GENETIC AND MOLECULAR INTERACTORS OF ABA INSENSITIVE1 GENE (abi1) OF ARABIDOPSIS THALIANA

by

Majid Ghassemian

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Botany
University of Toronto

© Copyright by Majid Ghassemian 2000
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-49826-3
Genetic and molecular Interactors of ABA insensitive gene 

(abil) of Arabidopsis thaliana

Doctor of Philosophy, 2000

Majid Ghassemian

Department of Botany, University of Toronto

ABSTRACT

The plant hormone abscisic acid (ABA) is important in numerous functions ranging from the establishment of seed dormancy and germination to protecting adult plants from a variety of environmental stresses. Previously in Arabidopsis, mutants with reduced sensitivity to applied ABA (abi) have been described. One ABA insensitive mutation, designated abil, identifies a gene that encodes a type 2C protein phosphatase suggesting the phosphorylation status of the plant is important for correct ABA action. To further understand the role of AB11 in ABA signal transduction and to identify new genes involved in ABA action, an abil mutant background was used to screen for mutations that further reduce ABA responsiveness in Arabidopsis. This abil enhancer (abe) screen resulted in the isolation of mutations in 6 genetic loci. Three complementation groups define the previously
identified ABA response loci ABI3, ABI4 and ABI5. Mutations at two loci identify new genes designated ABE1 and ABE2 and the last complementation group was found to be allelic to the CTR1 gene, a known negative regulator of ethylene signaling in Arabidopsis. This last result suggests that ABA and ethylene may have overlapping interactions that define the sensitivity of the plant seeds to these growth factors. Interestingly, in a parallel study it was discovered that a number of known mutants that reduce ethylene sensitivity also show altered ABA responsiveness. Since mutations that confer ethylene insensitivity cause seed ABA hypersensitivity and mutations that confer constitutive ethylene response cause increased seed ABA insensitivity, I proposed that ethylene signaling functions in the negative regulation of seed ABA responses. Furthermore, studies involving the interaction between ABA and ethylene in the root suggest ABA may signal directly through ethylene signal transduction pathway in an ethylene independent manner.
This thesis work is dedicated to my wife Negar for her endless love and support.
Acknowledgements

I would like to thank Peter McCourt for his support and encouragement through out my degree. I would also like to thank members of the Department of botany, which for the past eight years have contributed greatly to my education. I am grateful to Peter McCourt, Dario Bonetta, Robin Cameron, Nancy Dengler, and John Coleman for their critical review of this thesis and Thomas Berleth for stimulating discussions on various topics in the thesis. Dr. Eiji Nambara deserves unlimited credit for his contributions to chapter four of this thesis. last but not the least, many complements to my friends, Dario, Judith, Eiji, Fernando, Sara, Ashkan, Duka, Sean, Sarah, Gil, Andrea, Greg, Nocha, Sandy, Tamar, etc. for their support and friendship.

This work was partly funded by an NSERC PGSB grant.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>ABI</td>
<td>ABA insensitive locus</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cM</td>
<td>centi Morgans</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyl methyl ammonium bromide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethlenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>ERA1</td>
<td>enhanced response to ABA locus</td>
</tr>
<tr>
<td>F1</td>
<td>first generation offspring</td>
</tr>
<tr>
<td>F2</td>
<td>second generation offspring</td>
</tr>
<tr>
<td>FW</td>
<td>fresh weight</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>Ler</td>
<td>Landsberg erecta ecotype</td>
</tr>
<tr>
<td>LSM</td>
<td>least squares mean</td>
</tr>
<tr>
<td>Mcol</td>
<td>Meyerowitz Columbia ecotype</td>
</tr>
<tr>
<td>min(s)</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulphonic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
</tbody>
</table>
PCR  polymerase chain reaction
PP2C  protein phosphatase 2C
RAB/LEA  responsive to ABA/late embryogenesis abundant
RFLP  restriction fragment length polymorphism
RNA  ribonucleic acid
RT  room temperature
SDS  sodium dodecyl sulfate
T1  Transformants generation 1
T2  Transformants generation 2
T3  Transformants generation 3
TAE  tris-acetate/EDTA electrophoresis buffer
TBE  tris/borate/EDTA electrophoresis buffer
TE  Tris HCl/EDTA
UV  ultraviolet
vol(s)  volume(s)
wt  wild-type
w/v  weight per volume
v/v  volume/volume
µE  micro Einsteins
TABLE OF CONTENTS

ABSTRACT ........................................................................................................ ii

CHAPTER 1

ABA SIGNALING IN PLANTS .............................................................................. 1

Connecting environmental signals to plant growth and development .................. 2

A) REGULATION OF ABA ACCUMULATION ......................................................... 4

i) ABA biosynthesis .......................................................................................... 4

ii) ABA catabolism ............................................................................................. 7

iii) ABA release and transport .......................................................................... 8

B) ABA SIGNALING .............................................................................................. 9

i) Physiology and molecular biology of Arabidopsis seed ABA responses ............ 9

ii) Genetics and molecular biology of ABA signaling in Arabidopsis seeds .......... 11

C) GUARD CELL ABA SIGNALING ...................................................................... 19

i) Guard cell ABA responses ............................................................................. 19

ii) Molecular-Physiology of Guard cell specific ABA signaling ....................... 21

iii) Genetics of Guard cell ABA signaling ......................................................... 22

D) ABA SIGNALING IN ROOTS .......................................................................... 24

E) ABA SIGNALING IN ABA CELLULAR ACCLIMATION RESPONSES .......... 26
i) ABA induced genes and ABA cis elements .................. 26

ii) Genetic and molecular biological analysis of ABA signaling in vegetative tissue ............................................. 28

iii) The role of PP2Cs in signaling pathways .................... 28

iv) Molecular analysis of ABI1 and ABI2 .......................... 30

v) Roles for abil and abi2 in ABA signaling ..................... 32

F) OTHER GROWTH FACTOR SENSITIVITY MUTANTS WITH DEFECTS IN ABA RESPONSES 35

G) THE CHARACTERIZATION OF ABA SIGNAL TRANSDUCTION PATHWAY(S) REQUIRES MORE ABA RESPONSE MUTANTS................................................................. 36

CHAPTER 2

MATERIALS AND METHODS ............................................................. 38

Materials ................................................................. 39

Growth conditions .................................................. 39

RAB18-promoter-GUS transgenic lines ......................... 40

Preparation of transgenic lines expressing the Anti-sense ABI1 constructs ..................................................... 42

