THE ETHYLENE RECEPTORS IN *ARABIDOPSIS*
SHOW NON-REDUNDANT ROLES
IN DIFFERENTIALLY REGULATING SEED DORMANCY

A Thesis

presented to

The Faculty of Graduate Studies

of

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by

Donna Yee

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Abstract

The involvement of the plant hormone ethylene in controlling seed dormancy in *Arabidopsis thaliana* has been analyzed as part of a larger objective to understand the functional significance of hormone cross-talk in plants. For the first time, ethylene has been shown to have a definitive role in dormancy. Furthermore, distinctive non-redundant roles for two ethylene receptors have been established for seed development. Specifically, the ethylene receptors ERS1 and ETR1 were found to control the entrance and exit into seed dormancy, respectively. Recent advances in small molecule profiling have allowed for the unprecedented ability to simultaneously monitor the levels of abscisic acids, auxins, cytokinins, gibberellins, and several metabolites of these hormones. Applying a recently developed liquid chromatography-electrospray ionization tandem mass spectrometry method analysis of hormones and metabolites to seed development has allowed the visualization of a more complex network of hormone cross-talk that likely occurs during dormancy.

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"...seeds are invisible. They sleep deep in the heart of the earth's darkness, until some one among them is seized with the desire to awaken. Then this little seed will stretch itself and begin - timidly at first - to push a charming little sprig inoffensively upward toward the sun."

(translated excerpt from Antoine de Saint-Exupery's 'Le petit prince (The Little Prince)')

Table of Contents

Abstract	iii
Acknowledgements	iv
List of Tables	xi
List of Figures.....	xii
List of Figures.....	xii
Abbreviations.....	xiv
Abbreviations.....	xiv
1 INTRODUCTION	1
1.1 What are Seeds?	1
1.2 Seed Development	2
1.3 The Purpose of Seeds	3
1.4 Seed Dormancy	4
1.5 The Importance of Dormancy – Purpose and Advantages	5
1.6 Correlating Dormancy with Seasonal Variation.....	6
1.7 Breaking Dormancy	7
1.8 Germination – the End of Dormancy.....	8
1.9 <i>Arabidopsis</i> as a Model Plant.....	8
1.10 Five Groups of Plant Hormones.....	9
1.11 Processes Influenced by Ethylene	10
1.12 Interplay of Hormonal Cross-talk.....	11
1.13 The ABA-GA Balance in Seed Dormancy & Germination – An Initial Model of Simple Hormone Interactions	12
1.14 Beyond the Linearity of ABA-GA Balance in Seed Dormancy & Germination – Developing a More Complex Web of Hormonal Interactions	13
1.15 Ethylene Triple Response	14

1.16	Ethylene Receptors – Signal Perception.....	14
1.17	CTR1 – Signal Response Regulator.....	16
1.18	Two Types of Mutations Can Disrupt Normal Ethylene Signalling	19
1.19	Model of Ethylene Signalling – from Perception to Response Activation	22
1.20	How Dominant Gain-of-Function Mutants Signal to Confer Ethylene-Insensitivity	25
1.21	Assessing the Function of Individual Ethylene Receptors: Redundancy or Not?	28
1.22	The Potential Role of Ethylene in Cross-talk During Seed Development.....	29
1.23	Simultaneous Analysis of Plant Hormone Classes – the Advantages of HPLC/ESI-MS/MS.....	30
2	MATERIALS AND METHODS.....	33
2.1	Plant Strains.....	33
2.2	Initial Seed Preparation and Plant Growth Conditions	33
2.3	Ethylene Sensitivity.....	35
2.4	Plant Material and Staging.....	35
2.5	Days After Pollination Determination	37
2.6	Germination Potential Assay.....	37
2.7	Metabolite Analysis	38
2.7.1	Sample Collection	38
2.7.2	Metabolite Extraction.....	39
2.7.3	Extract Purification	39
2.7.4	Analysis Preparation	40
2.7.5	HPLC/ESI-MS/MS Analysis.....	40
3	RESULTS	42
3.1	Phenotypic Differences between Ethylene-Insensitive Mutants	42
3.2	Silique and Seed Development to Maturity	46
3.3	Days After Pollination Determination	49
3.4	Germination Potential	52

3.5	Plant Hormone and Metabolite Analysis	69
4	DISCUSSION	85
4.1	Phenotypic Differences Supporting Non-Redundancy in Ethylene- Insensitive Mutants	85
4.1.1	Various phenotypic traits are different between ethylene- insensitive mutants.....	85
4.1.2	Phenotypic traits can be associated to the role of ethylene	86
4.1.3	There are phenotypic differences during silique and seed development.....	87
4.1.4	Previously performed protein sequence alignment analysis of ethylene receptors supports non-redundancy	88
4.2	Days After Pollination Illustrating Photoperiod Differences	89
4.2.1	The influence of maternal plants on progeny development	89
4.2.2	The typical annual life cycle of Arabidopsis.....	90
4.2.3	The interrelation of photoperiod and season	91
4.2.4	The relationship between stratification and cold acclimation..	92
4.2.5	The possibility of a semi-annual cycles in Arabidopsis.....	93
4.3	Germination Potential and its Demonstration of Seed Dormancy	100
4.3.1	Wild-type germinability can be used to demonstrate dormancy in seeds	100
4.3.2	Pre-viable seed phase.....	103
4.3.3	Precocious germination phase	103
4.3.4	Dormancy phase	104
4.3.5	Seeds in dormancy-breakable phase are ready for germination	108
4.3.6	ers1 and etr1-1 show contrasting dormancy phenotypes .	110
4.3.7	ers1 and etr1-1 can be used to model dormancy control by ethylene.....	111
4.3.8	ctr1-1 mutant supports ethylene signalling in the dormancy pathway.....	114
4.4	Hormone and Metabolite Profiling and its Demonstration of Cross- talk	115

4.4.1	Interesting trends extracted from the hormone and metabolite profiles of wild-type and ethylene insensitive plants.....	117
4.4.2	Considerations during the interpretation of hormone and metabolite data.....	123
4.4.3	The arising complexity behind understanding hormonal cross-talk.....	124
5	REFERENCES	126

List of Tables

Table 1: The age of wild-type and ethylene-insensitive mutant <i>Arabidopsis</i> grown under long-day and short-day conditions when the transition from vegetative to reproductive growth occurred.	45
Table 2: The six stages of silique development related to days after pollination (DAP) of wild-type <i>Arabidopsis</i> grown under long-day and short-day conditions.	52

List of Figures

Figure 1: Schematic of the functional domains of the <i>Arabidopsis</i> ethylene receptor gene family showing variations in the architecture of their structural protein.	17
Figure 2: Model of ethylene signalling in <i>Arabidopsis</i>	23
Figure 3: Model of ethylene signalling in gain-of-function mutants of <i>Arabidopsis</i>	26
Figure 4: Sensitivity of <i>Arabidopsis</i> ethylene-insensitive mutant seedlings to continual ethylene exposure during etiolated germination.....	43
Figure 5: Colouration schematic of the pericarp and testa appearance of <i>Arabidopsis</i> siliques during the maturation period of seed development.	47
Figure 6: Days after pollination (DAP) timeline of the silique development through the six seed maturation stages in wild-type <i>Arabidopsis</i>	50
Figure 7: Germination potentials of wild-type <i>Arabidopsis</i> seeds developing through the six stages of seed maturation.....	54
Figure 8: Germination potentials of <i>ers1</i> mutant <i>Arabidopsis</i> seeds developing through the six stages of seed maturation.....	57
Figure 9: Germination potentials of <i>etr1-1</i> mutant <i>Arabidopsis</i> seeds developing through the six stages of seed maturation.....	60
Figure 10: Germination potentials of <i>ers2-1</i> mutant <i>Arabidopsis</i> seeds developing through the six stages of seed maturation.....	63
Figure 11: Germination potentials of <i>etr2-1</i> mutant <i>Arabidopsis</i> seeds developing through the six stages of seed maturation.....	65
Figure 12: Germination potentials of <i>ein4-1</i> mutant <i>Arabidopsis</i> seeds developing through the six stages of seed maturation.....	67
Figure 13: Germination potentials of <i>ctr1-1</i> mutant <i>Arabidopsis</i> seeds developing through the six stages of seed maturation.....	70
Figure 14: Hormone and metabolite profiles during the six seed development stages of wild-type <i>Arabidopsis</i> grown under long-day conditions.	73
Figure 15: Hormone and metabolite profiles during the six seed development stages of <i>ers1</i> mutant grown under long-day conditions.	75

Figure 16: Hormone and metabolite profiles during the six seed development stages of <i>etr1-1</i> mutant grown under long-day conditions.....	77
Figure 17: Hormone and metabolite profiles during the six seed development stages of wild-type <i>Arabidopsis</i> grown under short-day conditions.	80
Figure 18: Hormone and metabolite profiles during the six seed development stages of <i>ers1</i> mutant grown under short-day conditions.	82
Figure 19: A semi-annual life cycle of <i>Arabidopsis</i> with seeds over-wintering in a dormant state.	95
Figure 20: A semi-annual life cycle of <i>Arabidopsis</i> with plants over-wintering in a vegetative state.	98
Figure 21: A linear representation of the germinability patterns of short-day grown wild-type plants as seen in Figure 7-B with seed development phases.....	101
Figure 22: Conceptual representation of seed development.	106
Figure 23: Schematic representation illustrating the possible role of ethylene in entering and exiting seed dormancy.....	112
Figure 24: Summary of selected hormone and metabolite profiles during the six seed development stages of wild-type and mutant <i>Arabidopsis</i> grown under long-day conditions.	119

Abbreviations

2iP	isopentenyladenine
7'OH-ABA	7'-hydroxy-ABA
ABA	abscisic acid
ABA-GE	ABA glucose ester
ACC	1-aminocyclopropane-1-carboxylic acid
CK	cytokinin
CTR (or ctr)	constitutive triple response
DAP	days after pollination
DPA	dihydrophaseic acid
DW	dry weight
EIN (or ein)	ethylene insensitive
ERS (or ers)	ethylene response sensor
ESI	electrospray ionization
ETR (or etr)	ethylene response
FW	fresh weight
GA	gibberellic acid / gibberellin A
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
IAAsp	indole-3-acetyl-aspartate
IPA	isopentenyladenosine
IS	internal standard(s)
LD	long-day
M&S	Murashige & Skoog
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NS	non-stratification
PA	phaseic acid
RT	room temperature
S	stratification
SD	short-day
WT	wild-type
Z	zeatin
ZR	zeatin riboside

1 INTRODUCTION

1.1 What are Seeds?

A seed is a mature fertilized plant ovule consisting of a fully formed embryo and extra-embryonic tissues as possible food sources, bounded by a maternally derived protective coat or testa. The storage of nutrients for embryonic development and/or post-embryonic seedlings is derived from a combination of extra-embryonic tissues (perisperm, endosperm) and/or cotyledons and is species dependent. Often the seed(s) are contained in a maternal-derived fruit structure during maturation and are dispersed still contained in the fruit (indehiscence) or released spontaneously from the fruit at maturity (dehiscence).

The formation of seeds begins at the flower with pollination – a sequence of events starting with pollen contact, adhesion, and attachment of compatible pollen on the stigma; followed by pollen hydration, germination, and penetration; followed by the fertilization of the egg cell within ovules after pollen tube growth through the stigma, style, and ovary. The fertilized egg cell develops into the embryo consisting of an axis (the future body of the seedling/plant) and the cotyledon(s). The ovule integuments (the maternal tissues surrounding the ovule during seed development) differentiate into the testa (seed coat). Fertilized ovules undergo development to become mature seeds. The ovary wall (to which the ovule(s) are attached via a funiculus) develops to become the fruit. In all

cases, the end result is the same: seeds are dispersed with all the elements required to survive.

1.2 Seed Development

The development of seeds to maturity is much more complex internally than deduced from the external structures. What is recognized of seed development is the concept of two distinct periods, the first where morphogenesis occurs and the second where the maturation processes occur. In the former, the embryo initially develops through active cell division, forms embryonic organs and tissues, and establishes the plan of the embryo body. This includes the (pre-globular, globular, heart, torpedo, walking stick, upturned-U, fully-developed) stages of embryo development. In the latter, the maturing embryo accumulates nutrient reserves, and acquires various survival traits (ex. desiccation tolerance, cold tolerance), before the mature embryo continues through to desiccation and dispersal. All these processes occur in either a sequential, independent or inter-related manner. Koornneef and Karssen (1994) summarized the macromolecular content changes in carbohydrates, proteins (storage and others), lipids and hormones associated with reserve accumulation. Stored reserves are critical as nutritional sources until seedlings have established autotrophic growth. Again, the end result of undergoing seed development is the generation of mature seed with sustainable viability prior to dispersal.

1.3 The Purpose of Seeds

Even with the wide variety of seed and fruit types seen across different plant species, they all represent an elegant design to store and disperse plant progeny. Thus, the purpose of plants producing seeds is plant propagation. The ability to make seed (seed habit) is not a universal characteristic of all plants but is a defining characteristic of higher plants, and there are several advantages to being a seed plant. The objective of plant reproduction is to ensure the strong survival of the species and this can be readily accomplished by generating high quality progeny through viable seeds. Seeds are essentially 'packaged progeny' easily dispersible, with evolved traits (often acquired during seed development) to survive the elements after separation from the maternal plant. The seeds of some leguminous species that can survive acidic conditions during passage through the gut of an animal, as well as the seeds of most pine species that can survive the high temperatures of fire are some examples of how seeds have adapted to extreme environments.

Seed adaptation is closely related with proper germination timing, where the timing of seed germination is an important element for successful plant propagation. The survival of the species is dependent on correctly controlling seed germination. This assures that plants do not grow when conditions are unfavourable and, thus, do not ineffectively waste the energy and resources spent in producing seed. Plants accomplish this survival tactic through seed dormancy. Correspondingly, the plant must have a very tight control on seed dormancy in order to ensure the success of the progeny, and hence the species.

1.4 Seed Dormancy

Following the fertilization of the egg cell and the subsequent formation of the embryo, the development of the seed (and its ability to germinate) can be arrested for a number of reasons. In the most (over-) simplified terms, dormancy is where growth of an organ (like the embryo) is temporarily arrested. In the most (over-) complicated terms, dormancy has been classified in comprehensive systems (e.g. primary innate non-deep physiological dormancy) as those utilized by Baskin and Baskin (2001). Under favourable environmental conditions, the dispersed seed can have a natural developmental arrest known as primary seed dormancy. This type of dormancy can be imposed by the seed coat (a physical barrier to seed germination) or by the embryo (in which internal factors inhibit the seeds ability to germinate). In unfavourable environmental conditions (light, temperature, oxygen, and/or moisture), the dispersed seed can go through a period of quiescence similar in appearance to that of dormancy.

