *ers1* developing seeds had opposite phenotypes compared to wild-type seeds, such that the *etr1-1* seeds were more dormant and the *ers1-1* seeds were less dormant than wild-type seeds. We postulated that these two receptors could therefore have distinct and opposite functions with respect to regulating seed dormancy. Genetic and biochemical evidence suggests that these two receptors interact with CTR1 to transmit their signal through the ethylene signal transduction pathway. How then could both proteins interact with CTR1 to affect the appropriate response? The LOF *ctr1-1* mutant was used to investigate the paradigm that all, or at least these two receptors, signal through CTR1. The *ctr1-1* developing seeds did not show the same germination phenotype as wild-type seeds (Figure 3). The seeds were able to germinate precociously at stage 2, but opposite to wildtype, they had increased germination at stage 3 and stage 4. Stage 5 and stage 6 seeds exhibited almost 100% germination. It appears that these seeds do not enter into primary seed dormancy during seed development. If the ETR1 protein is involved in seed germination (or the release if dormancy), as supported by the GOF *etr1* and LOF *etr1* data, and it signals through CTR1,

then a LOF *ctr1* mutant should resemble a LOF *etr1* mutant. As the strong LOF *etr1* alleles and the LOF *ctr1-1* mutant exhibited an increased germination percentage throughout seed development, ETR1 must signal through CTR1 to regulate seed germination (release of dormancy) (summarized in Figure 4). The results of the *ctr1-1* germination phenotype did not support the notion that the ERS1 protein signals through CTR1 to regulate seed dormancy, as the LOF *ctr1-1* mutant and the LOF *ers1* mutant do not exhibit similar germination patterns throughout seed development. Instead, they may suggest that this signal is transmitted through a CTR1-independent signalling pathway. ERS1 is the only conserved His kinase without a fused receiver domain in Arabidopsis (Schaller *et al.*, 2002) and may be sufficient for a CTR1-independent signalling pathway. If this were true then it is peculiar that the ERS1 protein has been reported to interact with CTR1 and that double mutant analysis between ERS1 and CTR1 suggests that CTR1 operates downstream of ERS1. ERS1 may signal in both CTR1-dependant (triple response) and CTR1-independent (seed dormancy) pathways. Due to the different ecotypes used in this study, and the differences in the strategy used to isolate the LOF mutants of each receptor, the understanding of the role ERS1 plays in activating seed dormancy is not complete and will require this study to be performed again with a suitable LOF *ers1* mutant. To determine whether ERS1 may signal through a CTR1-independent signalling pathway, it would be useful to determine what other proteins this receptor may interact with at the endoplasmic reticulum. Any array of strategies to determine

Figure 4: Hypothetical model of ETR1, ERS1 and CTR1 signalling for seed dormancy.

Data is based on GOF *etr1-1* and GOF *ers1-1* germination profiles, LOF *etr1-1* and LOF *ctr1-1* germination profiles but does not include LOF *ers1-3* germination profiles (see discussion). Mutations are represented by black circles.

The ethylene receptors form homodimers at the endoplasmic reticulum (ER). A copper ion (Cu), which mediates ethylene binding, associates with the N-terminal hydrophobic domain of the receptors.

In the absence of ethylene, the C-terminal His kinase (black) region of the receptors interact with the N-terminal domain of CTR1 (dark grey). The kinase domain of CTR1 actively represses downstream ethylene responses.

Upon ethylene (light grey) binding, the interaction between the ethylene receptors and CTR1 changes such that the kinase activity of CTR1 is reduced and the ethylene response pathway is de-repressed.

GOF mutations in the ethylene receptors alleviates copper co-factor association and ethylene never binds to such receptors. Therefore in the presence of ethylene, the GOF receptor mutants maintain their interaction with CTR1, such that the kinase domain of CTR1 actively represses downstream ethylene responses.

Assuming the LOF *etr1* mutations form stable proteins, the resulting proteins would be truncated version of the GOF *etr1-1* protein. These truncated proteins do not contain the protein domains required for association with CTR1.