GUS staining ........................................................... 42

Screen for enhancers of abil ............................................. 43

Seed Fatty Acid Composition ...................................... 44

Water Loss Assay ..................................................... 44

Root growth sensitivity assays .................................... 45
CHAPTER 3

ISOLATION AND CHARACTERIZATION OF ENHANCERS OF ABI1 (ABE) MUTATION

INTRODUCTION

RESULTS

Seed and root ABA responses of transgenic plants carrying the ABII anti-sense construct

Isolation of enhancers of abil

Genetics of ena mutants in abil background

Seed ABA sensitivities of ena lines in the absence of the abil mutation

abi3, abi4 and abi5 the 3 classes of predicted enhancers of abil are recovered in abil enhancer screen

ena mutants identify ctrl and 2 new genetic loci

The enhancement of abil seed ABA insensitivity by ctrl is also influenced by the maternal tissue

abe mutants do not enhance guard cell and root growth insensitivity of abil seedlings
abe mutants have altered responses from abil in the up-regulation of certain ABA induced gene markers. ............ 74
ABA induced repression of β-tubulin and chlorophyll binding protein is shows defects in abe2 mutant .................. 76
Cross growth factor sensitivities in abe mutants ............ 76

DISCUSSION......................................................................................................................................................... 81

CHAPTER 4
INTERACTIONS OF ABA AND ETHYLENE SIGNAL TRANSDUCTION PATHWAYS IN ARABIDOPSIS SEED GERMINATION AND ROOT GROWTH................................................................. 88

INTRODUCTION.......................................................................................................................................................... 89

RESULTS................................................................................................................................................................. 95
Genetic analysis of era3 mutants .............................. 95
era3 vegetative tissues have increased ABA levels ........ 96
era3 is normal for ABA, RAB18 induction and guard cell responses, but shows ABA insensitivity at root growth ...... 104
Inhibition of root growth by ABA requires functional ethylene signal transduction ........................................ 106
Ethylene is a negative regulator of ABA action in Arabidopsis seeds ................................................................. 107

DISCUSSION........................................................................................................................................................... 112
Interactions between ethylene and ABA seed responses ...... 113
Interactions between ethylene and ABA vegetative responses . 115

GENERAL CONCLUSIONS AND FUTURE PROSPECTS ........................................... 120

APPENDIX 1

CHARACTERIZATION OF TRANSGENIC ARABIDOPSIS LINES CARRYING A CONSTRUCT OF
RAB18 PROMOTER FUSED TO THE GUS CODING REGION ..................................... 124

Embryonic and young seedling RAB18 expression .............. 126

The ABA induction of RAB18 ........................................ 126

APPENDIX 2

ISOLATION OF PROTEIN INTERACTORS OF MUTANT ABI1 ISOFORM IN A .................. 133

YEAST TWO-HYBRID SYSTEM ........................................................................ 133

BIBLIOGRAPHY .......................................................................................... 142
LIST OF TABLES

Table 2.1  PCR specifications for RAB18 amplification  48
Table 2.2  DNA probes  48
Table 2.3  EREBP3 and C-20 CAPS marker  49
Table 3.1  Genetic segregation of ABA sensitivity in ena mutants  60
Table 3.2  Genetic segregation of ABA sensitivity in ena mutants in AB11 background  61
Table 3.3  Genetic complementation analysis for ena lines  68
Table 3.4  A summary for ena lines  69
Table 4.1  Genetic segregation of ABA sensitivity in era3-1 mutant  97
TableAPN2.1 List of Abil interactors  138
LIST OF FIGURES

Fig. 1.1  Schematic showing ABA biosynthesis in plants  5
Fig. 1.2  Schematic diagram of Arabidopsis seed development  12
Fig. 1.3  Schematic diagram representing ABA genetic pathways in seeds  20
Fig. 1.4  Schematic diagram representing ABA action in guard cells  25
Fig. 1.5  Schematic diagram representing genetic pathways leading to ABA-regulated gene expression in the vegetative tissue  34
Fig. 3.1  ABA induction of RAB18 in ABI1 antisense  58
Fig. 3.2  ena seed ABA sensitivity at germination  59
Fig. 3.3  Eicosenoic acid (20:1) content of ena seeds  62
Fig. 3.4  AtEm6 and RAB18 transcript analysis of ena seeds  70
Fig. 3.5  Chromosomal position of ABE1 and ABE2  72
Fig. 3.6  Kinetics of water loss and root ABA sensitivity in abe mutants  78
Fig. 3.7  Transcriptional analysis of ABA inducible and β-TUB genes in abe mutants  79
Fig. 3.8  Transcriptional analysis of CAB in abe mutants  80
Fig. 3.9  An integrated model representing genetic pathways leading to ABA-regulated gene expression in the vegetative tissue  87
ABA seed sensitivity of era3-1 98
ABA induced proline accumulation in era3-1 99
Transcript analysis of ABA1 in era3-1 100
ABA-inducible RAB18 expression in era3-1 101
era3-1 root growth responses to ABA, ACC and kinetin 102
era3-1 and etr1-1 root growth responses to ABA 109
Wild-type root growth responses to AVG and AgNO3 110
Wild-type seed germination on ACC 111
Models for ABA and ethylene signal transduction cross-talk 119
A general model for ABA pathways in Arabidopsis 123
RAB18 expression in seed and young seedlings 128
ABA-inducible RAB18 expression in Wild-type seedlings 129
ABA-inducible RAB18 expression in abil 130
ABA-inducible RAB18 expression in era1-2 131
RAB18 induction by ABA application or dehydration treatment 132
Transcript analysis of INA1 139
Abil/ABI1 and INA1 protein interactions yeast 140
Protein phosphatase assay of INA1 141
CHAPTER 1

ABA SIGNALING IN PLANTS
Connecting environmental signals to plant growth and development

The pattern of growth and development in any organism is determined by two primary sets of interacting factors: the genetic potential or genotype of the organism and the environment in which it is growing. At the level of growth, it is easy to see how the rates of various genetically encoded metabolic processes are generally determined by limiting environmental factors. However, in many cases the role of the environment in the development of an organism is not fully appreciated. The influence of the environment on development is perhaps more easily observed in plants since much of plant development is post-embryonic. For example, physical factors such as light and temperature can have profound affects on whether a seed will germinate or when a plant will flower. Because of the ease of linking a specific developmental response to an environmental cue, for the past century plant biologists have attempted to identify compounds which could act as mediators of environmental inputs on developmental outputs. This avenue of research led to the identification of a small group of molecules termed plant growth factors or phytohormones. Unlike animal hormones, however, phytohormones are not produced by well-defined endocrine glands and they differ in both their chemical nature and metabolic roles. Furthermore, although plant hormones can be
transported to other parts of the plant this does not necessarily exclude their effects on the cell where they were synthesized. These differences have suggested that plant growth factors may play different roles than those suggested for animal growth factors.