Because of the subtle difference between dormancy and quiescence, it is common for these terms to be misused in the literature. The difference can be distinguished when environmental conditions become favourable again: quiescent seeds germinate when growth-restrictive conditions are lifted, while dormant seeds do not. Often, quiescent seeds exposed to prolonged conditions that are unfavourable for growth are re-induced into dormancy. This re-induction into dormancy is called secondary dormancy. In this thesis, I will exclusively

discuss primary seed dormancy and will not consider the factors that affect the seed in unfavourable environmental conditions.

1.5 The Importance of Dormancy – Purpose and Advantages

Proper germination timing is closely related with seed dormancy.

Generally, imposing dormancy is a means to ensure the survival of the mature embryo (in the mature seed) in the time spent between dispersal from the maternal plant to new seedling establishment. Dormancy is a well-adapted mechanism to ensure seedlings do not develop until conditions are favourable for growth of the new plant. The result of properly timing germination is the successful completion of the life cycle, producing viable seed that will develop into the next plant generation and thus again ensuring the survival of the species. There are several advantages of dormancy: 1) it plays a role in the timing of germination so that fitness (seed production) of the resulting plant is maximized; 2) it is an inherited life cycle trait that maximizes species fitness in a particular habitat; 3) it prevents seedlings from competing with the mother plant and/or siblings, thus increasing the maternal reproductive success since sibling competition is prevented; 4) it ensures species persistence in a risky environment through long-term soil seed banks (seed buried in the soil that allow annual species to persist in randomly unpredictable environments that may prevent seed set); and 5) it can be an adaptation for survival during a season when environmental conditions are unfavourable for seedling establishment (Beardmore, personal communication).

1.6 Correlating Dormancy with Seasonal Variation

The use of dormancy to adapt survival to seasons is strategic since seed fitness may not be maximized in all environments at the same time. During the cycling of seasons, environmental conditions represented by light levels, temperatures, and moisture levels are also constantly changing. Seasonal conditions can range from the cold, wet, and short light-length days of winter to the hot, dry, and long light-length days of summer. Dormancy/quiescence based on seasons could reasonably be imposed to delay germination until conditions appropriate to maximize fitness are present.

Annual plants are typically categorized as winter annuals or summer annuals. Winter annuals are characterized by seed germination occurring in autumn, while summer annuals are characterized by seed germination occurring in spring. The seasons occurring prior to the two categories of germination favoured in annuals are noticeably the seasons where conditions could be deemed unfavourable for plant growth. A strategic advantage would be for seed to adapt to avoid germination in these growth-limiting seasons. The imposition of dormancy/quiescence could accomplish this. Correspondingly, the requirement of seeds to actually undergo an interval under such unfavourable conditions to break dormancy/quiescence would ensure germination occurred at the right moment (after removal of limiting environmental factors). In other words, summer annuals germinate in spring because the seasonal dormancy/quiescence imposed on seeds prior to dispersal needs conditions

typically found in winter (wet and cold) to break dormancy/quiescence.

Likewise, winter annuals germinate in autumn because the seasonal quiescence/dormancy imposed on seeds prior to dispersal need conditions typically found in summer (dry and warm) to break quiescence/dormancy.

1.7 Breaking Dormancy

In many plants, seed dormancy requires specific environmental signals to break dormancy and allow the seed to germinate. For those plants with embryo-induced and/or seed-coat-induced dormancy, dormancy can be broken through stratification. Also called chilling or moist chilling, stratification involves the incubation of the seed in cool wet conditions, which mimics late winter/early spring conditions. In these plants, stratification causes swelling and cracking of the seed coat (thereby breaking seed-coat-imposed dormancy) and activates specific processes within the seed (breaking embryo-imposed dormancy). In *Arabidopsis*, stratification requires a 4-7 day dark storage on a moist substrate at 2-4°C. In plants that have strong seed-coat-imposed dormancy, the seed needs to go through scarification where extreme environmental conditions are needed to mimic such processes as passage through the gut of an animal, soil abrasion, forest fires, microbial action or winter freezing. There is also a natural process within some seeds that can break seed dormancy, and this is called afterripening. While afterripening is a term that encompasses a wide range of processes that can occur in the seed after development, in *Arabidopsis* it appears that afterripening causes the seeds to lose their dormancy so that they

can germinate without the need of stratification. The transition of a seed from a dormant state to a more germinable state is gradual in afterripening. In *Arabidopsis*, afterripening occurs after the seeds are dispersed and when they are stored in warm dry conditions after harvest.

1.8 Germination – the End of Dormancy

During germination, water enters the dry mature seed (imbibition) and the arrested embryo resumes its growth. As a result, the embryonic axis elongates, overcoming testa-imposed mechanical restraint, and the radicle of the embryo protrudes from the seed coat. At this point, the seed is considered germinated. The beginning of germination therefore signals the end of seed dormancy. Because there is no direct and non-invasive way to measure the degree of dormancy and because dormancy and germination are so closely correlated, the quantification of germination can alternatively be used to study dormancy. The proportion of seeds that germinate under given sets of conditions quantifies seed germination. The term germinability is used to denote the capacity of a seed (embryo) to germinate when prevailing conditions are germination-favourable and takes into account dormancy as it relates to germination.

1.9 *Arabidopsis* as a Model Plant

Arabidopsis is well suited to be the model for studying the physiological mechanisms of dormancy because of the similarity of the dormancy control and germination responses of this plant to many other plants with commercially important seeds. *Arabidopsis thaliana*, a member of the large Brassicaceae

family, is a small herbaceous plant whose use as a model for plant research has been exponentially growing in the last two decades, courtesy of its amenity to genetic experimentation. *Arabidopsis* functions well as a plant model system due to several characteristic features (small size, ease of growth, short generation time, and extremely large seed production), its small genome size, and amenability to various molecular techniques (ex. mutagenesis). The entire *Arabidopsis* genome has been sequenced (The Arabidopsis Genome Initiative, 2000) and there are thousand of mutants available for experimentation. There are also numerous other resources and tools readily accessible for genetic and molecular analyses and experimentation. Coupling these advantages with a high throughput of compelling information consistently being generated, the use of *Arabidopsis* to study fundamental plant biology is justifiable.

Although much is known about the morphological events governing dormancy, germination, and dormancy-breaking methods such as stratification and afterripening, little is known about the underlying physiological mechanisms controlling dormancy in plants. What is justifiably presumed is that seed development through dormancy to germination is likely controlled by phytohormones (plant hormones) in a similar manner to how physiological processes in animals are influenced by hormones.

1.10 Five Groups of Plant Hormones

Unlike animals, which have numerous different hormones regulating development, plants have only a relatively small number of plant hormones

(phytohormones). The five major types of plant hormones are abscisic acids (ABA), auxins, cytokinins (CK), gibberellins (GA), and ethylene. Unlike animals, which have hormones with very specific function, the physiological functions of plant hormones often overlap and interact. Because each of the plant hormone groups have been shown to influence a wide variety of events in plant developmental life, categorizing each group with a specific functional purpose is difficult. This categorization undermines the multiplicity of hormonal effects with the inaccurate perception that a particular plant process is solely influenced by one hormone. Our current understanding of the functional roles of plant hormones are limited to the characterization of specific processes, and thus are generalizations that do not necessarily exclude the possible influence of the hormone group on other plant developmental processes. For example, the plant hormone ethylene is generally considered to be involved in stress responses in plants but it is also important for many other plant processes.

1.11 Processes Influenced by Ethylene

The phytohormone ethylene is a small gaseous (and thus readily diffusible) molecule produced endogenously within the plant by a well-characterized biosynthetic pathway (reviewed by Kende, 1993). Briefly, there are two committed steps that lead to the production of ethylene from S-adenosyl-L-methionine (SAM). ACC synthase converts SAM to 1-aminocyclopropane-1-carboxylic acid (ACC), and ACC oxidase, also known as the ethylene-forming enzyme, is responsible for the oxidation of ACC to ethylene. Agriculturally,

ethylene is notable for its fruit ripening effects, where growers have used the gas to ripen harvested edible fruits. Commonly, ethylene is noted for its decay-inducing effects of susceptible leaf vegetables like lettuce, spinach, radicchio, and cabbage, when stored with climacteric (ethylene-producing with ripening) fruits like bananas, apples, and tomatoes.

To illustrate the multiplicity of phytohormone effects, ethylene has been shown to be involved in a wide range of cellular processes including inhibition or promotion of cell elongation and cell division; developmental and physiological processes influencing flowering, fruit ripening, seed dormancy and germination, root epidermis cell-fate determination (such as root hair, root nodule, and adventitious root formation); various morphogenetic effects (such as leaf epinasty (downward-curved morphology), apical hook formation, and gravity response); responses to abiotic (ex. wound) and biotic (ex. pathogen attack) stress conditions; induction of abscission and senescence of plant organs such as leaves and flowers (summarized in Abeles *et al.*, 1992). In many cases, ethylene interacts with other hormones to generate these events in the plant life cycle.

1.12 Interplay of Hormonal Cross-talk

Plant hormones have been studied for some time and it is the classical studies that have demonstrated that each plant hormone type affects a wide variety of developmental processes. But information behind the interactions occurring between plant hormones is still being uncovered, therein demonstrating multiplicity of phytohormone action, where developmental processes can be

influenced by more than one hormonal group. Termed hormone cross-talk, interaction, interplay, inter-connection or inter-signalling, they all essentially describe the same concept: the bioactivity of one hormone group is not entirely independent from the others. In a traditional sense, cross-talk describes the effect of one hormone on the synthesis of another, but a recurring description of cross-talk is in the context of shared signalling components or common downstream targets.

1.13 The ABA-GA Balance in Seed Dormancy & Germination – An Initial Model of Simple Hormone Interactions

In its most simple form, cross-talk could be represented by a linear pathway where, for example, one hormone regulates the initiation of one physiological event and another completes or terminates it. The latter example can be (initially) illustrated with dormancy. The two hormone groups strongly implicated in regulating seed dormancy and germination have been abscisic acids (ABAs) and gibberellins (GAs). To briefly generalize, ABAs establish dormancy in developing seeds during the maturation phase of embryos and GAs break ABA-induced dormancy. The antagonistic roles of ABA and GA on seed dormancy and germination may be well established (reviewed in Koornneef and Karssen, 1994) but contributions from other plant hormones are not as well understood. Although there is a comparative lack of information available on the involvement of the other three plant hormone groups in seed dormancy and germination, they should not be excluded from being considered as valuable components in overall seed development and merit further study.

1.14 Beyond the Linearity of ABA-GA Balance in Seed Dormancy & Germination – Developing a More Complex Web of Hormonal Interactions

Another hormone that may also be involved in seed dormancy and germination is ethylene. Exogenous ethylene has been shown to rescue the germination defects of GA-deficient mutants grown in light (Karssen *et al.*, 1989). Similarly, exogenous GA treatment of an ethylene-insensitive mutant stimulated germination (Bleecker *et al.*, 1988). Because ethylene and GAs can thus replace each other during germination (to a certain extent), and because GAs and ABAs have an opposing action during dormancy control, ethylene can also be antagonistic to abscisic acid's role in regulating seed dormancy. Correspondingly, it appears that the negative regulation of ABA signalling in seed germination occurs through the ethylene pathway (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000).

With emerging evidence supporting ethylene as another phytohormone involved in controlling seed dormancy and germination, the linear control pathway implied by ABA-GA balance in seed dormancy may be better illustrated as a network model, where hormone interactions involve more complexity. Through similar connections, auxins and cytokinins could also be involved in dormancy and germination processes, however, such a network in hormonal cross-talk has yet to be definitively established. The possibility of understanding how ethylene may interact with ABAs and GAs during seed development is enhanced by the extensive biochemical and genetic knowledge of the

components of the ethylene signal transduction pathway, one of the best-characterized hormone signalling transduction pathways in plants.

1.15 Ethylene Triple Response

One notable morphological response specifically attributed to ethylene is the 'triple response'. Grown in the continuous presence of ethylene, etiolated seedlings (seeds germinated in the dark) exhibit short and thick roots, short and thick hypocotyls, and an exaggerated curvature of the apical hook (all relative to etiolated seedlings grown without continuous ethylene exposure). These three features occur because of the effect of ethylene on inhibiting stem elongation (to produce short hypocotyls), inducing radial stem swelling (to produce thick hypocotyls), and removing normal geotropic response (to produce exaggerated apical hooks). All three of these features together form the 'triple response' effect. It was this triple response assay that allowed mutants in the ethylene signal transduction pathway to be identified in mutagenized seedling populations (Guzman and Ecker, 1990; Bleecker *et al.*, 1988) and provided a practical technique to study the *in vivo* processes on which ethylene has been attributed influential.

1.16 Ethylene Receptors – Signal Perception

From the identification of ethylene-insensitive mutants from chemically mutagenized *Arabidopsis* seed stocks (Guzman and Ecker, 1990; Bleecker *et al.*, 1988), the first ethylene receptor gene (*ETR1*, Chang *et al.*, 1993) was cloned, and the remaining 4 ethylene receptor genes were subsequently identified by

cross-hybridization (*ERS1*, Hua *et al.*, 1995; *ETR2*, Sakai *et al.*, 1998; *EIN4* and *ERS2*, Hua *et al.*, 1998). Thus, ethylene perception in *Arabidopsis* is controlled by a small multi-gene family, whose members are putative ethylene receptor genes: *ERS1*, *ERS2*, *EIN4*, *ETR1*, and *ETR2*. Based on *ETR1*, the structure of the ethylene receptors is homologous to a family of 'two-component' regulators (Chang *et al.*, 1993), which are sensors of environmental stimuli and transducers of signals responsible for adaptive responses in bacteria (Parkinson and Kofoed, 1992). Two-component regulators are typified by multiple functional domains, where (in the sensor component) a sensor domain receives the stimulus signal, a catalytic transmitter domain autophosphorylates an internal histidine residue and passes on the phosphate to (the response regulator component with) a receiver domain on an aspartate residue, and an output domain mediates response (Parkinson and Kofoed, 1992). Likewise, the general ethylene receptor structure can be divided into similar functional domains: A) the sensor domain is located at the amino-terminal hydrophobic transmembrane segment where ethylene (the signal) binds; B) followed by the GAF domain (gamma-activated factor domain) which has an unknown function; C) followed by the histidine kinase domain that is presumed to be a catalytic signal transmitter; and D) the carboxyl-terminal receiver domain that is presumed to affect the activity of a factor (output domain) located downstream (summarized by Hua *et al.*, 1998).

Although closely related, the five members of the ethylene receptor family exhibit interesting variations in the domain architecture of their structural proteins (Figure 1). ERS1 and ERS2 lack a receiver domain while ETR1, ETR2, and EIN4 have a receiver domain, thus following the previously described functional domain outline (Hua *et al.*, 1998). In addition, ETR1 and ERS1 proteins possess 3 hydrophobic subdomains at the amino-terminus, each predicted to span a membrane, and a conserved histidine kinase domain containing all the residues essential for catalytic histidine kinase functionality at the carboxyl-terminus (Hua *et al.*, 1998). Meanwhile ERS2, EIN4, and ETR2 have an additional amino-terminal hydrophobic sequence extension of unknown function, but possess a degenerate histidine kinase domain because of an apparent absence of conserved elements required for catalytic activity (Hua *et al.*, 1998).