Therefore, in air or in the presence of ethylene, the kinase domain of CTR1 is reduced and the ethylene response pathway is de-repressed.

The LOF *ctr1-1* is the result of a point mutation that alleviates kinase activity.

Therefore in air, there is no kinase activity and the ethylene response pathway is de-repressed. (adapted from Gao *et al.*, 2003)

protein-protein interactions would be useful, such as the yeast two hybrid (Y2H) strategy or tandem affinity purification (TAP-tagging) strategy.

Appendix I: Interaction between ABA and ethylene during seed dormancy.

Introduction

ABA and ethylene interact in many different tissues to regulate different developmental events including seed dormancy/germination, root growth, and vegetative growth (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000; LeNoble *et al.*, 2004). During seed dormancy, ethylene has been proposed to act as a negative regulator of ABA signalling by decreasing sensitivity to endogenous ABA (Ghassemian *et al.*, 2000). Sugar has also been implicated in the interaction between ABA and ethylene as many sugar insensitive mutants have been shown to be allelic to ABA biosynthetic and ethylene signalling genes (summarized in Leon and Sheen, 2003). As well, high levels of exogenous sugar repress seed germination and early seedling development. This repression is thought to occur by increasing endogenous ABA levels (Gazzarrini and McCourt, 2001).

To investigate the interaction between ethylene and ABA during seed dormancy, we crossed the ABA-deficient mutants, *aba1-6*, *aba2-3* and *aba3-1* with the ethylene insensitive *etr1-1* and *ers1-1* mutants. The ABA-deficient mutants were generated by EMS and the ABA1- zeaxanthin epoxidase (Rock and Zeevart, 1991), ABA2 - xanthin oxidase (Schwartz *et al.*, 1997; Leon-Kloosterziel *et al.*, 1996) and ABA3 - molybdenum cofactor sulfurase (Schwartz *et al.*, 1997; Xiong *et al.*, 2001) proteins belong to the ABA biosynthesis pathway. Double mutants were isolated using a screen that took advantage of characteristics of the individual mutants. Specifically, ABA-deficient mutants are

able to germinate in the presence of high levels of exogenous sugar while ethylene-insensitive mutant seedlings do not show the characteristic triple response morphology when germinated in the presence of high levels of ethylene.

Methods and preliminary results

Plant Material

All plants used in these experiments were of the Columbia (Col-0) ecotype. All seeds were obtained from the Arabidopsis Biological Resource Center (Columbus, OH).

Plant growth conditions

Plant growth condition, seed sterilization and seed germination media were followed as previously described (Thurston *et al.*, Chapter 1). M&S media containing 0.3M glucose was used for screening ABA-deficient mutants. For determining non-stratified seed germination on M&S and agar media, approximately 200 seeds per genetic background were sterilized (as reported in Thurston *et al.*, Chapter 1) and plated. For determining non-stratified seed germination on M&S, glucose and agar media, approximately 100 seeds per genetic background were sterilized and plated. Germination plates were wrapped in aluminum foil (dark) and incubated at room temperature (25°C) for four days. Germination was scored by radicle protrusion.

Generating double mutants

For generating all double mutants, pollen from ethylene receptor mutants was transferred onto the flowers of ABA-deficient mutants in order to score successful crosses. As the ethylene receptor mutants are the result of dominant mutations, then a proportion seeds of the F_2 population exhibiting ethylene insensitivity during a triple response assay would indicate a successful cross.

Immature, un-opened ABA-deficient flower buds at the top of recently bolting inflorescence were selected for crossing. All siliques and unused flowers/flower buds were removed from the selected maternal plant. From the selected flower buds, the sepals and petals were carefully dissected under a dissecting microscope and the immature stamens and anthers were carefully removed. Under the dissecting microscope, mature anthers from opened ethylene receptor mutant flowers were used to brush pollen over the stigmatic tissue of the ABA-deficient flower bud. The 'crossed' flower buds were carefully wrapped in plastic wrap to prevent desiccation. Because the stigmatic tissue of these flower buds were immature at the time of dissection, another 'crossing' was required during the following two days post bud dissection. Successfully 'crossed' flowers developed siliques and were left develop seed.