Of the known plant growth factors, abscisic acid (ABA) appears to be the most responsive to environmental changes. For example, in an adult plant the endogenous concentrations of ABA can rise and fall 10- to 50-fold within hours of exposure to drought stress and rewatering conditions (Zeevaart and Creelman, 1988). This dramatic change in ABA concentrations has led to a large body of work on the relationship between ABA, a plant's water use efficiency, and its growth and development. In this regard, ABA biosynthetic mutants have implicated ABA in osmotic responses ranging from drought stress to the establishment and maintenance of seed dormancy and desiccation tolerance. ABA functions have also been attributed to inhibition of shoot and root meristem growth and promotion of root elongation (Davis and Zhang, 1991).

A substantial amount of information on the cellular responses that occur upon ABA induction has accumulated; however, little is known about the nature of the cellular components of ABA signaling. Genetic approaches which have been critical in the isolation and characterization of a number of growth factor signaling components in plants (McCourt, 1999) have had limited success in
characterizing the ABA signaling pathway(s). However, these genetic studies have suggested that ABA responses can be regulated at multiple levels, from ABA accumulation to its perception and signal transduction.

A) Regulation of ABA accumulation

ABA responses appear to be concentration dependent. For example, an allelic series of ABA auxotrophic mutations in Arabidopsis has demonstrated that seed dormancy correlates directly with the amount of ABA synthesized in the embryo (Karssen et al., 1983). However, a clear understanding of the mechanisms of ABA accumulation has been elusive. Obviously, biosynthesis, catabolism and transport all contribute to the overall ABA levels in a tissue, but the regulation and coordination of these processes is not known. Furthermore, both osmotic stress signals in vegetative tissues and developmental signals in seeds result in ABA accumulation. Thus there appear to be at least two mechanisms for ABA accumulation in plants (Zeevaart and Creelman, 1988).

i) ABA biosynthesis

ABA is a sesquiterpene that is derived from mevalonic acid. Some phytopathogenic fungi synthesize ABA by a direct pathway from farnesyl pyrophosphate, whereas ABA biosynthesis in higher plants
Figure 1.1 Schematic pathway of ABA biosynthesis and ABA catabolism in higher plants. The metabolic block in various ABA-deficient mutants is indicated.
Mevalonic acid → Farnesyl pyrophosphate → Zeaxanthin → all-trans-Violaxanthin → 9'-cis-Neoxanthin → Xanthoxin → ABA aldehyde → ABA → 8'-HydroxyABA

EPOXIDATION: At abe1, Np abe2
ISOMERIZATION: Zm vp14, Le metabilis
OXIDATION: At abe3, Np abe1, Le fleccs, sitiens
HYDROXYLATION
REDUCTION
ISOMERIZATION
proceeds primarily via an indirect pathway (Zeevaart and Creelman, 1988). Studies of the biochemical basis of genetic lesions in several mutants have confirmed the occurrence of an indirect pathway in plants. The viviparous vp2, vp5, vp7 and vp9 mutants are blocked in early stages of carotenoid biosynthesis (Taylor, 1991). The abnormal complement of carotenoids appears to result in both photo-bleaching and ABA deficiency in these mutants, suggesting an ABA precursor role for carotenoids. In higher plants, ABA is synthesized from oxygenated carotenoids such as zeaxanthin, violaxanthin and neoxanthin (Figure 1.1) (Li and Walton, 1990). The aba1 mutants of Arabidopsis thaliana and the aba2 mutants of Nicotiana plumbaginifolia are impaired in the epoxidation of zeaxanthin into antheraxanthin and violaxanthin (Duckham et al., 1991; Rock and Zeevaart, 1991). The oxidation cleavage of carotenoids, neoxanthin and violaxanthin, has been characterized by the vp14 mutants of maize and the notabilis mutant in tomato, and is considered to be the first committed step in ABA biosynthesis. (Burbidge et al., 1999; Schwartz et al., 1997; Tan et al., 1997). The next ABA biosynthetic step is the conversion of xanthoxin to ABA-aldehyde. aba2 mutants of Arabidopsis are the only known mutants with lesions that disrupt this step (Leon-Kloosterziel et al., 1996; Nambara et al., 1998; Schwartz et al., 1997). The final step in ABA biosynthesis is the oxidation of ABA-aldehyde to ABA,
which is characterized by several mutants isolated from tomato (flacca and sitiens), potato (droopy), barley (nar2a), tobacco (abal) and Arabidopsis (aba3) (Merlot and Giraudat, 1997).

The analysis of ABA biosynthetic mutants has provided some information into the regulation of ABA accumulation. The aba auxotrophic mutants of Arabidopsis are defective in both osmotic-stress and developmental ABA accumulation. This suggests that both regulatory modes of ABA accumulation influence the same ABA biosynthetic pathway. Isolation and characterization of zeaxanthin epoxidase genes from tobacco (ABA2) and Arabidopsis (ABAI) and the 9-cis-carotenoid dioxygenase (VP14) gene from maize, has provided some insight as to the transcriptional regulation of this pathway (Marin et al., 1996; Schwartz et al., 1997; Tan et al., 1997). An increase in transcript abundance of these genes has been observed in seeds and osmotically stressed vegetative tissues, which suggests, that developmental and osmotic stress signals may act through transcriptional regulation of ABA biosynthetic genes for ABA accumulation (Audran et al., 1998; Tan et al., 1997).

ii) ABA catabolism

The level of ABA in a tissue is determined not only by its rate of biosynthesis, but also by the rate of its catabolism. The major pathway for ABA degradation is hydroxylation of ABA to phaseic acid.
(PA) and diphaseic acid (DPA). In addition to ABA catabolism, ABA can be conjugated with glucose in the forms of ABA-glucoside, PA-glucose ester and DPA-glucoside for storage in vacuoles (Zeevaart and Creelman, 1988). Enzymes involved in ABA catabolism have yet to be identified; however the information available from ABA feeding experiments suggest that most plant tissues act as sinks for ABA. The rate of ABA hydroxylation increases vigorously as the tissue ABA content increases. This increase in ABA catabolism has been linked to increased activity of an ABA induced ABA-hydroxylase (Uknes and Ho, 1984).