1.17 CTR1 – Signal Response Regulator

In ethylene signalling, it appears that a CTR1 protein located downstream of the ethylene receptors is the output domain of the ethylene signal succession (Kieber *et al.*, 1993). The CTR1 downstream factor has been suggested to function as an ethylene-signal regulator. Although the CTR1 protein has been shown to be homologous to a family of Raf-like (Ras-activated factor-like) Serine/Threonine protein kinases (Kieber *et al.*, 1993), it has yet to be proven that there is a corresponding MAP kinase (mitogen-activated protein kinase) cascade to implicate the phosphorelay system in signalling from CTR1 to the

Figure 1: Schematic of the functional domains of the *Arabidopsis* ethylene receptor gene family showing variations in the architecture of their structural protein.

The general ethylene receptor 'two-component' regulator structure sequence is: an N-terminal membrane-bound sensor domain where ethylene binds, a GAF domain of unknown function, a histidine kinase domain presumed to be a catalytic signal transmitter, and a C-terminal receiver domain presumed to be an effector of output domain activity downstream (CTR1 – not shown in this diagram).

There are five members of the ethylene receptor family in *Arabidopsis*, each exhibiting variations from the typical two-component regulator structure. ERS1 and ERS2 lack a receiver domain. ERS2, EIN4, and ETR2 possess an extra hydrophobic subdomain in the sensor domain. Only ERS1 and ETR1 have all the residues needed for catalytic activity in the histidine kinase domain, while the remaining receptors lack conserved elements and thus have degenerate histidine kinase domains.

ethylene responses. Thus, details of the ethylene signal transduction pathway downstream of CTR1 remains enigmatic.

There also remains ambiguity in the ethylene signal transduction pathway upstream of CTR1. Although the possibility of a “his-to-asp” phosphorelay signalling in plant histidine kinase has been examined (Urao *et al.*, 2000), no intermediate components between the ethylene receptors and CTR1 have been identified. Interestingly, based on yeast two-hybrid assays the transmitter domain of ETR1 and ERS1 were capable of directly interacting with CTR1 (Clark *et al.*, 1998). Furthermore, the amino terminus of CTR1 has showed weak homology to the amino terminus of Raf kinase (Kieber *et al.*, 1993). Taken together, the divergence at the amino-terminus and the capacity to directly interact with upstream components instead of using indirect phosphorelaying, suggest that CTR1 may be signalled through a novel mechanism.

1.18 Two Types of Mutations Can Disrupt Normal Ethylene Signalling

A range of ethylene response mutants has been identified in *Arabidopsis*. Careful genetic analysis of these mutants has identified several components involved in the ethylene signalling pathway. Two distinctive mutations were observed: 1) ethylene-insensitive ethylene receptor mutants that did not display the triple response phenotype even in the presence of ethylene; and 2) constitutive triple response mutants (*ctr1*) that always displayed the triple response phenotype even in the absence of ethylene. Epistatic analysis, which involves making double mutants between mutations with clearly distinct

phenotypes, was performed between the ethylene-insensitive receptor mutants and *ctr1*. Because the double mutant plants displayed the triple response phenotype, the *ctr1* phenotype was deemed epistatic (completely masked the phenotype of another mutation with its own phenotype) to the ethylene receptor mutants. Through further epistatic analyses, the CTR1 protein was identified as a downstream component of the ethylene signalling pathway (Kieber *et al.*, 1993).

The phenotype of the *ctr1* mutant shows a (constitutive) triple response similar to the triple response of wild-type seedlings continuously exposed to ethylene, which indicated that the native CTR1 protein is a negative regulator of ethylene responses (such as triple responses) (Kieber *et al.*, 1993). Hence, the ethylene-insensitive mutant receptors were gain-of-function mutations because of the gain of (negative ethylene response regulation) signalling from CTR1; and the constitutive triple response mutants were loss-of-function mutations because of the loss of negative signalling from CTR1.

Having revealed the epistatic relationship between the phenotypically distinct ethylene-insensitive receptor mutants and *ctr1* mutants, and having established the negative regulatory role CTR1 has on ethylene responses, two possible models of ethylene signalling emerged (summarized by McCourt, 1999). In both models, CTR1 is actively repressing ethylene responses when ethylene is not detected and is inactive (and hence not repressing ethylene responses) when ethylene is detected by the ethylene receptors. The difference between the two

models is in the classification of ethylene receptors as negative regulators or as positive regulators of CTR1 function. In the former, ethylene response phenotypes corresponding to the hormone absence/presence are obtained when ethylene perception activates the negative regulation of receptors. In the latter, corresponding ethylene response phenotypes are obtained when ethylene perception inhibits the positive regulation of receptors.

The proper ethylene signalling model was deduced from the dominant or recessive nature of gain-of-function and loss-of-function mutants in the ethylene receptors. Careful analysis of the ethylene-insensitive receptor mutants revealed that the gain-of-function mutations were dominant, meaning a mutation in just one copy would functionally override the wild-type gene. Even more significantly, a gain-of-function mutation in one of the five ethylene receptor genes was enough to functionally override the remaining receptors to confer insensitivity to ethylene. Hua and Meyerowitz (1998) made additional mutations in the ethylene receptors in the hopes of finding suppressors of the ethylene-insensitive phenotype describe above. These additional mutants all resulted in loss-of-function mutations in another part of the protein. Careful analysis of the loss-of-function mutants revealed that the mutations were recessive and also the expected constitutive triple response phenotype could only be seen when a triple loss-of-function mutant was generated. These revelations could only be justified with the second model (described in the next section). The idea that the kinase activity of receptor could continually activate subsequent signalling components

and demonstrate inhibition with ligand binding is not standard but is possible (Parkinson and Kofoed, 1992).

1.19 Model of Ethylene Signalling – from Perception to Response Activation

Based on the research presented above, the following is the current model for ethylene perception and signal transduction. Ethylene is perceived by up to 5 different receptors – ERS1, ERS2, EIN4, ETR1, and ETR2 – and signal transduction eventually leads to ethylene-mediated responses, such as the triple response. Although many genes have been implicated in the signalling pathway of ethylene, many more details have yet to be elucidated. A simplified model of operation through the main components involved in ethylene signalling is described by Figure 2. CTR1 is a negative regulator of ethylene responses, thus ethylene responses like the ‘triple response’ are repressed when CTR1 is activated. The precise mechanism is not known but it is generally accepted that ethylene responses are negatively regulated by the ethylene receptors (Hua and Meyerowitz, 1998), such that in the absence of ethylene (Figure 2-A), receptors positively activate CTR1. In the presence of ethylene (Figure 2-B), however, the gas binds to the receptor altering the signalling so that it can no longer activate CTR1, and without CTR1, there is a de-repression of downstream ethylene responses. In this sense, ethylene perception is a negative regulator of CTR1, itself a negative regulator. Thus, through this double negative regulation, the perception of ethylene allows the ethylene responses to occur through the hormone signalling pathway.

Figure 2: Model of ethylene signalling in *Arabidopsis*.

In the absence of ethylene (**A**), ethylene receptors activate CTR1, which repress downstream ethylene responses. In the presence of ethylene (**B**), gas binding to the sensor domain inactivates ethylene receptors, which in turn inactivates CTR1. Ethylene responses (ex. triple response) occur consequent to de-repression from CTR1 inactivation. Solid arrows represent positive regulation and solid bars represent negative regulation. Striated bars represent the loss of positive regulation and striated arrows represent the loss of negative regulation.

1.20 How Dominant Gain-of-Function Mutants Signal to Confer Ethylene-Insensitivity

The first mutants identified in the ethylene receptors conferred dominant ethylene insensitivity. All the point mutations conferring dominant ethylene insensitivity were located in the sensor domain of ethylene receptors (Chang *et al.*, 1993). Biochemical studies demonstrated that dominant mutations conferring ethylene insensitivity showed reduced ethylene-binding activity in receptor proteins expressed in yeast (Hall *et al.*, 1999). Figure 3 illustrates the mechanism behind how the impaired ethylene perception of gain-of-function mutants could result in dominant ethylene insensitivity. Phenotypic differences in dominant gain-of-function mutants are not detected in the absence of ethylene (Figure 3-A vs. Figure 2-A), but are detected in the presence of ethylene (Figure 3-A vs. Figure 2-B). Because gain-of-function mutated sensor domains are unable to bind ethylene, the mutated ethylene receptors do not inactivate CTR1. In this case, CTR1 is continually activated by a mutant receptor and ethylene responses are continually repressed

The dominant gain-of-function mutants conferring ethylene-insensitivity are directly contrasted by loss-of-function mutants, whose point mutations conferring constitutive ethylene response were located in the histidine kinase domain of ethylene receptors (Hua and Meyerowitz, 1998). By hindering the ability of receptors to catalytically transmit the CTR1 activation signal, ethylene responses are constitutively expressed because CTR1 would never be activated to repress them. However, single loss-of-function gene mutants did not show

Figure 3: Model of ethylene signalling in gain-of-function mutants of *Arabidopsis*.

In the absence of ethylene (**A**), ethylene response repression is equivalent to wild-type signalling model. In the presence of ethylene (**B**), gas is incapable of binding to the sensor domain, thus ethylene receptors constitutively activate CTR1, which constitutively represses ethylene responses (ex. triple response). Therefore, mutations in the sensor domain confer gain-of-function ethylene-insensitivity. Solid arrows represent positive regulation and solid bars represent negative regulation. The constitutive insensitivity to ethylene requires only one mutated receptor to override the remaining functioning receptors. Gain-of-function mutations are dominant.

this constitutive ethylene response. Only by combining 4 loss-of-function mutations did the ethylene response of etiolated seedlings approach that seen in *ctr1* mutations (Hua and Meyerowitz, 1998).

1.21 Assessing the Function of Individual Ethylene Receptors: Redundancy or Not?

The presented model generally illustrates what is known of the components in ethylene signal transduction pathways; what is not well understood, however, is the ultimate function of the transducing signal. The present model implies that all five of the ethylene receptors signal through the same regulator (CTR1). Because the single dominant gain-of-function mutation of ethylene receptors each shows the same lack of ethylene triple response phenotype and because single recessive loss-of-function mutations do not show an expected constitutive ethylene responses, functional redundancy of the ethylene receptors has been proposed (Hua and Meyerowitz, 1998). If the function of each ethylene receptor were not redundant, it would be expected that each gain-of-function mutant would display distinct phenotypes. To date, some minor differences in gain-of-function mutants have been described suggesting that each receptor may be involved in distinct functions. There is also differential expression of ethylene receptors in various plant tissues suggesting unique requirements for these receptors during specific times of development (Hua *et al.*, 1998). In contrast, the overlap of ethylene receptor function is supported by the observation that a triple loss-of-function mutant produces a phenotype that mimics the triple response equivalent to *ctr1*. Thus, it is unclear whether the

ethylene receptors have redundant or unique functions in the plant, or perhaps a combination of both (i.e. some redundant and some unique functions).

Another interesting observation from the analysis of the multiple loss-of-function mutations is the finding that quadruple mutants display a phenotype that is more severe than the *ctr1* mutant phenotype (Hua and Meyerowitz, 1998). The observation of a stronger ethylene-response phenotype in plants with loss-of-function mutations in four of the receptor genes suggests that the receptors may also signal through another factor (i.e. in addition to CTR1) in ethylene signalling. To date the phenotypic analysis of ethylene responses has been restricted to the triple response and the not all components of the ethylene signalling transduction have necessarily been identified and functionally accounted for; furthermore, the conditions used to find unique gene functions for each receptor may be too restrictive, thus the restrictive assumption of the ethylene receptor redundancy may not be sustainable.

1.22 The Potential Role of Ethylene in Cross-talk During Seed Development

Primary dormancy is said to be imposed during the later maturation stages of seed development on the maternal plant, however, this statement is largely based on the observation that freshly matured seeds are dispersed dormant. Many studies focus on the afterripening phase of mature seeds, without much consideration of the influence seed development has on initiating dormancy. As previously mentioned, ethylene has already been shown to affect seed germination, thus, it would be reasonable to consider how ethylene could

possibly influence seed dormancy. Specifically, what is the purpose behind transducing signals from 5 ethylene receptors? Assuming ethylene does have a strong influence on seed development, the influence is likely linked to a larger more complex network of hormonal interactions.

1.23 Simultaneous Analysis of Plant Hormone Classes – the Advantages of HPLC/ESI-MS/MS

At the hormone control level alone, the assortment of hormone interactions is complex enough to tremendously complicate the study of how one hormone class influences one developmental event. One of the limitations associated with studying hormone cross-talk has been the methodology behind traditional techniques. Although much functional information has been obtained from experiments where hormones are exogenously applied, hormone interactions are more precisely established with current genetic and molecular studies, where hormone mutants are used to demonstrate hormone interactions. The latter technique can still be limiting, as the effects of mutants affected in sensing one hormone are often described in relation to only one other hormone. Thus, often studies concentrate on the effects of a single hormone to simplify the study of cross-talk without accounting for the influences of other hormones that may be present and active.

To start to understand how hormones could be interacting to control plant development, it would be useful to know the concentration of all hormones at any given moment of interest in plant development. As ideal as such an analysis seems for profiling hormone/metabolite fluctuations and interactions in signalling

networks, it implicates the generation of an extremely complex and difficult methodology. The variations in chemical properties between each hormone class alone makes extraction challenging. Plant hormones and their metabolites can be acidic, basic or neutral; structurally simple (like ethylene) or complex (like gibberellins); highly bioactive to minimally to none at all. In addition, phytohormones are present physiologically at very low concentrations amongst a variety of other compounds found in high abundance in plants. Taken together, the simultaneous extraction and analysis of all plant hormones is therefore a very complicated and challenging task. Only recently have technologies advanced enough to enable scientists to integrate general compound extraction, identification, and quantification into a comprehensive analytical method.

Chiwocha *et al.* (2003) have developed a highly selective and sensitive method allowing several plant hormones and their metabolites to be simultaneously analyzed. The combination of high performance liquid chromatography (HPLC) with positive and negative electrospray ionization (ESI) with tandem mass spectrometry (MS/MS) represents a promising approach to comprehensively and simultaneously analyze of a wide range of plant hormones and metabolites. This versatile method first involves a general chemical extraction, accommodating the efficient extraction of hormones and metabolites with diverse chemical properties. Deuterium-labelled internal standards are added to extracts to allow the identification of the precursor-to-product ion

transition appropriate for the proper compound detection from multiple reaction monitoring (MRM). Simultaneously, the high specificity and sensitivity of MRM bypasses the need for a comprehensive compound resolution during extraction and permits several plant hormones and metabolites to be analyzed in a single run, under differing functions to account for differences in chemical properties. This liquid chromatography-electrospray ionization tandem mass spectrometry method was successfully used to follow the hormone and metabolite levels associated with seed dormancy and germination in lettuce (Chiwocha *et al.*, 2003). Similarly, the quantitative analysis of these fifteen compounds will be used in this thesis to yield preliminary information on the hormone and metabolite profiles of *Arabidopsis* seeds in wild-type plants and ethylene-insensitive mutants.