For each potential double mutant cross, F_1 seeds from three independent parental plants were sterilized and stratified for 4 days as previously reported (Thurston *et al.*, Chapter 1). Seeds were sown directly into soil and F_1 plants were allowed to self fertilize. The siliques from individual F_1 plants were

harvested for screening of F₂ seeds. Therefore three pools of F₂ seeds per cross were collected for screening.

Screen for isolating double mutants

The ABA-deficient mutations are recessive while the ethylene receptor mutations are dominant. All F₁ seeds are heterozygous for each trait, while F₂ seeds, assuming Mendelian inheritance patterns, are 9:3:3:1 for ethylene insensitive and normal ABA biosynthesis : **ethylene insensitive and ABA-deficient** : normal ethylene sensitivity and normal ABA biosynthesis : normal ethylene sensitivity and ABA-deficient. Bold font represents the target double mutants. Therefore, 18.75% of F₂ seeds (3 out of 16) should be ethylene insensitive and ABA-deficient, however only 6.25% of F₂ seeds (1 out of 16) will be homozygous for the ethylene receptor mutation and the ABA-deficient mutation. Understandably, an efficient screen is valuable in isolating such double mutants.

The ABA-deficient mutations have reduced ABA content and non-stratified mature seeds germinate with a higher percentage than wild-type seeds (Figure 1). Wild-type seeds have approximately 60% germination, while the ABA-deficient *aba1-6* and *aba2-3* mutant seeds have approximately 100% and 80% germination, respectively. The ABA-deficient *aba3-1* seeds show wildtype-like germination at approximately 30% germination. This reduction in germination compared to the other ABA-deficient mutants may relate to the location of the ABA3 protein in the biosynthetic pathway of ABA. While the ABA1 and ABA2 proteins are further upstream in the ABA biosynthetic pathway, the molybdenum

Figure 1: Graphical comparison of the germination characteristic for various ethylene-insensitive and ABA-deficient non-stratified seeds on M&S+Agar media.

Non-stratified wild-type (Col), *etr1-1*, *ers1-1*, *aba1-6*, *aba2-3* and *aba3-1* mature seeds were plated onto M&S +Agar and were left to germinate at room temperature in the dark for four days. All seed types except *etr1-1* are able to germinate under these conditions. The *ers1-1* mutant has a reduced level of germination compared to wild-type. The ABA-deficient *aba1-6* and *aba2-3* alleles show an increased ability to germinate, attributed to a reduction in endogenous ABA content. The *aba3-1* mutant displayed a similar level of germination compared to wild-type. This observation may be explained by the location of the ABA3 protein in the ABA biosynthetic pathway. ABA3 is involved in the conversion of ABA-aldehyde into ABA, and therefore ABA-aldehyde is likely exhibiting biological activity. In general, ABA-deficient mutants show a greater percent germination when compared to the ethylene receptor mutants and wildtype under these conditions.

cofactor sulfurase (the *aba3* protein product) is involved in the conversion of ABA-aldehyde into ABA (Schwartz *et al.*, 1997) and thus it is likely that the ABA-aldehyde exhibits some degree of biological activity. The ethylene receptor *ers1-1* mutant seeds show approximately 20% germination while the *etr1-1* mutant seeds do not germinate under these conditions. Previously, ABA-deficient and ethylene-insensitive double mutants of *Arabidopsis* (*aba2-1;etr1-1*) were isolated using a screen that took advantage of two characteristics of the *etr1-1* mutation (LeNoble *et al.*, 2004). Since the *etr1-1* mutant displays an increase in seed dormancy and does not germinate without stratification, LeNoble and colleagues isolated double mutants based on the ability of the ABA-deficient mutation to recover germination in an *etr1-1* background. Germinated seedlings on media containing ACC and displaying a lack of triple response were selected as likely double mutants. (Unfortunately, these authors did not reply to our requests for these seeds. Instead, we used a similar approach to isolate our three double mutant lines containing the *etr1-1* mutation.) We did not use this strategy for crosses involving the *ers1-1* mutation as increased seed dormancy is not a characteristic of the *ers1-1* mutant (See figure 1). Instead we used the sugar insensitive characteristic of ABA-deficient mutations along with the ethylene-insensitivity of the *ers1-1* mutant. ABA-deficient seeds are able to germinate in the presence of high levels of exogenous sugars that are inhibitory to wild-type germination (Figure 2) (Laby *et al.*, 2000). We chose to screen these seeds/seedlings directly by selecting double mutants on the basis of germination in the presence of high glucose (Glc) and insensitivity to ACC.