iii) ABA release and transport

Tissue specific ABA accumulation may be the result of ABA transport from other organs. For example, ABA mediated stomatal closure during water stress is believed to be the result of ABA synthesized in the roots (Davis and Zhang, 1991). These suggest under drought conditions, ABA may act as a long distance messenger between roots and aerial parts of the plant (Davis and Zhang, 1991). Since plant cells have a great capacity for ABA uptake and catabolism, a mechanism to safeguard ABA for its arrival to its site of action is also necessary (Davis and Zhang, 1991). Plants appear to achieve this task through a slight increase in their apoplastic pH. When roots of flacca mutants of tomato were
dehydrated, an increase in xylem sap pH was observed. Due to the lack of ABA biosynthetic ability in this mutant, no reduction in transpiration was observed. However, application of ABA to the xylem sap, resulted in reduced transpiration, but failed to effectively change transpiration in non-stressed control plants. The rise in apoplastic pH maintains ABA in the non-diffusible or deprotonated state which appears to contribute to long range ABA transport by reducing the ABA uptake and catabolism by the cells in the vicinity of the transport route. This allows the stress released ABA to remain in the xylem sap and by moving through the transpiration stream induce stomatal closure (Wilkinson et al., 1998; Wilkinson and Davies, 1997).

B) ABA signaling

i) Physiology and molecular biology of Arabidopsis seed ABA responses

Higher plants have incorporated a number of physiological processes during seed formation as a fitness strategy to ensure their survival in the next generation. Some of these adaptations include the accumulation of nutrient reserves, the acquisition of desiccation tolerance and, most importantly, the establishment of dormancy. Dormancy is usually induced late in embryogenesis and ensures that the seed will survive long periods of time in a
desiccated state until environmental conditions are favorable to resume growth.

In Arabidopsis seeds two peaks of ABA accumulation are observed (Koornneef et al., 1985). Genetic analysis indicates the first peak, which occurs in early embryogenesis, is of maternal origin and is important in seed coat mucilage production. Although the role of seed mucilage in Arabidopsis is not known, this pectinaceous slime is thought to be important in retention of water to aid germination in a number of plant species. The second ABA peak is of embryonic origin and appears to be involved in the induction of seed dormancy (Koornneef et al., 1985). This ABA peak occurs at the end of embryonic nutrient accumulation period and removal of embryos just prior to this stage results in the loss of dormancy and desiccation tolerance. Moreover, exogenous ABA application to these prematurely excised embryos can inhibit germination and stimulate the accumulation of certain late abundant reserve proteins (LEA) (Quatrano, 1995). It is generally thought that LEA proteins are required to protect the embryo during the desiccating conditions imposed on the seed during late embryo development. These findings have been used to support a function for ABA in the establishment of seed dormancy and the induction of seed programs leading to desiccation tolerance.
However, genetic analysis of severe ABA auxotrophic mutations suggests ABA is required for dormancy establishment, but is not required for seed desiccation and maturation programs. In addition, molecular studies indicate there is no direct evidence that induction of desiccation genes in the embryo requires ABA (Hughes and Galau, 1991). A detailed transcript analysis of an Arabidopsis desiccation-induced gene, RD22, for example, has demonstrated that while the vegetative expression of this gene is ABA-dependent, its embryonic expression is independent of ABA (Yamaguchi-Shinozaki and Shinozaki, 1993). This suggests that other developmental regulators, independent of ABA, may play a role in the induction of this and other LEA genes in Arabidopsis seeds.

ii) Genetics and molecular biology of ABA signaling in Arabidopsis seeds

Seed screens for ABA response mutants have yielded a number of mutants with altered seed ABA sensitivities. The seed ABA insensitive (abi) screens, which have targeted lesions in components of ABA signaling pathway(s), have recovered abi mutants that represent five genetic loci in Arabidopsis (Finkelstein, 1993; Finkelstein, 1994; Koornneef et al., 1984). Based on their characterized ABA related defects, these can be divided into two groups. The first group is the semi-dominant abi1 and abi2 mutants.
Figure 1.2  A schematic diagram of Arabidopsis seed development. Each bar represents the period in seed development in which a specific developmental process takes place. ABA-related mutant loci which affect these developmental processes are noted above the bars.
Arabidopsis Embryo Development

- **Storage accumulation**: abi3
- **ABA peaks**: aba1 aba2 aba3
- **Dormancy**: aba1,2,3 aba1,2,3 era1
- **LEA protein accumulation**: abi3 abi4 abi5
- **Chlorophyll degradation**: abi3
- **Desiccation**: abi3

Days Post Anthesis (DPA)
These two response mutants mimic aba auxotrophic phenotypes with respect to dormancy establishment and water relations; however, as expected, application of ABA can not rescue their ABA related phenotypes. ABI1 and ABI2 share extensive sequence identity and belong to the protein serine/threonine phosphatase type 2C (PP2C) gene family (Leung et al., 1994; Leung et al., 1997; Meyer et al., 1994). Furthermore, abi1 and abi2 mutants share the same mutation that converts a glycine residue in their catalytic domain into an aspartic acid. Epistatic analysis using abi1/abi2 double mutants suggest these mutants play roles in similar ABA signaling pathway(s) in Arabidopsis seeds. However, due to the semi-dominant nature of these mutations and lack of loss-of-function alleles, it is difficult to assign genetic relationships for ABI1 and ABI2 in seed ABA signaling.

The second group of ABA insensitive mutations defines the ABI3, ABI4 and ABI5 genes. These recessive mutations only appear to reduce seed ABA sensitivity and do not affect vegetative ABA responses. The most extensively characterized of these seed specific mutants, abi3, also shares the loss of seed dormancy phenotype observed in aba biosynthetic mutants and abi1 and abi2 response mutants. Severe alleles of abi3 fail to complete a number of seed maturation programs such as defects in seed storage protein accumulation, chlorophyll degradation, late embryogenesis protein
accumulation and desiccation tolerance (Figure 1.2). Analysis of
abi1/abi3 and abi2/abi3 double mutant seeds show reduced ABA
sensitivity when compared to their parental types, suggesting ABI3
defines a second independent ABA signaling pathway from ABI1 and
However, since a weak abi3 allele was used in this analysis and
abi1 and abi2 are semi-dominant mutations, these relationships
should be viewed with caution.