Studies, so far, have yet to establish what kind of influence ethylene has on processes that occur during seed development. Because previous studies have implicated ethylene to be involved during dormancy and have implicated dormancy to occur during seed development, there remains a possibility that ethylene receptors may not show redundancy in their function during this instance of seed development. Furthermore, there is a potential for hormonal cross-talk to be established during seed development. Through the use of simultaneous analysis of several plant hormones and metabolites, the potential interactions of such cross-talk can be monitored quantitatively.

2 MATERIALS AND METHODS

2.1 Plant Strains

The background of wild-type and mutant lines of *Arabidopsis thaliana* used in this study was of the Columbia ecotype. All wild-type, ethylene-insensitive mutants (*ers1*, *ers2-1*, *ein4-1*, *etr1-1*, *etr2-1*) and constitutive triple response mutant (*ctr1-1*) seeds were obtained from the Arabidopsis Biological Resource Center (ABRC).

2.2 Initial Seed Preparation and 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 thoroughly rinsed with sterile distilled water (10 times). For wild-type seeds, stratification (treatment of cold, wetness, darkness) to break dormancy and to synchronize germination was carried out by keeping the seeds in the last rinse water, covering the tube with tinfoil, and storing at 4°C for 4-7 days.

Stratified and surface-sterilized wild-type seeds were suspended in sterile 0.1% (w/v) agar and sown directly (via Pasteur pipette) onto sterilized soil. For the gain-of-function ethylene receptor mutants, a triple response assay was performed to screen for ethylene insensitivity in the etiolated seedlings (description immediately follows). Surface-sterilized mutant seeds were sown on petri plates (50-100 seeds per plate) with growth medium consisting of Murashige and Skoog (M&S) basal salt mixture (Sigma, Oakville, Ontario, Canada) buffered to pH 5.7-5.8 with 1 M KOH, solidified with 0.8% (w/v) agar,

and supplemented with 50 μ M 1-aminocyclopropane-1-carboxylic acid (ACC-HCl; Sigma, Oakville, Ontario, Canada) after the media was autoclaved. ACC is an ethylene synthesis precursor and, when present in the media, will cause an accumulation of ethylene in the plant tissues. ACC stock solutions were prepared in sterile distilled water. As a control, surface-sterilized wild-type seeds were also sown on each plate to ensure that the media was able to induce the triple response. Seeds were stratified for 4-7 days before incubation at room temperature in the dark for 4 days, during which time germination occurred. Etiolated mutant seedlings with long hypocotyls and roots (compared to wild-type) confirmed ethylene-insensitivity and were chosen for continued growth. ACC-screened etiolated ethylene-insensitive mutant seedlings were delicately transplanted (via curved forceps) into sterilized soil. Likewise, the constitutive triple response mutant was screened by plating on M&S media lacking ACC and identified as those seedlings that displayed the triple response in the absence of ethylene. All plants were acclimated by covering the trays with plastic humidity domes (Kord Products Inc., Brampton, Ontario, Canada) until seedlings had expanded true leaves. To prevent cross-pollination and seed contamination, and to facilitate mature bulk seed harvest, Aracons (Lehle Seeds, Round Rock, Texas, USA) were used.

All plants were grown to maturity in autoclaved soil (Pro-Mix BX, Premier, Riviere-du-Loup, Quebec, Canada) in AC60 *Arabidopsis* growth chambers (Enconair Ecological Chambers Inc., Winnipeg, Manitoba, Canada) at 22°C. The

two photoperiods (light/dark regime) used were long-day (16h light / 8h dark) conditions or short-day (8h light / 16h dark) conditions. White fluorescent lighting provided a light intensity of 130-190 $\mu\text{E}/\text{m}^2/\text{s}$ at the rosette level. Plants were individually grown in soil-packed traditional 48 cell-pak inserts in traditional open thermoformed and injection-molded ribbed trays (Kord Products Inc., Brampton, Ontario, Canada) and daily watering was done by sub-irrigation followed by complete drainage of excess water.

2.3 Ethylene Sensitivity

The hypocotyl lengths of etiolated ethylene-receptor mutants and wild-type seedlings grown with continual exposure to ethylene were used to gauge sensitivity of the plants to the hormone ethylene. Seeds were surface-sterilized before being sown onto M&S-agar square petri dishes with 50 μM ACC. After 7 days of stratification, the tin-foiled covered dishes were transferred to room temperature and seeds were vertically grown in the dark for 7 days. Only germinated seeds were considered for measurement. Data was compiled from two separate experiments and analyzed with one-way ANOVA.

2.4 Plant Material and Staging

When the adult plants bolted and the inflorescence produced siliques (seed pods), the latter provided the seeds needed for the germination potential assays and metabolite analyses. Six distinct stages were identified based on silique and seed phenotype and are described below from youngest (stage 1) to oldest (stage 6).

Stage 1 – silique was flaccid, not fully elongated, and pericarp had a pale green colouration; seeds were small, white and fragile.

Stage 2 – silique was plump, fully elongated, and pericarp had a bright green colouration; seeds had reached full size and ranged from a white testa with a visible green embryo to a completely bright green coloured seed coat.

Stage 3 – siliques were in the process of losing the brightness of the green colouration with signs of yellowing evident at the tips; seeds had also lost their bright green colour and were showing signs of browning.

Stage 4 – silique pericarp had a dull yellow colouration; seeds had a tinge of green colour remaining as they continued to brown.

Stage 5 – siliques were a tan in colour with no signs of desiccation visible; seeds were completely brown but were still plump from lack of drying.

Stage 6 – 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.

Because *Arabidopsis* has an inflorescence meristem, up to 50 flowers can be formed sequentially on each branch. In a fully extended stem, the oldest flower is found at the base of the stem, and proceeding up the stem, each flower is younger until you reach the stem apex where the flowers are the youngest. The identification of siliques in each developmental stage was facilitated by this progressional method maturing siliques developed along the stem length. All

experiments were done using these six distinguishing stages to represent an immature seed undergoing development to maturity.

2.5 Days After Pollination Determination

To establish an approximate timeline for each seed development stage, the number of days after pollination (DAP) for the stages was tracked for wild-type plants grown in long-day and short-day conditions. Selected flowers were labelled around the petiole on the day of pollination (the date was included on the label). Pollination was considered to have occurred when the stigma was just starting to be visible through the closed petals and sepals of the flower. Flowers were tagged for 15 consecutive days for long-day conditions and the timing of each stage was recorded for 12 days. Under long day conditions, the seeds quickly matured so stage recordings were done twice daily. Under short-day conditions, the flowers were tagged for 23 consecutive days and the stages of the seeds were recorded on a daily basis for 32 days.

2.6 Germination Potential Assay

To identify stages of seed development and dormancy, the germination potential of seeds from each stage was assessed for wild-type and mutant plants. Six siliques were used for each stage and eight seeds were used from each silique (for a total of 48 seeds) and were plated (without sterilization) on each of two replicate square petri dishes with media that only contained 0.8% (w/v) agar. Seeds were carefully separated from their siliques under a dissecting microscope and seeds from each stage were visually confirmed prior to transfer

to the medium surface. Individual replicates were either subjected to stratification as previously described or not stratified before being incubated in continuous light at RT in a humidified polystyrene mini-desiccator cabinet (Sanplatec Corp., Osaka, Japan). For the plate being subjected to stratification, the plate was covered with tinfoil and stored at 4°C for 4-7 days and then stored in the humid box as above. After 14 days of growth in conditions allowing germination, 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 embryo radicle was seen emerging from the imbibed seed coat.

2.7 Metabolite Analysis

Siliques were removed from wild-type and mutant plants at each stage of development and freeze-dried prior to sample preparation and analysis. Analysis with high-performance liquid chromatography (HPLC) coupled with positive and negative electrospray ionization-tandem mass spectrometry (ESI-MS/MS) was performed for simultaneous metabolite profiling and quantification at the National Research Council of Canada's Plant Biotechnology Institute in Saskatoon, Saskatchewan. Chiwocha *et al.* (2003) described the procedure in detail and a summary with appropriate modifications follow.

2.7.1 *Sample Collection*

Because hormone levels can fluctuate throughout the day, siliques were harvested at the same time every day. Wild-type, *ers1* and *etr1-1* mutant plants

were grown under both long-day and/or short-day conditions and the siliques were harvested and sorted. To ensure proper staging, siliques were dissected to reveal the appropriate appearance of the seed contents before collection in a 1.5 ml microtube. Siliques were frozen in liquid nitrogen and stored at -80°C until enough plant material had been collected. Because a minimum of 50 mg of dry weight material was used per replicate, 150 mg of dry weight was required for a triplicate run of each stage. Because water loss ranged from 20% to 80% (the younger the silique, the higher water loss) in these tissues, a fresh weight of approximately 0.75 g was isolated for each stage.

2.7.2 *Metabolite Extraction*

When all material had been collected and frozen, samples were lyophilized (FreeZone Freeze Dry System, Labconco, Kansas City, Missouri, USA) for 48 hrs in the dark. The dried sample was then ground into a coarse powder (FastPrep BIO101, Thermo Savant, Milford, Massachusetts, USA) 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.

2.7.3 *Extract Purification*

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, Canada) 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, Canada) was used to facilitate column elution.

2.7.4 *Analysis Preparation*

To evaporate solvents from the column eluate, the purified extract was dried in a Speed-Vac (CentriVac Concentration System, Labconco, Kansas City, Missouri, USA). 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.

2.7.5 *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, Canada) coupled to an electrospray ion source (ESI) linked quadrupole tandem mass spectrometer (MS/MS) (Quattro

Ultima, Micromass, Manchester, UK). 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, UK). 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. Because the precise dry weight and average water loss from each sample was recorded, it was possible to calculate the concentration of plant hormone and metabolites on a dry weight and fresh weight basis. However, because of similar trends were seen in both, only profiles based on dry weights were considered in this thesis. All HPLC conditions, MS conditions, calibration curve generation, and deuterium-labelled compounds production was carried out exactly as detailed by Chiwocha *et al.* (2003).

3 RESULTS

3.1 Phenotypic Differences between Ethylene-Insensitive Mutants

The first indication that the ethylene receptors could function differentially in developmental processes came from the analysis of full-grown gain-of-function mutants. In *Arabidopsis*, growing leaves arrange themselves in an overlapping whorl to form a rosette, the center from which emerges the flowering bolt. In wild-type plants, the leaves are initially small and round with a smooth margin, and eventually become larger with a more spatulate shape and coarsely serrated margin. The leaves are a deep green in colour and will turn purplish in very old plants as anthocyanins accumulate. The leaves of *ein4-1*, *etr2-1* and *ers2-1* developed like wild type both in shape and colour while the leaves of *ers1* and *etr1-1* were not serrated but had a smooth margin and the leaves did not accumulate anthocyanins but instead appeared pale green or yellow (Kozela, 2003).

The dominant gain-of-function receptor mutants also varied in their response to ethylene. Figure 4 shows the variation in hypocotyl length between the 5 ethylene-insensitive mutants and the wild-type seedlings germinated under etiolating conditions in the presence of ethylene. While the wild-type plants displayed the classical triple response, the mutants had varying insensitivity to ethylene. Using the average length of the hypocotyls as an indicator of ethylene insensitivity, *ers2-1* seedlings were the most insensitive, followed by *ers1* and *etr2-1* seedlings. The *ein4-1* and *etr1-1* seedlings appeared to have the greatest

Figure 4: Sensitivity of *Arabidopsis* ethylene-insensitive mutant seedlings to continual ethylene exposure during etiolated germination.

The hypocotyls of etiolated ethylene-insensitive mutant and wild-type (WT) seedlings germinated in the continual presence of ethylene were measured. The wild-type seedlings showed the triple response and the receptor mutant seedlings lacked the triple response phenotype. One-way ANOVA analysis between ethylene insensitive mutants yielded a P-value < 0.01 .

sensitivity to ethylene. The only mutant to show a triple response similar to the wild-type seedlings was *ctr1-1* (data not shown).

Another phenotype that varied in the gain-of-function ethylene receptor mutants was the timing of the transition of the vegetative meristem to a floral meristem. A bolted flowering stalk indicates the transition to flowering and it was observed that flowering times varied in the ethylene-insensitive mutants. Table 1 summarizes the vegetative to floral meristem transition in mutant and wild-type plants under differing photoperiods.

Table 1: The age of wild-type and ethylene-insensitive mutant *Arabidopsis* grown under long-day and short-day conditions when the transition from vegetative to reproductive growth occurred.

Mutant (days)	Long-day Conditions (16h light / 8h dark)	Short-day Conditions (8h light / 16h dark)
WT	30.9 ± 1.4	68.5 ± 3.5
<i>ers1</i>	26.3 ± 1.0	66.1 ± 1.3
<i>ers2-1</i>	28.9 ± 1.3	87.4 ± 5.6
<i>ein4-1</i>	33 ± nd	nd
<i>etr1-1</i>	34.6 ± 0.4	> 131.5 ± 7.4
<i>etr2-1</i>	30.0 ± 3.0	nd
<i>ctr1-1</i>	32 ± nd	nd

Abbreviations: WT = wildtype; nd = no data

Photoperiod length is one of the factors affecting the timing of transition to flowering. Generally, a long-day photoperiod promotes bolting while a short-day photoperiod delays bolting. For the long-day photoperiod, flowering time transitions appeared to occur within a few days of each other, with *ers1* bolting

slightly earlier than wild-type and *etr1-1* and *ein4-1* showing a slight delay. A much greater variation was detected during short-day conditions where *ers1* plants again bolted slightly ahead of wild-type, and *ein4-1* and *etr1-1* plants showed a considerable delay in flowering transition. The bolting time of 131 days for *etr1-1* short-day was conservative as only 2 of 6 trays had plants that bolted after 141 days of observation. Furthermore, the flowers from the primary bolts of *etr1-1* did not fully develop. The lengthy time *etr1-1* short-day plants took to flower made it impractical for subsequent experimentation in this thesis.

3.2 Silique and Seed Development to Maturity

After pollination, the silique of *Arabidopsis* goes through phenotypic changes indicating the progression through maturation of its seed contents. After the fertilization of each seed within the flower's carpel, the petals, sepals, and stamens abscise and the elongating and maturing green silique emerges. The silique is comprised of 30-50 seeds packed into two locules separated by a false septum. Six stages of silique and seed development were defined, starting with immature seeds and ending with dispersal-ready mature seeds. Figure 5 schematically illustrates the silique (fruit pod) colouration changes of the pericarp (fruit coat) and testa (seed coat) during maturation through the six stages of seed development. At the earliest stage (stage 1), the siliques were not fully elongated and the seeds were undersized, white and fragile. When the siliques were fully elongated and the pericarp was a bright green colour, the seeds were at stage 2. The appearance of the seeds in this stage ranged from a white seed

Figure 5: Colouration schematic of the pericarp and testa appearance of *Arabidopsis* siliques during the maturation period of seed development.