Figure 2: Graphical comparison of the germination characteristic for various ethylene-insensitive and ABA-deficient non-stratified seeds on M&S + 0.3M glucose + Agar media.

Non-stratified wild-type (Col), *etr1-1*, *ers1-1*, *aba1-6*, *aba2-3* and *aba3-1* mature seeds were plated onto M&S +Agar +0.3M glucose and were left to germinate at room temperature in the dark for four days. All seed types demonstrate a reduced ability to germinate in the presence of high levels of sugar. The ABA-deficient alleles are able to germinate on such media. Therefore to screen for double mutants carrying the ethylene insensitive and ABA-deficient mutations, we sought to screen the potential double mutant seeds on media containing high levels of sugar (for the ABA-deficient mutation) and screen for the ethylene insensitive mutation by selecting mutants exhibiting a lack of the triple response when germinated in the presence of ACC.

Dormancy pattern of ABA-deficient mutant developing seeds

Siliques and seeds were staged as previously reported (table 1, Thurston *et al.*, Chapter 1). Briefly, siliques corresponding to the appropriate stage were dissected and seeds corresponding to the appropriate stage were transferred to germination media consisting of water and agar (0.8% w/v) media pH 5.7-5.8. Six siliques per stage were used and eight seeds from each silique were plated onto replicate plates (48 seeds/stage). Plates were wrapped in parafilm and one replicate plate was used for analysis of germination after stratifying conditions (7 days incubation at 4°C in the dark) the other was used for analysis of germination under non-stratifying conditions. Germination was scored by radicle protrusion after 14d incubation at 22°C under constant light. As expected, all ABA-deficient single mutants exhibited a non-dormant phenotype throughout seed development (Figure 3).

Anticipated Results

The *etr1-1* mutant is hypersensitive to ABA (Beaudoin *et al.*, 2000). Accordingly this mutant has comparable ABA levels in developing siliques and seeds compared to wildtype, however these developing mutant seeds exhibit an increased dormancy (Thurston *et al.*, Chapter 1). Therefore, a double mutant including *etr1-1* with any of the ABA-deficient mutants should have a less dormant phenotype than *etr1-1* due to the predicted decrease in endogenous ABA biosynthesis. It is probable that the *etr1-1; aba1-6* and *etr1-1; aba2-3* double mutants will show the strongest double mutant phenotype, while the

Figure 3: Graphical representation of developing seed germination patterns for ABA-deficient mutants. Staged seeds were harvested from their corresponding siliques and plated on germination media. Germination plates were either stratified or not stratified and grown under constant light conditions for 14 days. Seed germination counts were scored on the fourteenth day. Germination was scored by radicle protrusion. Black bars represent non-stratified seeds, grey bars represent stratified seeds. A. *aba1-6*, B. *aba2-3* and C. *aba3-1*. Notice that all seed stages germinate. The four phases of seed germination of wildtype Col are not observed in these mutants.

A. **B.** **C.**

etr1-1; *aba3-1* double mutant will probably show an *etr1-1*-like phenotype due to the structural similarities to ABA and ABA aldehyde, as previously seen with the non-stratified single mutant germination patterns (Figure 1). The germination pattern of these double mutant seeds will be valuable in determining the contribution of endogenous ABA and ETR1 throughout the later stages of seed development

The *ers1-1* mutant has not been described as being hypersensitive to ABA. Our previous hormone profiles showed this mutant accumulating higher than wildtype ABA and ABA metabolite levels during our first two stages of siliques and seed development (Thurston *et al.*, Chapter 1). Interestingly, these seeds were able to germinate throughout the six stages of seed development and did not enter into dormancy. Therefore it will be interesting to determine the germination profiles of these double mutant seeds and it is likely that we will see germination for every seed stage in any *ers1-1*; ABA-deficient double mutant.