The ABI3 gene was found to be homologous to the maize seed-
specific ABA-response gene, VP1 (Giraudat et al., 1992). A number
of findings suggest that these proteins function as transcription
factors involved in gene expression. Their protein sequence
analysis has revealed that several regions of ABI3 and VP1 amino
acid sequences share similarities to transcriptional activation
domains. In fact, the VP1 protein possesses in vitro
transcriptional activation and DNA binding properties (McCarty,
1995; Suzuki et al., 1997). Furthermore, ectopic expression of ABI3
in vegetative tissues of Arabidopsis plants trans-activates various
promoters in response to ABA (Parcy and Giraudat, 1997).

A summary of abi3 mutant phenotypic analysis and ABI3 molecular
functions leads to a model in which ABI3 functions downstream in
ABA signaling, acting as a transcriptional regulator of seed ABA
specific programs. However, the lack of characterized defects in
embryo maturation programs of *Arabidopsis aba* auxotrophic mutants has challenged this model and has resulted in additional models to accommodate ABI3's role in ABA signaling (Bonetta and McCourt, 1998). As a transcription factor, ABI3 may be involved in the induction of additional ABA independent pathways leading to desiccation tolerance in seeds. Alternatively, the desiccation tolerance of aba mutants may be the result of leakiness in ABA biosynthesis in these mutants; thus, the residual ABA produced may be sufficient to induce embryo-maturation programs. It is also possible that the role of ABI3 in ABA signal transduction is indirect. Possibly, ABI3 may function to induce competence to respond to ABA, by promoting the establishment of an ABA signaling pathway in the embryo. In this scenario, abi3 mutants would lack the required components for ABA signaling in the seed and hence become ABA insensitive. Support for this model comes from ABI3 ectopic expression studies in the guard cells of abil mutants (Parcy and Giraudat, 1997). Although these cells should be non-responsive to ABA due to the abil lesion, ectopic expression of ABI3 reverted this phenotype and re-established ABA responsiveness. The kinetics of guard cell responses to ABA suggests gene expression does not play a role in ABA guard cell responses; therefore, the ABI3 transcription factor should not alter guard cell response to ABA. Possibly, the ectopic expression of ABI3 up-
regulates the expression of existing ABA signaling components, which in turn overcome the semi-dominant effects of the ab1 mutation. Alternatively, ectopic expression of ABI3 expands the domain of embryo-specific gene expression to the leaf, which suggests this gene is instructive in determining the developmental fate of the cell rather than being directly involved in ABA signaling. ABI3 appears to be selecting the cells which will become sensitive to the hormone, rather than being directly involved in signaling. Whichever is the case, resolution of these models will require identification of downstream ABI3 targets.

The abi4 and abi5 mutations confer the weakest ABA insensitive phenotypes with respect to exogenous ABA seed sensitivity (Finkelstein, 1994). These mutants show no detectable reduction in seed dormancy and appear normal for most characterized embryo maturation programs. The only characterized embryo maturation defect in abi4 and abi5 that is also shared with abi3 mutants is the accumulation of LEA AtEm6 mRNA transcripts. Double mutant analysis of abi3/abi4 and abi3/abi5 seeds places abi4 and abi5 genetically in ABI3 ABA response pathway(s) (Finkelstein, 1994). Furthermore, the lack of severe embryonic phenotypes may suggest roles for ABI4 and ABI5 in ABA-dependent pathway(s) downstream of ABI3. This model finds some support in the double mutant analysis of abi2/abi4. This double mutant does not display the additive ABA
insensitivity effects observed in $abi1/abi4$, $abi1/abi5$ and $abi2/abi5$ double mutant seeds. These results suggest a role for $ABI4$ downstream from $abi2$. Since $ABI3$ and $abi2$ have been placed in independent pathways, however, it is difficult to understand how $ABI4$ is shared between these pathways. One explanation is the presence of a third minor ABA signaling pathway in the seeds, where $abi2$, $ABI3$, and $ABI4$ all play a role (Figure 1.3). However, since it has not been determined if $abi4$ and $abi5$ alleles are true null allele mutations, genetic relationships between these mutant alleles should be viewed with caution.

The $ABI4$ gene has been molecularly isolated and characterized in *Arabidopsis*. At the protein sequence level, $ABI4$ has homologies to the DNA binding domain of the AP2 family of transcription regulator proteins (Finkelstein et al., 1998). The AP2 family of genes play important roles in a number of plant signal transduction pathways and based on this homology, $ABI4$ may be a transcriptional regulator involved in ABA signaling in the embryo (Riechmann and Meyerowitz, 1998). However, $ABI4$ may also have roles outside the embryo. $ABI4$ transcripts are not only present in embryonic tissue, but are also found in roots and shoots of mature plants. Phenotypic characterization of $abi4$ mutants suggested no obvious vegetative phenotypes, which may suggest a redundant function for this gene in vegetative tissues. A more careful transcript analysis of ABA
induced genes in these organs could determine ABI4 roles in vegetative ABA signaling.

An alternative genetic approach to distinguish components involved in ABA signaling is the identification of mutants with enhanced responsiveness to ABA (era). Aside from broadening the spectrum of new ABA response mutants, supersensitive screens also identify negative regulators of ABA signaling. Such screens in Arabidopsis have resulted in the isolation of recessive mutants at three genetic loci (Cutler et al., 1996). Among these, the era1 mutants are the most extensively characterized. In addition to seed ABA hypersensitivity, era1 mutants also display increased seed dormancy, but show no other seed phenotypes. The era1/abil double mutant seeds have restored ABA responsiveness and also a degree of dormancy in their seeds compared to wild-type seeds. This suggests that ERA1 may function in a similar ABA signaling pathway to that of abil in Arabidopsis seeds (Cutler et al., 1996). However, since abil is a semi-dominant mutation these interpretations should be viewed cautiously.

The ERA1 gene encodes a β-subunit of protein farnesyl transferase in Arabidopsis (Cutler et al., 1996). These enzymes catalyze the attachment of a hydrophobic farnesyl group (15-carbon) to the C-terminus of a variety of proteins, which facilitate their membrane targeting and/or protein-protein interaction (Schafer and
Rine, 1992). In mammalian cells farnesylation plays a critical role in sub-cellular localization and function of a number of signaling proteins such as the RAS GTPase super family and trimeric G-proteins (Schafer and Rine, 1992). The biochemical basis of the ERA1 gene in combination with the ABA supersensitive phenotype observed in era1 loss-of-function mutants suggests a role for a farnesylated protein in negative regulation of ABA signaling in Arabidopsis seeds.