The appearance of the ovules attached along the ovary wall in two locules is outlined before dissection **(A)** and shown after dissection along the false septum **(B)**. The green silique containing immature pre-viable seeds (stage 1) fully elongates (Stage 2) before turning completely yellow (Stage 4) starting at the tips (Stage 3), then turning brown (Stage 5), prior to seed desiccation (Stage 6) in preparation for seed release.

coat with a visible green fully-grown embryo developing within, to a bright green testa. The siliques then turned yellow beginning at the tip at stage 3. Seeds at this stage had lost their colour brightness, showing a muted green with a brown tinge; furthermore, dissection of the seed coat in this and subsequent stages revealed a yellow embryo. Siliques continued to yellow to the base by stage 4 and seeds at this stage were a light brown with a tinge of green remaining. At stage 5, the siliques turned brown and the seeds had lost all signs of a green colour. By stage 6, the silique valves desiccated in preparation for the spontaneous opening of the locules (dehiscence) to disperse the dried, seed coat-hardened dark brown seeds contained within. At this last stage, the siliques would easily shatter and release seeds when touched.

3.3 Days After Pollination Determination

Arabidopsis is considered self-compatible pollen from the stamen has the capacity to pollinate the stigma and subsequently fertilize the ovules of the same flower. Flowers are ready to be pollinated when the green papillae of the stigma emerge from the tip of the still closed petals and sepals. To gain an understanding of the duration of each seed development stage described above, the time to the beginning of each stage, recorded as days after pollination, was determined. Table 2 lists the approximate start date of each developmental silique stage while Figure 6 shows the timeline of the stages of silique development related to days after pollination (DAP).

Figure 6: Days after pollination (DAP) timeline of the silique development through the six seed maturation stages in wild-type *Arabidopsis*.

Plants were grown under long-day (16h light / 8h dark) and short-day conditions (8h light / 16h dark). Day of pollination was recorded by stigma visibility through closed flower petals and sepals and staging was solely based on exterior pericarp colouration.

Table 2: The six stages of silique development related to days after pollination (DAP) of wild-type *Arabidopsis* grown under long-day and short-day conditions.

Stage (DAP)	Long-day Conditions (16h light / 8h dark)	Short-day Conditions (8h light / 16h dark)
1	2.8 ± 0.1	4.8 ± 0.1
2	4.4 ± 0.2	7.5 ± 0.3
3	12.5 ± 0.1	13.1 ± 0.2
4	13.6 ± 0.1	15.4 ± 0.1
5	14.6 ± 0.1	16.5 ± 0.2
6	15.7 ± 0.2	18.9 ± 0.3

In comparing the timing of each developmental stage between plants grown under long-day conditions and plants grown under short-day conditions, in general each short-day stage was almost twice as long as each long-day stage, with the exception of stage 2. For example, in long days, stage 3 lasted from day 12.5-13.6, essentially one day, while in short-day conditions stage 3 lasted from day 13.1-15.4, more than two days. The anomaly was stage 2, which was approximately 3 days longer in long days than stage 2 of short-day grown plants. After stage 2 turnover through the remaining stages occurred daily for long-day grown plants. Interestingly, from the beginning of stage 1 to the onset of stage 6, short-day plants take approximately 14 days while long-day plants take approximately 13 days.

3.4 Germination Potential

To assign a function to each developmental stage, the ability of seeds to germinate with and without stratification was tested for wild-type and mutant

plants. Starting with results from the wild-type plants in short-days (Figure 7-B), the seeds at stage 1 were unable to germinate in either stratified or non-stratified conditions. This indicates that the seeds at this stage have not completed enough embryo development and are unable to survive without the maternal tissue. At stage 2, seeds were able to germinate, with a high percentage of seeds germinating in the non-stratifying conditions and far fewer germinating after stratification. These results indicate the end of embryo development and entrance into a stage known as precocious germination. Seeds in this stage have developed enough to survive without the maternal tissue, but have yet to acquire cold tolerance so they are typically able to germinate in non-stratified conditions but not after a cold treatment. At stages 3 and 4 there was minimal germination potential in both stratified and non-stratified conditions. At these stages, the seeds have entered dormancy, during which the seeds go through additional developmental processes to prepare the seed for dispersal. The dormancy cannot be broken by stratification or non-stratification conditions. By stages 5 and 6 the seeds were again able to germinate, marking the potential end of dormancy. In this phase, the highest germination potential was found in the stratified seeds. This indicates that the seeds have acquired cold tolerance and remain viable during cold treatment. Since there was a lower germination potential in the non-stratification conditions at this stage, this indicates that the seeds have also acquired the need to be stratified before germination can occur. Thus, four distinct seed phases can be identified based

Figure 7: Germination potentials of wild-type *Arabidopsis* seeds developing through the six stages of seed maturation.

Plants were grown under long-day conditions (**A**) and under short-day conditions (**B**) and the percentage of germinated seeds were determined after 14 days.

on this analysis, pre-viable (stage 1), precocious germination (stage 2), dormancy (stage 3-4), and dormancy-breakable (stage 5-6). The same phases could also be detected in wild-type seeds grown under long-day conditions (Figure 7-A) but with slight variations in germination potentials. Most noticeably, there was greater germination (less dormancy) seen in stages 3 and 4 of long-day plants than of short-day grown plants. Although not as low as in short-day grown plants, the germination potential of these stages did show a marked decrease in both stratified and non-stratified conditions, indicating that dormancy, although more shallow, was induced. The favouring of germination of non-stratified seeds over stratified seeds indicates that cold tolerance has not been fully developed in the seeds of these stages.

The same tests were performed on the gain-of-function ethylene receptor mutants and the constitutive triple response mutant. *ers1* and *etr1-1* were two mutants that displayed very different germination potential results compared to wildtype. The remaining three receptor mutants bore a close resemblance with some variations to the results of wildtype.

The results for the *ers1* mutant grown under long-day and short-day conditions are shown in Figure 8 respectively. Stage 1 seeds were unable to germinate as was found in the wild-type plants. Similarly, at stage 2, the seeds were able to germinate under non-stratified conditions, and to a lesser extent under stratified conditions. The most striking difference occurred at stages 3-4 where the seeds were still able to germinate, unlike the wild-type seed, which

Figure 8: Germination potentials of *ers1* mutant *Arabidopsis* seeds developing through the six stages of seed maturation.

Plants were grown under long-day conditions **(A)** and under short-day conditions **(B)**.

had entered dormancy at this time. This indicates that the *ers1* seeds have lost the ability to enter seed dormancy. At stages 5-6 the *ers1* seeds again resembled the wild-type seeds. After stage 2, there were higher germination potentials in stratification conditions, again indicating the acquisition of cold tolerance and the necessity to be stratified before germination can occur.

The results for the *etr1-1* plants grown under long-day conditions are shown in Figure 9. Short-day results were not obtainable due to the extreme delay in flowering time in this mutant. Compared to wild-type seeds, the stage 1 seeds were also unable to germinate, and stage 2 seeds were able to germinate. The seeds appeared to enter a deep dormancy at stage 3-4 and consequently there was no germination potential at these stages in both stratified and non-stratified conditions. The most striking difference in the *etr1-1* seeds occurred in stages 5-6. Unlike wild-type seeds, the *etr1-1* seeds very poorly germinated at these stages in either stratified or non-stratified conditions. This germination failure suggests that the dormancy stage was prolonged in this mutant or the seeds in this mutant are unable to exit dormancy normally. The deeper dormancy was even observed during general germination of *etr1-1* seeds from stored (afterripened) seed stocks used for growing adult plants for experiments or for seed stock bulking. Another striking difference in the *etr1-1* strain also occurred. Only in this mutant was the germination potential higher in stage 2 (precocious germination) than in stage 6 (mature), indicating that something occurred during stages 3 and 4 that reduced germination of mature seeds.

Figure 9: Germination potentials of *etr1-1* mutant *Arabidopsis* seeds developing through the six stages of seed maturation.

Plants were grown under long-day conditions (16h light / 8h dark).

Because the germination potentials were so reduced, it was difficult to infer whether cold tolerance was being established, but because germination potential of stratified seeds in stage 6 were higher than that for unstratified seeds, some cold acclimation was assumed to have occurred.

The germination results from the *ers2-1* mutant were identical under long-day and short-day conditions (Figure 10). The results from *ers2-1* were also similar to results from wild-type plants grown under short-day conditions. Stage 1 seeds were unable to germinate, and stage 2 seeds did germinate but could only do so at a very low potential. As expected from the wild-type results, the *ers2-1* seeds were completely unable to germinate during stages 3-4, with or without germination. This inability to germinate was again attributed to dormancy. Again, the ability for the seed to germinate resumed in stage 5 and 6. Interestingly, the seeds were only able to germinate at stage 5-6 under stratified conditions suggesting that these seeds were more dependent on stratification than seeds of the same age in wild-type.

The germination results for *etr2-1* (long-day) plants (Figure 11) were essentially the same as for those of *ers2-1*. The only noticeable difference was seen in stage 2, where the overall germinability was higher in *etr2-1* mutants and stratified seeds germinated to a greater extent than did non-stratified seeds. The results for the final ethylene-insensitive mutant *ein4-1* (long-day) (Figure 12) had similar germination potentials to long-day grown wild-type plants. Overall, stage 1 seeds did not show capacity to germinate, stage 2 seeds showed moderate

Figure 10: Germination potentials of *ers2-1* mutant *Arabidopsis* seeds developing through the six stages of seed maturation.

Plants were grown under long-day conditions **(A)** and under short-day conditions **(B)**.

Figure 11: Germination potentials of *etr2-1* mutant *Arabidopsis* seeds developing through the six stages of seed maturation.

Plants were grown under long-day conditions (16h light / 8h dark).

Figure 12: Germination potentials of *ein4-1* mutant *Arabidopsis* seeds developing through the six stages of seed maturation.

Plants were grown under long-day conditions (16h light / 8h dark).

germination, stages 3 and 4 showed a slight decline in potential due to dormancy, before the germination potentials once again increased, with stratification, in stages 5 and 6. Cold tolerance and stratification-dependence to overcome the relatively shallow dormancy in stages 5 and 6 were once again characteristic of the germination results of *ein4-1*. The most notable feature of the germinability pattern of *ein4-1*, was the clearest demonstration of accumulating cold tolerance after the precocious germination of stage 2 (from germination pattern in stratified data) and the gradual loss of germination in non-stratified seeds until there was strong dependence of stage 6 seeds on stratification to allow germination.

The results of the germination potential for seeds from the *ctr1-1* mutant under long-day conditions are shown in Figure 13. Similar to the *ers1* results, seeds were able to germinate in stages 2-6 indicating a bypass of dormancy at stages 3 and 4. The most striking similarity between the germination results of *ctr1-1* and *ers1* is the reduced dependency on stratification for seeds to germinate. This dependency on stratification was typically seen in wildtype and the other ethylene receptor mutants.

3.5 Plant Hormone and Metabolite Analysis

One of the “drawbacks” of metabolite profiling is the large amount of data that is generated. While likely all possibly relevant to some aspect of seed development, I will restrict my discussion to a subset of results with interesting trends that appear to be related to seed dormancy. Starting with the results from

Figure 13: Germination potentials of *ctr1-1* mutant *Arabidopsis* seeds developing through the six stages of seed maturation.

Plants were grown under long-day conditions (16h light / 8h dark).

wild-type plants grown under long-day conditions (Figure 14), abscisic acid levels were generally high at the start of seed maturation (stage 1) before gradually declining to the lowest levels, seen at the end of seed maturation (stage 6). For the auxins, IAA levels also showed gradual decline from the start of seed maturation, while IAAsp levels remained relatively stable (with a decrease at stage 2). Profiles of cytokinins showed that ZR was predominant and in gibberellin profiles, GA₃ was predominant, especially from stages 1-3.

The results from *ers1* grown under long-day conditions (Figure 15) showed both similarities and differences to those of wildtype. Absciscic acid levels were also initially high in the first two seed development stages before quickly declining to relatively low levels. The most striking difference was the high levels of ABA and DPA seen in both of the first two stages in *ers1*. The trends and levels seen in the auxin analysis of *ers1* were extremely similar to those of long-day wildtype. In the profiles of cytokinins, the predominant compound was IPA in *ers1* long-day results. In the gibberellin profiles, the overall levels were lower. The predominant compound was again GA₃ but more so in stages 3-5.

The results from long-day *etr1-1* (Figure 16) also showed both similarities and differences to wildtype. Absciscic acid levels were very much the same, with the gradual decline occurring at stage 2. The trends in auxin profiles were similar, but most striking was the very large decrease in IAA levels.

Figure 14: Hormone and metabolite profiles during the six seed development stages of wild-type *Arabidopsis* grown under long-day conditions.

Hormones quantified were abscisic acids **(A)**, auxins **(B)**, cytokinins **(C)**, and gibberellins **(D)**. Monitored abscisic acid compounds were: abscisic acid (ABA), phaseic acid (PA), dihydrophaseic acid (DPA), 7'-hydroxy-abscisic acid (7'-OH-ABA), and abscisic acid glucose ester (ABA-GE). Auxin compounds monitored were: indole-3-acetic acid (IAA) and indole-3-acetyl-aspartate (IAA_{sp}). Monitored cytokinin compounds were: zeatin (Z), zeatin riboside (ZR), isopentenyladenine (2iP), and isopentenyladenosine (IPA). Gibberellins (GA) monitored were: GA₁, GA₃, GA₄, and GA₇. Results are on a dry-weight (DW) basis.

Figure 15: Hormone and metabolite profiles during the six seed development stages of *ers1* mutant grown under long-day conditions.

Hormones quantified were abscisic acids **(A)**, auxins **(B)**, cytokinins **(C)**, and gibberellins **(D)**. Monitored abscisic acid compounds were: abscisic acid (ABA), phaseic acid (PA), dihydrophaseic acid (DPA), 7'-hydroxy-abscisic acid (7'-OH-ABA), and abscisic acid glucose ester (ABA-GE). Auxin compounds monitored were: indole-3-acetic acid (IAA) and indole-3-acetyl-aspartate (IAA_{sp}). Monitored cytokinin compounds were: zeatin (Z), zeatin riboside (ZR), isopentenyladenine (2iP), and isopentenyladenosine (IPA). Gibberellins (GA) monitored were: GA₁, GA₃, GA₄, and GA₇. Results are on a dry-weight (DW) basis.

Figure 16: Hormone and metabolite profiles during the six seed development stages of *etr1-1* mutant grown under long-day conditions.