Appendix II: Gene expression profiling during seed development in wildtype and two ethylene-insensitive mutants

Introduction

Previously, we have generated hormone profiles during seed and silique development in wildtype and two ethylene receptor mutants, *etr1-1* and *ers1-1*. The hormone profiles revealed that the *ers1-1* mutant has altered ABA and cytokinin levels while the *etr1-1* mutant has altered auxin and gibberellin levels. These two mutants exhibit opposite phenotypes when developing seeds are assayed on their ability to germinate. Briefly, developing *ers1-1* seeds appear to by-pass the dormant state seen in wild-type seeds while the *etr1-1* developing seeds appear to have an increased dormancy. We analysed the global gene expression patterns in two key stages of seed and silique development, namely the stage prior to the entrance into seed dormancy (Stage 2) and the stage after the exit of seed dormancy (Stage 5). These expression profiles will ultimately corroborate the hormone profiles, but will also be used to for hypothesis generation. This data will also be useful for the investigation of other non-hormone related gene expression changes with quantitative polymerase chain reaction.

Materials and Methods

Plant Material

All plants used in these experiments were of the Columbia (Col-0) ecotype. All seeds were obtained from the Arabidopsis Biological Resource Center (Columbus, OH).

Plant growth conditions

Plant growth conditions were followed as previously described (Thurston *et al.*, Chapter 1).

Silique and Seed Staging

Silique and seed staging was followed as previously described (table 1, Thurston *et al.*, Chapter 1). Samples were collected for only one replicate of wildtype, *etr1-1* and *ers1-1* siliques and seeds at stage 2 and stage 5. Siliques were dissected to verify proper seed stage, then frozen in liquid nitrogen and stored at -80°C until use.

RNA preparation

Total RNA from wildtype, *etr1-1* and *ers1-1* stage 2 and stage 5 siliques and seeds was isolated according to the protocol published by Suzuki and colleagues with some modifications (Suzuki *et al.*, 2004). Samples were ground to a fine powder with a mortar and pestle under liquid nitrogen for ten minutes. At least twenty volumes of extraction buffer (100mM Tris-HCl (pH 9.5), 10mM EDTA (pH 8.0), 2% (w/v) lithium dodecyl sulphate, 0.6M sodium chloride, 0.4M trisodium citrate, 5% (v/v) 2-mercaptoethanol) was added to ground samples and mixed immediately. Samples were incubated at room temperature (25°C) for

forty-five minutes to one hour. Samples were spun at 12000 x gravity for five minutes at room temperature. One volume of chloroform:isoamyl alcohol (CIA, 24:1) was added to the supernatant and mixed by inversion. Samples were then centrifuged at 12000 x gravity for ten minutes at 4°C. One volume of TRIzol reagent (protocol suggests phenol mixture: water saturated phenol containing 35%(w/v) of guanidinium thiocyanate and 0.1 volume of 2M sodium acetate (pH 4.0) – which is the similar to TRIzol) was added to the supernatant, mixed vigorously by hand and left to incubate at room temperature for forty-five minutes to one hour. CIA was added to samples at one half volume of the TRIzol reagent. Samples were mixed vigorously by hand and left to incubate at room temperature for thirty minutes. Samples were centrifuged at 12000 x gravity for ten minutes at 4°C. 0.3 volume of isopropanol and 0.3 volume of a salt solution (0.8M sodium citrate, 1.2M sodium chloride) was added to the upper aqueous layer, mixed by hand and incubated at room temperature for forty minutes. Samples were centrifuged at 12000 x gravity for five minutes at 4°C. The pellets were washed with an aliquot of 75% (v/v) ethanol, vortexed briefly and centrifuged at 7500 x gravity for five minutes at 4°C. Pellets were air-dried for five to ten minutes at 55°C. Pellets were dissolved in fifty microlitres water and 0.25 volume of 8M lithium chloride was added to the samples, mixed by hand and left to precipitate overnight at -80°C. Samples were melted at room temperature and centrifuged at 12000 x gravity for thirty minutes at 4°C. The pellets were dissolved in fifty microlitres of water and 0.1 volume of 3M sodium acetate (pH 5.2) and 1 volume of isopropanol were added, mixed and incubated