C) Guard cell ABA signaling

i) Guard cell ABA responses

The closure of stomates in all Arabidopsis ABA auxotrophic mutants is defective, suggesting an important role for ABA in this process. Biophysical and physiological studies have suggested that activation of slow anion channels in guard cells is the rate-limiting step in ABA-induced stomatal closing (Pei et al., 1997; Schmidt et al., 1995; Schroeder and Hagiwara, 1989; Schroeder et al., 1993). These channels are activated within seconds of ABA application, suggesting that their activation is not transcription-dependent (Schroeder and Keller, 1992). Patch clamp studies in Vicia fava guard cells have shown that slow anion channels are strongly activated by elevation of cytosolic Ca" and by
Figure 1.3 A schematic diagram representing genetic pathways that influenced by ABA during Arabidopsis seed development. Arrows indicate the pathways affected by each gene.
A Genetic model for Seed ABA pathways
phosphorylation events (Schmidt et al., 1995; Schroeder and Hagiwara, 1989). When slow anion channels are activated, the resulting sustained efflux of anions from guard cells would result in long-term depolarization, which is a measured guard cell response to ABA (Thiel et al., 1992). Depolarization in turn can activate outward-rectifying K⁺ channel currents, which mediates K⁺ efflux and this effect is also enhanced by ABA application (Armstrong et al., 1995; Lemtiri-Chlieh and MacRobbie, 1994; Schroeder et al., 1987; Schwartz et al., 1994). It is thought that the resulting simultaneous efflux of K⁺ and Cl⁻ lowers the turgor and the volume of guard cells, resulting in closure of stomatal pores (Raschke, 1979).

**ii) Molecular-Physiology of Guard cell-specific ABA signaling**

In terms of perception, physiological and biochemical studies suggest the presence of ABA receptor proteins in the plasmalemma of *Valerianella locusta* guard cells (Hartung, 1983). However, *in vivo* ABA cross-linking experiments have also localized cytosolic ABA binding proteins in addition to proteins in the plasmalemma of *Vicia faba* guard cell protoplasts (Hornberg and Weiler, 1984). Although a number of years have passed since the release of this report, the identity of these receptors has not been determined. This raises questions about the validity of this approach and since
these assays were carried out in vitro systems, their in vivo relevance remains unclear.

Once ABA is perceived a range of biochemical changes take place in the cytoplasm of the guard cells prior to changes in membrane channel activities and stomatal closure. These include an elevation in cytoplasmic calcium concentrations, phosphorylation and dephosphorylation events. An overwhelming number of reports support a role for ABA stimulated increase in cytoplasmic Ca\(^{2+}\), in stomatal closure (Gilory et al., 1991; Grabov and Blatt, 1998; McAinsh et al., 1990). However, there are also reports of stomatal closure with no changes in cytoplasmic Ca\(^{2+}\) concentrations (Allan et al., 1994). These contradictory findings may suggest roles for calcium-dependent and independent ABA signaling pathways in guard cells. In many eukaryotic systems the elevation in cytosolic Ca\(^{2+}\) levels is often followed by activation of calcium-dependent protein kinases (CDPKs) (Bush, 1995). Biochemical analysis of Vicia faba guard cells have identified the activity of a Ca\(^{2+}\)-dependent protein kinase and two Ca\(^{2+}\)-independent protein kinases upon ABA application in this system (Li and Assmann, 1996; Mori and Muto, 1997).

iii) Genetics of guard cell ABA signaling

A number of the Arabidopsis ABA-response mutants mentioned above also display altered guard cell ABA responses. Electro-
physiological analysis of *Arabidopsis abil* and *abi2* guard cell protoplasts has shown that these mutations inhibit the activities of the slow anion channels in this system (Pei et al., 1997). However, transgenic tobacco plants expressing the *Arabidopsis abil* mutant allele, reduced outward K⁺ channel currents but had no measurable effect on anion currents (Armstrong et al., 1995). This may reflect species differences in guard cell channel regulation, in which case activation of slow anion channels is not the crucial event in stomatal closing in tobacco.

*abil* and *abi2* mutants show some differences in how they affect guard cell ABA signaling. Protein kinase inhibitors were able to revert the ABA insensitive phenotype in *abil*, but not in *abi2* (Pei et al., 1997). This suggests that these mutants act at different steps or different signal transduction pathways in guard cell ABA signaling. The reversion of the *abil* phenotype by a protein kinase inhibitor also suggests a role for protein kinases in ABA signaling downstream from *abil* (Pei et al., 1997). The inhibition of protein kinase activity by ABA signaling appears to be required for the slow anion channel activation which suggests a negative regulatory role for protein kinase(s) in guard cell ABA signaling (Figure 1.4).

The *eral* mutants of *Arabidopsis* show a reduction in water loss when assayed for dehydration tolerance (Pei et al., 1998).
Patch-clamp studies of eral guard cell protoplasts have linked this phenotype to ABA hypersensitivity of slow anion channels. As with the seed eral hypersensitivity phenotype, it appears that a farnesylated protein is acting as a negative regulator of ABA signaling in guard cells. Double mutant analysis of ABA signaling mutants have been useful in determining the order of ABA signaling events in guard cells. Patch-clamp analysis of eral/abil and eral/abi2 double mutants has shown that eral restores the guard cell ABA sensitivity in these mutants. This finding demonstrates the role for farnesylated modulators at or downstream of abil and abi2 in guard cell ABA signaling (Figure 1.4) (Pei et al., 1998).