Hormones quantified were abscisic acids **(A)**, auxins **(B)**, cytokinins **(C)**, and gibberellins **(D)**. Monitored abscisic acid compounds were: abscisic acid (ABA), phaseic acid (PA), dihydrophaseic acid (DPA), 7'-hydroxy-abscisic acid (7'-OH-ABA), and abscisic acid glucose ester (ABA-GE). Auxin compounds monitored were: indole-3-acetic acid (IAA) and indole-3-acetyl-aspartate (IAAsp). Monitored cytokinin compounds were: zeatin (Z), zeatin riboside (ZR), isopentenyladenine (2iP), and isopentenyladenosine (IPA). Gibberellins (GA) monitored were: GA₁, GA₃, GA₄, and GA₇. Results are on a dry-weight (DW) basis.

The general trends and levels of cytokinins profiles were very similar to long-day wild-type plants. A very striking difference between long-day wildtype and *etr1-1* results were in the gibberellin profiles. The levels of GA₃ were similar, but there was a sudden high level of GA₄ and GA₇, which were found in very low levels or not at all in wildtype and *ers1*.

Hormone and metabolite profiles from short-day grown wild-type and *ers1* plants also showed interesting trends. Short-day results for *etr1-1* plants were not possible due to the extreme delay in flowering time in this mutant. In short-day wild-type plants (Figure 17), the drop in abscisic acid levels found in long-day grown wildtype was also seen in short days, but the decline was more dramatic after stage 1. Auxin profiles were also very similar between short-day and long-day plants, where IAA levels gradually declined (with a large decrease in stage 6), and IAAsp levels remained relatively stable (with a slight decrease at stage 2). Cytokinin profiles showed the predominant compound to be IPA but most strikingly showed high levels of ZR and IPA in stage 1. In the gibberellin profiles, GA₃ was again the predominant compound but appreciable levels in the other GAs were also detected.

The results from short-day grown *ers1* plants (Figure 18) were also comparable to short-day grown wildtype and long-day grown *ers1*. Like short-day wildtype, there was a sharp decrease in overall abscisic acid levels after stage 2. Like long-day *ers1*, there were strikingly high levels of ABA and DPA

Figure 17: Hormone and metabolite profiles during the six seed development stages of wild-type *Arabidopsis* grown under short-day conditions.

Hormones quantified were abscisic acids **(A)**, auxins **(B)**, cytokinins **(C)**, and gibberellins **(D)**. Monitored abscisic acid compounds were: abscisic acid (ABA), phaseic acid (PA), dihydrophaseic acid (DPA), 7'-hydroxy-abscisic acid (7'-OH-ABA), and abscisic acid glucose ester (ABA-GE). Auxin compounds monitored were: indole-3-acetic acid (IAA) and indole-3-acetyl-aspartate (IAA_{sp}). Monitored cytokinin compounds were: zeatin (Z), zeatin riboside (ZR), isopentenyladenine (2iP), and isopentenyladenosine (IPA). Gibberellins (GA) monitored were: GA₁, GA₃, GA₄, and GA₇. Results are on a dry-weight (DW) basis.

Figure 18: Hormone and metabolite profiles during the six seed development stages of *ers1* mutant grown under short-day conditions.

Hormones quantified were abscisic acids (**A**), auxins (**B**), cytokinins (**C**), and gibberellins (**D**). Monitored abscisic acid compounds were: abscisic acid (ABA), phaseic acid (PA), dihydrophaseic acid (DPA), 7'-hydroxy-abscisic acid (7'-OH-ABA), and abscisic acid glucose ester (ABA-GE). Auxin compounds monitored were: indole-3-acetic acid (IAA) and indole-3-acetyl-aspartate (IAA_{sp}). Monitored cytokinin compounds were: zeatin (Z), zeatin riboside (ZR), isopentenyladenine (2iP), and isopentenyladenosine (IPA). Gibberellins (GA) monitored were: GA₁, GA₃, GA₄, and GA₇. Results are on a dry-weight (DW) basis.

seen in both of the first two stages. Unlike long-day *ers1*, the levels of auxins were much decreased. Unlike either short-day wildtype or long-day *ers1*, the predominant compounds in cytokinin profiles were ZR and 2iP. Like long-day grown *ers1* to long-day grown wildtype, the overall gibberellin level of short-day grown *ers1* was lower than short-day grown wildtype.

4 DISCUSSION

4.1 Phenotypic Differences Supporting Non-Redundancy in Ethylene-Insensitive Mutants

When the 5 ethylene receptors were identified in *Arabidopsis*, it was assumed that they had redundant functions since gain-of-function mutations in each caused dominant insensitivity to ethylene. Analysis of some classical ethylene responses, confirmed that each receptor was involved in the triple response, since gain-of-function mutations in any one receptor rendered the seedling insensitive to ethylene (Chang *et al.*, 1993; Hua *et al.*, 1995; Sakai *et al.*, 1998; Hua *et al.*, 1998). Thus, at least at the level of the triple response, all receptors appeared to be involved, and therefore had redundant functions during this process. Previous research had shown that throughout the course of plant development, the ethylene receptors appeared to have more distinct roles (Kozela, 2003). Leaf shape and colour were distinctively different amongst the gain-of-function mutants, with three mutants resembling wildtype with dark green curled leaves that accumulate anthocyanins in older plants, and two mutants having light green flat leaves that turn yellow in older plants. These preliminary results were the first indication that the receptors could have unique as well as redundant functions, and this thesis has tested whether some of these unique functions could be related to seed development.

4.1.1 *Various phenotypic traits are different between ethylene-insensitive mutants*

Redundancy in protein function would generally presume that there would be no differences in the phenotype of plants mutated in the same protein domain. The mutants *ers1*, *ers2-1*, *ein4-1*, *etr1-1*, and *etr2-1* contain gain-of-function mutations in the ethylene-binding domain of ethylene receptors, causing these mutants to be ethylene-insensitive. Although presumed to be redundant, signalling through each ethylene receptor produces different mutant phenotypes – an observation that dispels the presumption of complete functional redundancy. Additional phenotypes that varied in these mutants included stress response, leaf senescence, “triple response” degree, and flowering time.

4.1.2 *Phenotypic traits can be associated to the role of ethylene*

For a few of the qualitative phenotypic traits used to assess each ethylene receptor mutant the appearance of the phenotype could be related to one specific role of ethylene known in plants. Ethylene is generally considered a stress hormone and has been shown to be involved in responses to wounding, flooding, chilling, disease, and light/temperature/drought stress (Abeles *et al.*, 1992). Since anthocyanins are produced in response to stressful plant growth conditions (Abeles *et al.*, 1992), the absence of anthocyanin in the older leaves of some mutants (*etr1-1* and *ers1*) suggests that these receptors are involved in anthocyanin accumulation in the leaf.

The triple response exhibited by plants grown in the presence of ethylene is believed to mimic the accumulation of ethylene by seedlings when trying to emerge from physically restraining or obstructed soil. The restriction causes

stress ethylene production (Abeles *et al.*, 1992), and 'triple-responsive' short and sturdy seedlings result from shoot/root thickening to surpass the mechanical impedance during growth. Since the ethylene-insensitive mutants showed variations in their response to ethylene during triple response testing (Figure 4), this suggests that these receptors are involved, to varying degrees, in how responsive they are to stressful conditions that can occur during soil emergence.

Ethylene has also been shown to be involved in altering flowering time (Abeles *et al.*, 1992). In altering the timing of the irreversible transition from vegetative to reproductive growth, ethylene signalling ensures that plants do not commit to making seeds when stressful conditions are present. Thus similarly to anthocyanin production and triple responsiveness, variations in flowering time of ethylene-insensitive mutants (Table 1) also suggests that these receptors, specifically ETR1 and EIN4, are involved in influencing the transition to reproductive growth. This preliminary search of phenotypic differences between ethylene-insensitive mutants indicates that all of the functions of these receptors are not redundant.

4.1.3 *There are phenotypic differences during silique and seed development*

There were slight differences in the appearance of seeds and siliques between *ers1*, *etr1-1*, and wild-type plants. Generally, *etr1-1* and wild-type plants produced siliques of similar length and colour, while *ers1* plants yielded shorter siliques with a lighter tan colour in the late silique stages. Since the number of

seeds contained within the silique dictates the silique length, the shorter silique of *ers1* had a lower seed yield than the wild-type plants. Another small difference in seed phenotype was found in the *etr1-1* mutants, in which the stage 2 seeds were more translucent than the seeds of the same stage in the wild-type plants. Thus, developmental differences between some ethylene-insensitive mutants also do not show redundancy.

4.1.4 *Previously performed protein sequence alignment analysis of ethylene receptors supports non-redundancy*

Further evidence supporting non-redundant functional roles of ethylene receptors has been generated by alignment analysis of ethylene receptor protein sequences (Kozela, 2003). Previous studies by Hua *et al.* (1998) showed that the sequences of the five ethylene receptor were distinct with ETR1 and ERS1 forming separate groups, and the remaining receptors (ERS2, EIN4, and ETR2) falling into a separate group. Extensive analyses with all available ethylene receptor sequences from plants found the same distinct groups, ETR1-like, ERS1-like and third group that was less distinct, containing all ETR2-, ERS2- and EIN4-like sequences. Within the ERS1 and the ETR1 groups, the sequences were highly similar, indicating a slow evolution of these sequences. Conversely the ERS2/EIN4/ETR2 group contained more divergent sequences suggesting a faster evolution in this group. Because ERS1 and ETR1 were identified to be evolving more slowly and separately from the relatively fast evolving ERS2, EIN4, and ETR2 sequences, it might be predicted that the function of the ERS1 and ETR1 proteins are more essential for plant development, otherwise their

sequences would have been able to evolve more freely. Although the precise function of the ERS1 and ETR1 ethylene receptors cannot be solely elucidated from the use of protein alignment analyses, the observation that they consistently group into three clusters, suggests at least three different functions and therefore non-redundant roles.

4.2 Days After Pollination Illustrating Photoperiod Differences

The timing of each seed development stage (measured by DAP) revealed a distinct difference between long-day and short-day grown plants (Figure 6). For almost all stages, the time between each stage was generally twice as long in short-day conditions compared to long-day conditions. The only difference was the length of stage 2, which was substantially shorter in short-day grown plants. Interestingly, this discrepancy in the length of stage 2 compensated for variations in other stages such that the overall time from stage 1 to stage 6 was almost equal in short-day and long-day grown plants (14 days and 13 days, respectively). The different lengths of the stages, does not appear to pose a specific benefit since both long day and short day grown plants produce mature seeds with 100% germinability after stratification in wild-type (Figure 7), *ers1* (Figure 8), and *ers2-1* (Figure 10) plants.

4.2.1 *The influence of maternal plants on progeny development*

The idea that maternal tissues can influence the development of seed is not new. It is also expected that the maternal plant is influenced by environment, and this in turn can influence how and when the seed develops. The ability to

sense and respond to environmental cues (such as light, water, temperature) is integral to a plant's survival and hence ability to propagate. Daylength is one of the environmental signals sensed by plants to cue internal biological and physiological processes affecting seed fitness. Thus, it is expected that daylength could affect seed development and this effect is seen in the shift in maturation time in seed stages between long-day and short-day grown plants. In the next section, these results will be considered in relation to the seasonal variation of seed germination.

4.2.2 *The typical annual life cycle of Arabidopsis*

Arabidopsis is an example of the winter annual plant, germinating in the autumn, over-wintering in the vegetative state (rosette), flowering in the longer days of spring, and setting seed and dying in late spring/early summer. In wild-type *Arabidopsis*, however, the transition from vegetative to flowering state is not exclusive to long-day photoperiods (as seen by short-day flowering in Table 1 and implied by short-day fruit production in Figure 7-B). Therefore, *Arabidopsis* is a facultative long-day plant (prefers to flower in long-day but capable of flowering in short-day conditions). *Arabidopsis* can also be considered a facultative winter annual: preferring the life cycle sequence as described for winter annuals, but capable of germination in the spring, flowering soon afterwards, setting seed, and dying in the late spring/summer (the life cycle described for summer annuals). Thus, *Arabidopsis* is able to adapt its life cycle to suit environmental conditions.

4.2.3 *The interrelation of photoperiod and season*

During the changing of the seasons, plants are exposed to different photoperiods. Long-day conditions are essentially found in the spring and summer, while short-day conditions would be essentially found in autumn and winter. To insure the survival of the seed, it would be reasonable to expect that the plant would adjust seed development (perhaps by changing the types of stored reserves) to suit the growth conditions. In this manner, the maternal photoperiod can be expected to have an effect on the germinability of the seed.

Studies done by Munir *et al.* (2001) found that maternal plant photoperiod influenced the dormancy of seed offspring. In terms of stratification requirements, the majority of seeds developed under long-day conditions did not need stratification, while the majority of seeds developed under short-day conditions did require stratification. Thus, daylength affects the development of the seed such that it either does (short-day) or does not (long day) require stratification. Previously studies utilized afterripened seeds (Munir *et al.*, 2001), which, in comparison to this study, would be seeds well past the pre-dispersal seed development stages (stage 6) presented here. Nonetheless, our results on the germination responsiveness of seeds from the two photoperiods generally resembled their findings. The germinability of non-stratified long-day wild-type seeds (Figure 7-A) generally remained the same through seed development through the stages (with a moderate increase in the last stage) and the increased responsiveness of non-stratified seeds over stratified is best exemplified in the middle stages 3 and 4. The increased responsiveness of stratified seeds over

non-stratified under short-day conditions was best exemplified by short-day grown *ers1* plants (Figure 8-B). A possible explanation for why short-day wild-type seeds did not show a preference towards stratification treatment will be discussed in Section 4.3.5.

4.2.4 *The relationship between stratification and cold acclimation*

In the above section, it was demonstrated that varying maternal photoperiod appears to influence the need for stratification of seeds, and in this section, these results will be discussed in terms of its role in germination of seeds in different seasons. To quickly review and generalize, seed dormancy is necessary to delay germination until favourable growth conditions occur and stratification is a means of artificially breaking dormancy. Cold stratification requires a damp and chilled environment for a consecutive number of days (precise number being plant specific), conditions that typically occur in a winter season. For plants with a high mortality rate during the winter, germination in autumn or winter would be impractical and germination in the spring would be favoured. Thus, seeds with an enforced need for cold stratification would bypass the less favourable winter season and ensure germination in the spring (as described by winter annuals). For plants capable of surviving the summer, autumn germination (as described by summer annuals) would enable early spring flowering or flowering at a larger plant size, both favourable traits in seed production (Baskin and Baskin, 2001).

The ability to endure stratification treatment requires that the seed has also developed some cold tolerance. Cold tolerance is an example of a seed characteristic obtained by the cold acclimation process where plants, when exposed to certain environmental conditions, acquire the ability to withstand cool temperatures. Low temperatures and a short photoperiod have been shown to independently trigger cold acclimation in temperate zone woody plants (Welling *et al.*, 2002). The effects of cold acclimation induction are found in physiological and biochemical processes in plants and assuming these changes are expressed in the developmental stages of seeds, different durations in seed stages can be a result of plants environmentally sensing and responding to photoperiod (and hence season) in order to gain cold tolerance when necessary. Seeds that have been produced and dispersed under maternal long-day (spring) conditions would germinate in autumn and thus the development of cold tolerance would be unnecessary, as the summer is not cold. Conversely, seeds produced under short-day (fall) conditions would germinate in the spring and would need to have acquired cold tolerance to survive the winter. Thus, based on photoperiod, the seed can be programmed to either acquire cold tolerance and stratification requirement to germinate in the spring or not develop cold tolerance and not require stratification to germinate in the fall.