at room temperature for ten minutes. Samples were centrifuged at 12000 x gravity for fifteen minutes at 4°C. The pellets were washed with an aliquot of 75% (v/v) ethanol, vortexed briefly and centrifuged at 7500 x gravity for five minutes at 4°C. Pellets were air-dried briefly for five to ten minutes at 55°C. Pellets were dissolved in ten to twenty microlitres water. One microlitre of each sample was used to estimate RNA quantity and quality on a 1% (w/v) agarose gel. Quality was checked using a spectrophotometer and optical density (OD) was determined using wavelengths of 280 and 260 nanometers. OD_{260/280} for WT stage 2 = 2.0, stage 5 = 2.3, *ers1-1* stage 2 = 1.9, stage 5 = 2.2, *etr1-1* stage 2 = 2.0, stage 5 = 2.0 .

Affymetrix Gene Chip processing

Sample labelling, hybridization and reading was performed by the Gene Chip facility hosted by the University of Toronto, Dr. Nicholas Provart.

Thesis Conclusion

Previously, the five ethylene receptors in Arabidopsis were proposed to function redundantly to signal ethylene. Based on our numerous distinct observations of ethylene receptor mutants across plant development (such as leaf shape and size, flowering time, trichome formation to name a few), as well as a detailed analysis of the expression of each receptor across development, we have concluded that the receptors perform non-redundant roles throughout development. In this thesis, distinct roles for the ETR1 and ERS1 receptors during seed development were investigated using a multi-disciplinary approach involving genomics, bioinformatics, metabolomics, classical genetics and developmental physiology. When wild-type seeds are assayed on their ability to germinate throughout six stages of seed development, four distinct phases are observed. Immature stage 1 seeds do not germinate; stage 2 seeds germinate precociously but have not developed cold tolerance as stratified stage 2 seeds have a marked reduction in germination; stage 3 and stage 4 seeds are dormant and have an overall reduced germination; stage 5 and stage 6 seeds are mature seeds and are germination ready as stratified seeds display close to 100% germination. We have determined the germination characteristics of developing ethylene receptor mutant seeds and these data reveal unique roles for the ETR1 and ERS1 receptors in regulating seed dormancy. Ethylene has been implicated in having a positive effect on seed germination, as seeds treated with ethylene gas display increased germination levels. The ethylene insensitive GOF *etr1-1*

mutant has been reported as having an increased dormant state in mature seeds. However, to our knowledge, this is the first study that has identified components of the signal transduction pathway (receptors) as having required roles in regulating dormancy throughout seed development. The germination pattern of developing GOF *etr1* and GOF *ers1* mutant seeds indicated that the ETR1 receptor is required for exiting seed dormancy/initiating germination while the ERS1 receptor is required for the entrance into primary seed dormancy. To confirm the role of the ETR1 and ERS1 receptors in regulating the release and entrance into seed dormancy we investigated the ability of LOF *etr1* or LOF *ers1* mutant seeds to germinate across seed development. The LOF *etr1* mutants seeds germinate throughout almost every stage of seed development. Therefore it is very likely that the ETR1 receptor is required for the release from seed dormancy. Unfortunately, a suitable LOF *ers1* allele does not currently exist and further analysis with a suitable LOF *ers1* allele will be required to determine the role ERS1 fulfills during seed dormancy. The LOF *ers1* mutant used in this study was in a different ecotype from the GOF *ers1* mutant and as such, many questions remain as to the role of ERS1 during seed dormancy. The germination data for the GOF *ers1-1* and GOF *etr1-1* mutants was also very interesting from the perspective of the early signalling events in ethylene signal transduction pathway. As all receptors were shown to signal through CTR1, at least with respect to the triple response assay, it was perplexing that the two GOF receptor mutants could result in opposite germination patterns. The germination pattern of developing LOF *ctr1-1* mutant seeds was used to investigate the possibility