D) ABA signaling in roots

During extended drought periods a reduction in growth is essential to the plant’s survival. ABA appears to influence this process by inhibiting cell division in shoot apical and root meristems (Davis and Zhang, 1991). The ABA inhibition of cell division at root meristem is carried out in part by transcriptional inhibition of a key cell cycle regulator, CDC2A (Hemerly et al., 1993). Although cell division is decreased in root meristem during mild drought periods, it appears that root growth persists in this tissue. Root growth under mild drought conditions is the
**Figure 1.4** Schematic diagrams for ABA activation of slow anion channels in wild-type *Arabidopsis* guard cells. A) In the absence of an ABA signal, an active protein kinase inhibits the activity of the slow anion channel (S-channel). B) In the presence of an ABA signal a protein phosphatase is activated which in turn inactivates the protein kinase responsible for inhibition of S-channel, hence resulting in activation of the S-channel. C) A model for *abil* dominant-negative role in ABA signaling pathway leading to S-channel activation. In this model *Abil*'s interaction with the activated protein kinase interferes with the function of other protein phosphatases. This would maintain the protein kinase in an active state where it inhibits S-channel activity. The M protein represents an ERA1 prenylated protein acting as a negative regulator of ABA signaling. The M protein function may be for the proper cellular localization of a negative regulator of ABA signaling, in this case a protein kinase. In era1 mutants due to lack of farnesylation the negative regulator may be mislocalized and result in guard cell ABA hypersensitivity.
**A**

No signal

Inactive phosphatase  
Active kinase  
Inactive S-channel

**B**

ABA signal

ABA  
Activated phosphatase  
Inactivated kinase  
Activated S-channel

**C**

ABA signal in *abil*

ABA  
*abil*  
Activated phosphatase  
Active kinase  
Inactive S-channel
result of cell expansion in the elongation zones of roots (Davis and Zhang, 1991). ABA appears to influence this process by stimulating the activity of the cell wall loosening enzymes, xyloglucan-endotransglycosylases (XET), in these zones (Davis and Zhang, 1991). Interestingly, the activity of XETs in the aerial tissues is inhibited by ABA (Davis and Zhang, 1991; Hemerly et al., 1993). The mechanism for this preferential activation of XETs in roots by ABA is unknown. However, an ABA induced root specific kinase (ARSK1) has been isolated from Arabidopsis that has a spatial pattern of RNA expression similar to that observed for XET activities. Such root-specific kinases could potentially act as activation triggers for XETs in the root (Hwang and Goodman, 1995).

E) ABA signaling in ABA cellular acclimation responses

i) ABA induced genes and ABA cis elements

ABA plays a major role in the induction of osmotic stress responses in plants. ABA-induced cellular osmotic stress tolerance includes the accumulation of compatible solutes and a broad range of proteins that function to protect cellular components from dehydration stresses. The accumulation of these proteins can also be induced by exogenous application of ABA. A number of ABA-induced
genes from many plant species have been isolated in this manner (Leung and Giraudat, 1998; Skriver and Mundy, 1990). Most ABA-induced genes also accumulate during seed development, cold stress and drought. This information has been used to support ABA’s roles in these processes. However, a more detailed transcript analysis of these genes under various stress conditions and the utilization of ABA biosynthetic mutants in these analyses have shown that a number of these genes can also be induced by ABA-independent signaling pathways (Shinozaki and Yamaguchi-Shinozaki, 1996; Shinozaki et al., 1998). These pathways include the osmotic stress signaling and cold stress signaling pathways.

The merger of such diverse signaling cascades at the point of gene expression requires the presence of sophisticated promoters. A number of cis-acting promoter elements have been identified that may play a role in ABA specific gene expression. The first promoter type element is the Rab16 LEA gene motif from rice, and the Em1a motif of the Em LEA gene from wheat (Guiltinan et al., 1990; Ono et al., 1996; Skriver et al., 1991). These sequences share a G-box core motif ACGT, designated ABRE, for ABA response element. ABRE-related sequence motifs are present in many other ABA-inducible genes. Multimerized copies of ABREs can confer ABA responsiveness to minimal promoters (Ono et al., 1996, Skriver, 1991). A single copy of ABRE, however, is not sufficient for ABA responsiveness. In
fact, to confer embryonic ABA responsiveness to barley HVA22 and HVA1 genes, one ABRE appears to be substituted for one of the 2 unrelated G-box sequences; CE1 and CE3 (Shen et al., 1996, Shen, 1995). These results indicate that a diverse group of ABRES participates in ABA gene induction.

ii) Genetic and molecular biological analysis of ABA signaling in vegetative tissue

Genetic analysis of vegetative ABA signaling, in contrast to seed ABA signaling, suffers from a short list of vegetative ABA response mutants. In all, two semi-dominant mutations; abi1 and abi2 and mutations in one recessive locus, era1, represent the available mutants. Since era1 mutants do not display defects in their ABA gene induction pathway(s), abi1 and abi2 are the only remaining ABA response mutants with defects in these pathways. Because PP2Cs play roles in many eukaryotic signaling pathways a careful phenotypic analysis of abi1 and abi2 mutants at the molecular and whole plant level can provide insights into plant ABA signaling.

iii) The role of PP2Cs in signaling pathways

PP2Cs constitute a diverse group of regulatory enzymes. They have been implicated in many essential metabolic pathways, stress
signaling and sex determination pathways in eukaryotes (Chin-Sang and Spence, 1996; Hunter, 1995). PP2Cs are single subunit, Mg²⁺ cofactor requiring enzymes with no known chemical inhibitors. Since PP2Cs are resistant to all known protein phosphatase inhibitors, genetic and molecular biological approaches have been the only fruitful approaches in determining their functions in eukaryotes. In *Schizosaccharomyces pombe*, three PP2C types (*PTC1,2,3*) account for almost all PP2C activity in this organism. Although single mutant analysis of these loci failed to determine their role(s) in cellular processes, double and triple PP2C mutant combinations result in defects in osmo-regulation, suggesting a redundant role for these proteins in *Schizosaccharomyces pombe*. Genetic screens for suppressors of Δ*ptc1/Δptc3* mutants have resulted in identification of the *WIS1* locus, which encodes a mitogen-activated protein kinase kinase (MEK) homologue. *WIS1* is closely related to the PBS2 protein of *Saccharomyces cerevisiae*, a MEK central to the mitogen-activated protein kinase (MAP) kinase cascade for high-osmolarity glycerol biosynthesis (HOG) in this organism (Shiozaki and Russell, 1995). In fact, similar PP2Cs have been shown to function in osmo-regulatory pathways of *Saccharomyces cerevisiae* and also in stress activated pathways for mammals (Hanada et al., 1998; Maeda et al., 1993; Maeda et al., 1994). These findings suggest a regulatory role for PP2Cs in stress activated signaling
pathways. Since ABA also functions in the induction of cellular stress pathways in plants it is not difficult to imagine roles for ABI1 and ABI2 in ABA signaling.

iv) Molecular analysis of ABI1 and ABI2

ABI1 and ABI2 share considerable identity at the amino acid level (approximately 86%). The N-terminal domains of these proteins show sequence homologies to the EF hand, Ca$^{2+}$ binding motifs (Nakayama and Kretsinger, 1994). However, the phosphatase activity of ABI1 and ABI2 show no increase in protein phosphatase activity with the addition of Ca$^{2+}$. (Bertauche et al., 1996; Leung et al., 1997). In fact, the removal of the Ca$^{2+}$ binding domain results in an increased catalytic activity of ABI1, suggesting a negative regulatory role for the N-terminus on the phosphatase activity (Sheen, 1998).