4.2.5 *The possibility of a semi-annual cycles in Arabidopsis*

The annual life cycle (winter vs. summer annuals) previously presented and ascribed to *Arabidopsis* does not completely coincide with why maternal

photoperiod could be used to adjust stratification requirements (and hence seed fitness) to affect subsequent germinabilities. The term 'annual' implies that *Arabidopsis* is capable of a single cycle of growth per year. But the rapid generation time of *Arabidopsis*, a characteristic that makes it attractive as a model system, allows an entire plant life cycle (the time taken for one seed to germinate, grow, reproduce, and disperse progeny) to take about 2-3 months in long-day grown plants and about 3-4 months in short-day grown plants. Factoring in time for stratification, a summer quiescence, and seed dispersal, it is therefore possible for *Arabidopsis* to have a semi-annual life cycle. There are two possible semi-annual life cycles that could occur depending on whether dormant seed or vegetative plants over-winter.

In the first semi-annual life cycle seen in Figure 19, seeds that had been set out in late autumn/early winter have been cold stratifying over winter and germinate in late winter/early spring. The growth and maturation of these stratified seeds in spring yield long-day plants that flower, set seeds and die by late spring/early summer. These seeds, having been maternally developed under long-day conditions, do not require cold stratification and did not acquire cold tolerance as germination in the following season would not need stratification. Quiescence assures no germination during the heat and dryness of summer. Germination occurs in late summer/early autumn when summer temperatures have lowered enough to allow favourable germination and growth conditions for the plants. The non-stratified seeds then flower and produce seed

Figure 19: A semi-annual life cycle of *Arabidopsis* with seeds over-wintering in a dormant state.

Seeds dispersed late in autumn or early in winter over-winter as dormant seeds and germinate after natural stratification in late winter/early spring. Plants grow and flower in the spring (long-day conditions). The seeds produced (without stratification requirement) from these plants remain quiescent over the (hot and dry) summer and germinate without stratification in milder conditions (ex. late summer). The plants grown and flowered in autumn (short-day conditions) produce seeds with stratification requirement, which again over-winter with dormancy.

under short-day conditions. The shortened daylength triggers the maternal plants to induce cold tolerance in the seed. Then, before the onset of winter frost, the short-day maternal plants die after setting seed that have acquired cold tolerance to favour germination after the cold stratifying conditions of winter.

In the second semi-annual life cycle seen in Figure 20, plants that had germinated in autumn remain in a vegetative state over the winter season. The lengthening daylight of spring triggers flowering. The maternal photoperiod is now considered long-day and the seed progeny produced and dispersed in late spring/early summer does not require stratification (no cold tolerance). Following brief summer quiescence, non-stratified seeds germinate in mid-summer when conditions are milder. By the time the transition from vegetative to flowering states occurs, the daylength is reduced to short-day conditions. The progeny from the maternal short-day photoperiod will develop cold tolerance. The cold tolerance allows the quick stratification of seeds in late autumn to yield plants over-wintering in the rosette state, which continues the life cycle.

These alternate semi-annual life cycles illustrate the possible adaptive flexibility of the growth of these plants that could allow it to respond to different environmental conditions. Germination-ready mature seeds could be released earlier or retained on the maternal plant for longer in response to local conditions occurring in the environment at the time. This flexibility in dispersal time could result in a subsequent shift in germination season (over-wintering seeds to over-

Figure 20: A semi-annual life cycle of *Arabidopsis* with plants over-wintering in a vegetative state.

Seeds that have germinated in autumn over-winter as a full grown vegetative plants and flower in the spring (long-day conditions). The seeds formed (without stratification requirement) from these plants remain quiescent in the (hot and dry) summer and germinate without stratification in milder conditions (ex. mid-summer). The plants grown and flowered in autumn (short-day conditions) produce seed with stratification requirement.

wintering vegetative plants, or vice versa) without significant disruption in *Arabidopsis* life cycling.

4.3 Germination Potential and its Demonstration of Seed Dormancy

Primary dormancy is typically defined as the dormancy seen in seeds after their desiccation and dispersal from the maternal plant. The majority of studies concentrated on this post-dispersal dormancy but it is also known that dormancy is a state that is induced while the seed is still developing. In the following sections, the maturation phases of seed development will be defined, and in particular the establishment of seed dormancy will be discussed.

4.3.1 *Wild-type germinability can be used to demonstrate dormancy in seeds*

The initial germination pattern through the stages of wild-type plants grown under long-day conditions (Figure 7-A) is essentially identical to stages defined by others (Raghavan, 2002). Under short day conditions, the stages become very well defined as: A) pre-viable; B) precocious germination; C) dormancy; and D) dormancy-breakable (Figure 21, linear representation of Figure 7-B). In the pre-viable phase, seeds have not undergone sufficient development to survive separation from maternal tissues (stage 1). In the precocious germination phase, seeds are fully developed and when artificially removed, are able to germinate (stage 2). In nature, however, it would be very unusual for the seeds to be removed at this stage. In the dormancy phase, seeds are suddenly unable to germinate even in the presence of favourable growth conditions and it is during this stage that seeds are truly dormant (stages

Figure 21: A linear representation of the germinability patterns of short-day grown wild-type plants as seen in Figure 7-B with seed development phases.

Four distinct seed developmental phases were inferred from the pattern of germination: pre-viable (stage 1), precocious germination (stage 2), dormancy-initiation (stages 3 and 4), and dormancy-breakable (stages 5 and 6). The light line represents the germination of non-stratified seeds; the dark line represents the germination of stratified seeds.

3 and 4). In the dormancy-breakable phase, seeds are capable of leaving the dormant state (stages 5 and 6). After stage 6 would be the completion of desiccation, which results in silique valves shattering to disperse germination-capable mature seed.

4.3.2 *Pre-viable seed phase*

In order to delineate all the stages of seed development, it was essential to find the end of embryo development. The stage 1 seed therefore represents the final stages of embryo development, where the seed is still incapable of surviving without the maternal tissues and is just one day (under long days) or two days (under short days) away from being able to survive on their own. After this stage, the seeds are not fully mature, but can generally survive if extracted from the silique. Germinating these seeds under appropriate conditions can identify the various phases of seed maturation.

4.3.3 *Precocious germination phase*

Immediately after the completion of embryo development, the seed is capable of germinating. This early germination ability is known as precocious germination and is similar to vivipary – the germination of immature seeds within the fruit (ex. silique) while still attached to maternal tissue (via the funiculus). Vivipary is the natural propagational method of mangroves and can sporadically occur in cultivated plants like corn in over wet conditions, and can be induced in cultured siliques in *Arabidopsis* (Raghavan, 2002). While both precocious germination and vivipary result in the germination of seeds that are not fully

mature, precocious germination is restricted to seeds that are removed from the maternal plant and maternal fruit coat. Meanwhile, vivipary is limited to seeds still attached to the maternal plant, or removed from the maternal plant but still attached to the maternal fruit coat, as was the case with cultured siliques in Raghavan (2002). Overall, the capacity for precocious germination to occur indicated that at this developmental point (stage 2), the seeds could germinate because enclosed embryos had developed as much as necessary to be viable, indicating that seed maturation is not necessarily needed for viable embryo formation. However, the percentage of germinable seed at stage 2 was not as high as fully mature seeds at stage 6, indicating that seed maturation increases seed germinability.

4.3.4 *Dormancy phase*

The lack of germination in stages 3 and 4 of short-day wild-type plants was indicative of a dormant phase where germination did not occur. According to Simpson (1990) seed dormancy is defined as “the temporary failure of a viable seed to germinate after a specified length of time in a particular set of environmental conditions that later evoke germination when the restrictive state has been terminated by either natural or artificial means”. The seeds isolated from the previous stage (stage 2) had already established that the seed was viable and looking ahead at the later stages (stages 5-6) the seeds eventually gain the ability to germinate, therefore, the stages 3-4 fit the criteria of dormancy. Specifically, the seeds of stage 3 appeared to have entered dormancy, those of

stage 4 maintained this dormant state, and those of subsequent stages could terminate the dormant state naturally (by afterripening) or artificially (by stratification). Dormancy occurring after embryo viability establishment (seed stage 2 in this study) is further supported by studies showing embryo growth is signalled to temporarily arrest during seed maturation processes, resulting in dormant seed that only resumes embryonic development after post-germinative growth (Raz *et al.*, 2001).

Although the primary dormancy found in dispersed seeds customarily implies metabolic quiescence, the dormancy initiated during seed development described here does not necessarily involve a drop in metabolic activity. During this dormancy various physiological and biochemical processes are occurring in response to endogenous and exogenous cues usually related to increasing seed health. To clarify understanding of events that occur during seed maturation, Figure 22 summarizes a broad interpretation of the seed development sequence as adapted from previously described profiles (Harada, 1997; Raz *et al.*, 2001) with the integration of the developmental stages and phases described in this study. Also incorporated are two examples of metabolic processes: cold acclimation and desiccation tolerance, whose positioning has been inferred from the observation of tolerant qualities during the seed development sequence.

Seed development can be divided into two major phases: embryo development and seed maturation. During embryo development (or early embryogenesis), the embryo's body plan is first established during

Figure 22: Conceptual representation of seed development.

The developmental stages and phases established in this thesis (Figure 21) was integrated into previously described representations of development (Harada, 1997; Raz *et al.*, 2001). Acquisitions of cold tolerance and desiccation tolerance are featured here as examples of metabolic processes that occur during seed development. This is a broad interpretation of seed development where dashed diagonal lines represent an approximation of the start/end of each event. Initial broken bars of the solid arrows represent an approximation of when tolerance is acquired. This was implied by the observation of plants showing cold and/or desiccation tolerance in subsequent stages (represented by solid arrows).

morphogenesis before embryo growth occurs until the embryo fills the seed sac. From the precocious germination seen in this study, the embryos were viable after embryo growth. Embryo growth from cell division is temporarily arrested (Raz *et al.*, 2001) during embryo maturation (or late embryogenesis). This embryo maturation represents the commencement of the seed maturation process – during which time cold acclimation and desiccation tolerance are believed to occur. The placement of cold and desiccation tolerance within the seed development sequence was arbitrarily determined from the germinability assay of *ers1* mutant plants that showed increased germination with stratification (Figure 8) and from previously reported germination percentages of dried ovules (Raghavan, 2002), respectively. Other metabolic processes that occur early during seed maturation include nutrient reserve accumulation. Based on the germination results of *ers1* and *ctr1-1*, it is clear that some of these metabolic processes can still occur even when the seed does not enter dormancy, since seeds in these plants still acquire cold tolerance but do not undergo dormancy. Thus, cold tolerance and seed dormancy appear to coincide in time, but may not be dependent on each other.

4.3.5 *Seeds in dormancy-breakable phase are ready for germination*

The final stage of seed maturation is a time when the seed has completed metabolic processes and can be released from dormancy. At this time, the dormancy of these seeds can be broken by stratification and typically at this stage the percentage of seeds that germinate with stratification is much higher

than without. In fact, the majority of the stratified results from these late seed development stages showed at or near 100% germination ability (exception of *etr1-1*). Following dormancy, late seed maturation continues until the vascular connection of the maternal plant is severed. As the seeds become progressively drier, their ability to break out of dormancy increases, first via stratification and later as the seeds are dispersed, via afterripening.

In relating back to the previous section concerning maternal photoperiod and seed progeny stratification requirement (section 4.2.4), the generation of seeds preferring non-stratification occurred during maternal long-days and was easily seen in the dormant stages of long-day wild-type plants (Figure 7-A). Short-day wild-type plants however, did not produce stratification-requiring seeds. This can be explained by the assumption that the display of dormancy was capable of masking the cold tolerance acquired from acclimation. In other words, short-day grown wild-type plants have a deeper dormancy than long-day grown plants, and did not demonstrate the expected cold acclimation trademark of short-day grown plants during seed dormancy. However, because cold tolerance is seen after dormancy is broken, cold acclimation has occurred.

In summary, immature seeds will not germinate before fully developing a viable embryo. Precociously germinating seeds have a fully developed embryo, but have not undergone either cold or desiccation tolerance. Coinciding with dormancy, the seed develops cold and desiccation tolerance in preparation for dispersal from the plant. The final products are fully viable seeds, matured

enough to tolerate and survive various environmental conditions after dispersal from the maternal plant, and which are therefore better equipped to germinate under a wider range of growth conditions.

4.3.6 *ers1* and *etr1-1* show contrasting dormancy phenotypes

Comparison of the germination potential of the ethylene insensitive mutants to the wild type reveals that two mutants (*ers1* and *etr1-1*) have dramatically different germination potential profiles. Compared to wild-type, the germination potentials of *ers1* revealed an ability to germinate at all stages after precocious germination. These results suggest that the seeds of the *ers1* mutant fail to undergo dormancy and therefore are able to germinate at all stages of development after precocious germination. In striking contrast, the germination potentials of *etr1-1* mutant plants revealed a drop in the germination potential of all seeds after precocious germination. The seeds from the last stage (stage 6) should have been capable of breaking dormancy but appeared to remain in dormancy.

The other ethylene receptor mutants displayed less striking germination potential profiles and the significance of those results still needs to be investigated. The remainder of this thesis will focus on the *ers1* and *etr1-1* mutants and the potential involvement of the ERS1 and ETR1 receptors on seed dormancy.

4.3.7 *ers1 and etr1-1 can be used to model dormancy control by ethylene*

Referring back to germination potential testing, the high percentages observed in all the seed maturation-related stages of *ers1* suggests the seeds were never signalled to enter dormancy. Similarly, the low to null percentages seen in all the post-precocious stages of *etr1-1* suggests the seeds were not or were weakly being signalled to exit dormancy. Therefore, in the context of the roles of ethylene receptors, ERS1 appears to be involved in seed entry into dormancy while ETR1 appears to affect seed exit out of dormancy, as schematically shown in Figure 23. This is the first clear demonstration that the ethylene receptors have distinct functions. In relating this finding back to the results of the sequence comparisons in section 4.1.4, Kozela (2003) had proposed that ETR1 and ERS1 likely played distinct roles in plant development. It was also predicted that these two receptors must play very essential roles in the plant since their sequences have evolved very slowly compared to the other receptor sequences. It is therefore possible that the distinct roles ERS1 and ETR1 play in controlling seed dormancy are the critical function that has restricted their evolution. Considering the wide range of developmental processes that occur in a plant, it is not hard to imagine that seed development is one of the most critical processes. And taking this further, the ability of the plant to ensure that the seed will enter and exit dormancy correctly is also an important task that apparently is partially controlled by ethylene and these two receptors.