that both receptors use CTR1 as a common signalling intermediate for the regulation of seed dormancy. The LOF *ctr1-1* mutant seeds exhibited a reduced state of dormancy throughout seed development and supported the notion that ETR1 signals through CTR1 to activate seed germination (release from dormancy). Since the LOF *ctr1-1* data did not support the notion that ERS1 signals through CTR1, it is possible that ERS1 may signal through a CTR1-independent mechanism. Further characterization of proteins associated with these receptors at the ER may reveal the presence of other CTR1-like proteins or perhaps other uncharacterized protein involved as signalling intermediates. Assuming that a CTR1-like protein is involved in transmitting the signal from ERS1, then a LOF CTR1-like protein would be expected to display a dormant phenotype with respect to seed germination. Such mutants could be isolated from mutagenized populations of seeds by screening for non-germinating seeds and subsequently transferring those seeds onto media supplemented with GA. Those seed may possibly manifest differences in the triple response and other specific ethylene related responses.

The two hormones, abscisic acid and gibberellic acid, have been implicated in the regulation of seed dormancy versus germination, respectively. Since the developing *etr1-1* seeds were more dormant than wild-type seeds, and since the *ers1-1* developing seeds were less dormant than wild type seeds, we might have expected that the *etr1-1* mutant would have an increase in ABA accumulation while the *ers1-1* mutant would have an increase in GA accumulation. We took a metabolomic approach to investigate whether the

presence of the GOF mutation altered the relative accumulation of ABA, GA, auxins or cytokinins. Surprisingly, the *etr1-1* mutation lead to an increase in GA and a reduction in auxin accumulation while the *ers1-1* mutation lead to an increase in ABA and cytokinin accumulation. As the GA and ABA content of the mutant seeds were opposite to those expected, it is possible that the signal from each receptor may be affecting the sensitivity of the seed to the ABA/GA balance. To investigate this possibility, we crossed the ABA-deficient mutants, *aba1-6*, *aba2-3* and *aba3-1* into the *etr1-1* and *ers1-1* genetic backgrounds. By reducing the seeds endogenous ABA content, we should be able to determine if the *etr1-1* or *ers1-1* mutations are affecting the seeds sensitivity to the ABA/GA balance, based on how the developing double mutant seed germinate throughout seed development. Future studies should include the generation of double mutants including the GA insensitive or GA synthesis mutants to support the presumption that the signal from the ETR1 or ERS1 receptors alters the seeds ability to perceive the ABA versus GA balance in regulating dormancy versus germination. We investigated the downstream genes that are modified at two key stages of seed development as a result of the GOF *etr1-1* or GOF *ers1-1* mutations using a microarray approach for global gene expression analysis. Stage 2 and stage 5, representing the stage just prior to the entrance into seed dormancy and the stage just after seed dormancy in wild-type seeds should manifest the greatest difference in genes affected by these mutations as the *ers1-1* mutant does not enter seed dormancy and the *etr1-1* mutant exhibits an increased dormancy. Since many changes in hormone metabolism were

observed in the mutant developing seeds and siliques compared to wild-type developing seeds and siliques, it is expected that some of the gene involved in these metabolic pathways will be differentially regulated in the GOF mutants. Genes of interest may include gene products known to be involved in DNA binding or transcriptional activation as well as previously uncharacterized genes. These changes in candidate gene expression should be verified using some method of following gene activity such as Northern analysis or quantitative PCR. Mutants of these candidate genes should be tested on the ability of their developing seeds to germinate across seed development in order to clarify the genes regulated downstream from the ETR1 and ERS1 proteins with respect to seed dormancy.

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