Figure 23: Schematic representation illustrating the possible role of ethylene in entering and exiting seed dormancy.

(A): *Role of ERS1 and ETR1 in controlling seed dormancy.*

The ERS1 and ETR1 ethylene receptors signal the seed to enter dormancy and to exit dormancy, respectively, leaving the seed ready for germination.

(B): *Germinability during seed development of wild-type, ethylene-insensitive mutants, and the constitutive triple response mutant.*

Because *ers1* mutant plants showed a high degree of germinability at the dormant phase, the ERS1 ethylene receptor appears to signal dormancy entry. Because *etr1-1* mutant plants showed a greatly lowered degree of germinability than in wild-type plants, the ETR1 ethylene receptor appears to signal dormancy exit.

Because the germinability of *ctr1-1* mutant plants closely resembled that of *ers1-1*, the constitutive negative regulation of CTR1 appears to be affected at the first stage where ethylene is needed to signal dormancy.

(C): *A model for different ethylene concentrations in controlling dormancy.*

Wild-type plants normally accumulate and sense ethylene levels appropriate to dormancy entry and exit as controlled by ERS1 and ETR1, respectively. *ers1* plants do not sense enough ethylene at altered ERS1 receptors and thus bypass dormancy. *etr1-1* plants sense appropriate ethylene levels to enter dormancy, but the altered ETR1 receptors do not sense appropriate levels to allow the exit out of dormancy. *ctr1-1* plants show a constitutive ethylene response typical of continual exposure of high levels of ethylene, thus the simulation of ethylene detection above dormancy-initiating levels, results in dormancy being bypassed as well.

4.3.8 *ctr1-1* mutant supports ethylene signalling in the dormancy pathway

The final germination potential assay yet to be discussed is that for *ctr1-1* mutant plants. The germinability patterns of *ctr1-1* appeared identical to *ers1* plants indicating that these seeds also failed to enter dormancy. Unlike the gain-of-function ethylene receptor mutants, however, the *ctr1-1* mutant continually signals an ethylene triple response. As previously stated, epistatic studies have placed CTR1 downstream of the ethylene receptors in the ethylene signal transduction pathway, and current evidence points to the receptors signalling through CTR1. Based on the germination results of the ethylene receptor mutants described above, the first stage in seed development where a requirement for ethylene can be detected is the entry to seed dormancy. It is therefore not unexpected that this same stage would show a phenotype in the *ctr1-1* mutant. Since the loss-of-function *ctr1-1* mutant continually signals the ethylene response, this might suggest that too much ethylene (signalling) could prevent the seed from entering dormancy. This, combined with the *ers1* data, points to the possibility that a specific concentration of ethylene is required to enter dormancy in wild-type plants.

In wildtype, the gradual accumulation of ethylene during seed development would signal (via ERS1) dormancy entry at one concentration and signal (via ETR1) dormancy exit at another concentration. By mutating the ERS1 receptor, the seed does not detect that it has acquired enough ethylene to enter dormancy, while by mutating the CTR1 protein, the seed senses that it has too

much ethylene. This skewed ethylene detection is schematically represented in Figure 23, where the level to enter dormancy has been arbitrarily chosen to be low. The *ers1* mutant avoids dormancy because the detected level of ethylene is too low, and the *ctr1-1* mutant avoids dormancy because the “detected” level of ethylene is too high. At the other end of dormancy, the level of ethylene needed to signal the seed to exit dormancy has arbitrarily been chosen to be high. In this case, the *etr1-1* mutant is unable to exit dormancy because the seed senses that the seed has not yet acquired enough ethylene. While the levels of ethylene needed to enter and exit could just as easily be high for entering and low for exiting, the model still points to receptors sensing different levels of ethylene. The idea that too much ethylene could prevent a seed from entering dormancy has also been seen in wild-type seeds that had been germinability tested in the presence of high concentrations of ACC, the precursor for ethylene (data not shown). These results suggest that the 5 ethylene receptors affect specific developmental pathways by detecting different levels of ethylene. Although at this point, it is not known how much ethylene is detected by each receptor, in the next section, we will see how altered signalling from the ERS1 and ETR1 receptors can affect the levels of other plant hormones.

4.4 Hormone and Metabolite Profiling and its Demonstration of Cross-talk

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. There are approximately five

laboratories in the world that can carry out mass spectrometry profiling of plant hormones, and one of the most advanced is Dr. Suzanne Abrams group at the NRC's Plant Biotechnology Institute (PBI) in Saskatoon. In collaboration with Dr. Abrams, the levels of abscisic acids, gibberellins, cytokinins and auxins were followed during seed development in wild-type seeds and in the two ethylene mutants with altered seed dormancy, *etr1-1* and *ers1*.

Although simultaneous profiling of hormones and metabolites is preferable over using traditional physiological and genetic studies to establish more complex hormone relations, this method is not without its limitations. This method of hormone profiling is a closed system, such that only those compounds, for which deuterium-labelled analogs have been generated, can be detected. The fifteen hormones and metabolites analyzed in this study are not the only compounds present in seeds that are capable of influencing seed development. Also, this method of profiling hormone and metabolites only shows accumulation at a given developmental time-point. This 'snapshot' does not account for how dynamic physiological and metabolic processes are.

Despite these limitations, this high throughput approach of simultaneously profiling plant hormones and metabolites has been successfully used to identify potential cross-talk between ethylene and other hormones during seed dormancy. Moreover, the signalling networks identified in this study between interacting hormone response pathways reveal new areas of research on seed development.

In this part of the discussion, I will first briefly review some interesting trends extracted from the hormone and metabolite profiles with some suggestions to what they may infer, before outlining other considerations in interpreting the metabolite data. Finally, I will briefly examine how understanding of hormonal cross-talk is becoming more complex.

4.4.1 *Interesting trends extracted from the hormone and metabolite profiles of wild-type and ethylene insensitive plants*

The overall observation that ethylene-insensitive mutants demonstrate variations in hormone and metabolite levels during seed development different from that of wildtype establishes that ethylene is involved in a hormonal cross-talk during seed dormancy. Had cross-talk not been involved in dormancy, the control of ethylene on dormancy entry and exit would not have shown effects in the levels of other hormones and would have been a pure ethylene effect. Thus, this research has successfully shown that seed dormancy is not controlled by a single hormone but is due to a more complex network of hormonal cross-talk. And more specifically, since the profiles are different for the two receptor mutants, this further supports the major theory of this thesis, that the ethylene receptors have distinct functions in plant development. Furthermore, the variation in hormones amongst the different stages supports the idea that dormant seeds are not metabolically inactive. Having established the existence of cross-talk between hormones during seed dormancy, there remains the task of sorting through the large amount of data generated by profiling and properly interpreting what information lies within.

Because the hormone and metabolite profiles were intended to be preliminary collection of data, it is too early for specific conclusions to be drawn from these results. Instead, a more appropriate approach to discussing the hormone and metabolite data would be highlighting some trends seen from long-day analyses that could be of some interest during subsequent data interpretation. To facilitate the comparison of hormone and metabolite profiles between wild-type and mutant plants, graphs based on selected compounds were generated (Figure 24). This summary of the profiling data enabled interesting trends to be more readily identified. As previously mentioned, abscisic acids and gibberellins are the two hormones that have been specifically implicated in seed dormancy and germination, respectively. Previously, ethylene has been indicated to be involved in seed dormancy through its signal interaction with the response pathway of abscisic acid (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000). In this thesis, a distinct role for ethylene in seed dormancy has been established, and using hormone profiling, potential interactions of ethylene with other hormones was identified.

Focusing first at the stages when the seed is just about to enter dormancy (stage 2), as expected ABA levels are generally high just before dormancy initiation (stage 3) and decrease gradually once the seed enters dormancy. This agrees with what is known about abscisic acids in inducing seed dormancy. Absciscic acid levels are initially high early in seed development to induce seeds into dormancy, and because abscisic acid levels no longer need to be high for

Figure 24: Summary of selected hormone and metabolite profiles during the six seed development stages of wild-type and mutant *Arabidopsis* grown under long-day conditions.

The profiles comparing wildtype (—●—) and dominant ethylene receptor mutants *ers1* (.....▽.....) and *etr1-1* (—■—) were individually compiled for selected compounds from levels seen in Figures 14-16. Hormones quantified were abscisic acids (**A**), auxins (**B**), cytokinins (**C**), and gibberellins (**D**). Selected abscisic acid compounds were abscisic acid (ABA) and dihydrophaseic acid (DPA). Selected auxin compound was indole-3-acetic acid (IAA). Selected cytokinin compounds were zeatin riboside (ZR) and isopentenyladenosine (IPA). Gibberellins (GA) selected were GA₃, GA₄, and GA₇.

the dormant state to be maintained, a gradual decrease after dormancy initiation occurs (Karssen *et al.*, 1983). In the *etr1-1* mutant, the seeds enter dormancy normally, and it is therefore expected that abscisic acid levels would decrease in this mutant as it does in the wildtype. In the *ers1* mutant that is blocked at the entrance to dormancy, the abscisic acid levels remain high in stage 2. This suggests that the lack of a signal through the ERS1 receptor has an effect on the accumulation of abscisic acids and could indicate that a molecule downstream of the ethylene signal from ERS1 is specifically regulating abscisic acid. The fact that the *etr1-1* mutant has normal abscisic acid levels at this stage indicates that this change in abscisic acid levels is not due to a general ethylene effect but is specific to the pathway downstream of ERS1.

To be partially responsible for the breaking of seed dormancy, at some point during seed development, gibberellins would be expected to rise. The profiles of gibberellins were not as clear-cut as those for abscisic acids, however. Considering total quantified levels of GA, the profiles of wild-type and *ers1* mutant were similar, but were much higher in the *etr1-1* mutant. The *etr1-1* mutant seeds enter a deeper or more prolonged dormancy and do not readily break dormancy at stages 5 and 6 as is found in the wildtype. Since it is not known what level of gibberellins is needed to break dormancy, these results could be interpreted in a couple of ways. First, the higher overall GA levels in the *etr1-1* mutant could reflect the seed's continued attempt to terminate dormancy by producing more gibberellins when lower levels are unsuccessful.

Alternatively, ETR1 could have a more direct effect on the level of gibberellins in the seed. If a specific amount of gibberellin were assumed to be needed to terminate dormancy, then these results could indicate that signalling from the ETR1 receptor is important in down-regulating or decreasing GA levels. When signalling from ETR1 is removed in the *etr1-1* mutant, the GA levels could rise due to the lack of control from this ethylene signal transduction pathway.

The examination follows upon the similarities/variations seen in overall hormone profiles of auxins and cytokinins. Auxins and cytokinins have not previously been implicated in seed dormancy or germination, but there were significant differences in the levels of these hormones between wildtype, *ers1*, and *etr1-1*. Specifically there were very high levels of bioactive IAA in wild type and *ers1* mutant and much lower levels in the *etr1-1* mutant. In cytokinin profiles, the most notable variation was the predominance of ZR in wildtype and *etr1-1* and of IPA in *ers1*. Both ZR and IPA are low bioactive conjugates of more bioactive Z and 2iP, respectively. The possibility of both auxins and cytokinins also being involved in seed dormancy is interesting, but will require further investigation to understand their potential roles and how they interact with abscisic acids, gibberellins, and ethylene.

Similarly, the results from short-day grown *ers1* and wildtype were both supportive and slightly contradictory to those from long-day. However, because of *etr1-1* data could not be generated under short-day conditions, a cursory examination can only be applied since the picture is incomplete. The increase in

abscisic acids and decrease in gibberellins in *ers1* all relative to wildtype, correspond strongly to the long-day profile results. However, the results seen from auxins and cytokinins are complicated. Because the data is so confounding, adequate interpretation cannot be done at this moment and further investigation is necessary.

Overall, old theories (ABA-GA balance) and predicted theories (ethylene) of seed dormancy and germination were supported by this study. But at the same time, new possibilities (auxins and cytokinins) emerged from the simultaneous profiling of all these hormones and their metabolites.

4.4.2 *Considerations during the interpretation of hormone and metabolite data*

During the course of our collaboration at PBI, several prominent comments were mentioned about the difficulty in interpreting information from the profiles of hormones and metabolites. The first was the relevance of hormone and metabolite trends when some of compounds analyzed ranged in stability (ex. 7'OH-ABA is highly unstable intermediate). Thus, this relates back to an earlier comment on how the 'snapshot' approach loses much of the dynamic metabolic momentum. The second was the incorporation of bioactivity into the profiles. While some of the compounds analyzed are highly bioactive, others are conjugates of these active compounds, and can be equally bioactive, partially so, or not have any bioactivity at all. For example, highly active ABA can be inactivated by conjugation to form inactive ABA-GE (potential storage form) or by oxidation to an unstable intermediate that subsequently converts to partially

active PA and inactive DPA (possible transport forms) (Taiz and Zeiger, 1998). Similarly, levels of biologically active auxin IAA can be regulated by conjugation to IAAsp (Tam *et al.*, 2000), as well as cytokinins Z and 2iP to their respective conjugates, ZR and IPA (McGaw and Burch, 1999). It is also possible that some compounds do not exhibit their effects immediately. In other words, a compound critical to an event like dormancy initiation may not demonstrate significant accumulation at initiation, but accumulation prior to initiation may have been decisive.

4.4.3 *The arising complexity behind understanding hormonal cross-talk*

Although the precise mechanism behind how hormone cross-talk could regulate plant developmental processes like seed dormancy has yet to be fully established, the question of why such a complicating network would be necessitated in plant processes remains. In order for a plant to properly develop, there must be a way to co-ordinate a wide variety of physiological processes to ensure the overall development is correct. Not much is known on how appropriate physiological responses result from such transduction of developmental signals, but plant hormones are often found to play important roles.

Further clouding the influence of hormone cross-talk are the recent findings that the 5 hormone classes used here are not necessarily the sole response regulators relaying information for plant growth and development. Other compounds have been implicated in cross-talk and/or seed

dormancy/germination. For example, brassinosteroids have been shown to play a role in promoting germination in *Arabidopsis* (Steber and McCourt, 2001) and cross-talk between sugar and ethylene signal transduction has been revealed (Zhou *et al.*, 1998; Leon and Sheen, 2003). Glucose as a signalling molecule has even been implicated as a new branch of ethylene signalling uncoupled from the signalling of the triple response (Zhou *et al.*, 1998).

The regulation of seed dormancy and germination by plant growth regulators (hormones, bioactive compounds, etc.) is undoubtedly complex. And as more research is conducted on how dormancy is influenced by the large web of molecular cross-talk, the complexity will undoubtedly also increase. Regardless of how multifaceted these networks of signalling become, some major regulatory pathways, such as abscisic acids, gibberellins and ethylene signalling in seed dormancy, will likely still emerge as major players in the complex network of molecular cross-talk. Future research in this area will undoubtedly need to include equally complex technologies to understand how plants develop.

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