The abil and abi2 mutants share the same point mutations and, based on the human PP2C crystal structure analysis, it has been hypothesized that this mutation might disrupt the conformation of metal-coordinating residues, resulting in reduced enzymatic activity (Das et al., 1996). In vitro analyses of abil and abi2 mutant isozymes has supported this hypothesis and revealed a =70% reduction in their protein phosphatase activity (Bertauche et al., 1996; Leung et al., 1997). The reduced protein phosphatase activity
in abi1 and abi2 might lead to cellular hyper-phosphorylation and cause the semi-dominant phenotypes observed in these mutants. However, the transformation of wild-type (ABI1/ABI1) plants with a single mutant copy of abi1 gene results in the mutant phenotypes, demonstrating that the semi-dominant effect of these mutations is not due to cellular hyper-phosphorylation events. The loss of protein phosphatase activity in abi1 and abi2 and their semi-dominant phenotypes suggests that abi1 and abi2 may function as dominant-negative type mutations.

The dominant-negative effects of abi1 and abi2 mutations are not unique to these proteins and have been characterized in other protein phosphatases. Certain mutations in calcineurin and in the corkscrew gene in Drosophila are examples where a reduction in protein phosphatase activity leads to dominant-negative effects (Perkins et al., 1992; Herbst et al., 1996; Shibasaki et al., 1996). The dominant negative effects may be the result of mutant enzymes forming poison complexes with their substrate(s). This complex would keep the target protein in a phosphorylated state by shielding it from other active phosphatases. The semi-dominant ABA insensitivity phenotypes in abi1 and abi2 plants may also be the result of neomorphic effects of these mutations. In this scenario the mutant isoform gains affinity for new cellular targets. Some recent findings dispute this possibility for abi1 and abi2
mutations. Over-expression of wild-type ABII protein in a maize protoplast system resulted in ABA insensitivity in gene induction, which led to the conclusion that ABII functions in ABA signaling as a negative regulator (Sheen, 1998). However, due to the promiscuous nature of protein phosphatases in other systems, any over-expression data for PP2Cs must be viewed very cautiously, since over-expression of PP2Cs may lead to inappropriate cellular localization or increased representation of these enzymes that may lead to gain-of-function effects.

v) Roles for abil and abi2 in ABA signaling

The abil and abi2 mutants share a number of defects in their vegetative ABA responses. These include defects in stomatal regulation, cold acclimation, ABA inhibition of root growth and the induction of specific osmotic stress ABA-regulated markers upon ABA application (Finkelstein and Somerville, 1990; Gilmour and Thomashow, 1991; Koornneef et al., 1984; Lang and Palva, 1992; Leung et al., 1994; Leung et al., 1997). However, not all ABA markers are affected equally in these mutants. The ABA induction of alcohol dehydrogenase (ADH) is defective in abi2 but appears normal in abil plants (Figure 1.5) (de Bruxelles et al., 1996). Conversely, the ABA-induced expression of ATHB-7 is reduced in abil
but is normal in abi2 (Soderman et al., 1996). Although abil and abi2 often overlap in the processes that they affect, these gene markers demonstrate these gene products do not always affect the expression of the same downstream genes (Figure 1.5.)

The ABA induction of a number of ABA-inducible genes is defective in abil and abi2 mutants. However, very little is known about the mechanism by which abil and abi2 cause these effects. Protoplast systems have had some success in the characterization of components of ABA signaling and abil in ABA gene expression. In these systems in addition to ABA, Ca^{2+}, mediators of cytoplasmic Ca^{2+} release, cyclic ADP-ribose (cADPR), and specific calcium-dependant protein kinases can activate the transcription of downstream ABA regulated promoters (Sheen, 1996; Wu et al., 1997). These findings have suggested roles for Ca^{2+} and Ca^{2+} signaling in ABA gene induction. Transfection of these protoplasts with abil inhibits the gene-inductive power of these molecules (Leung and Giraudat, 1998), which suggests that abil plays a negative regulatory role at or downstream from the most downstream component of calcium signaling such as the CDPKs.
**Figure 1.5** A schematic diagram representing genetic pathways leading to ABA-regulated gene expression in vegetative tissue. The model is based on the information obtained from transcript analysis of ABA-inducible genes *RAB18* (Gosti et al., 1995; Lang and Palva, 1993), *RAB21* (Gosti et al., 1995; Lang and Palva, 1993), *ADH* (de Bruxelles et al., 1996), *ATHB-7* (Soderman et al., 1996), and ABA-repressible gene *β-TUB* (Giani et al., 1998), in different mutant background. Mutations that affect the expression of the listed markers are marked on the hypothetical pathways(s).
F) Other growth factor sensitivity mutants with defects in ABA responses

*Arabidopsis* growth factor sensitivity mutant screens for auxin, methyl jasmonate, and brassinosteroids have yielded a number of mutants with multiple growth factor response defects. *axr2* dominant mutations, which were isolated in screens for reduced auxin sensitivity with respect to root growth (Wilson et al., 1990), also display reduced root sensitivity to ABA and ethylene. *jar1* and *jin4* are recessive mutations that confer resistance to methyl jasmonates and also result in seed ABA hypersensitivity (Berger et al., 1996; Staswick et al., 1992). *bri* and *sax* are recessive mutations that result in reduced brassinosteroid sensitivity and biosynthesis, respectively (Clouse et al., 1996; Ephritikhine et al., 1999; Ephritikhine et al., 1999). The roots of these mutants display an ABA hypersensitive growth phenotype. These mutants are especially important, since they may represent defects in the shared components between ABA and other growth factor signaling pathways. A similar function has been shown for the *Arabidopsis* ethylene sensitivity gene, *EIN2*. Mutations in the *EIN2* locus in *Arabidopsis* confer ethylene insensitivity in combination with a loss in jasmonic acid induced defense responses (Alonso et al., 1999). The molecular dissection of the *EIN2* protein has identified separate domains responsible for jasmonic acid responses or ethylene
responses, demonstrating EIN2’s role in linking ethylene and jasmonic acid signaling pathways. The multiple growth factor sensitivity phenotypes in these mutants can also be the result of mutant plants re-adjusting their ABA responses, by changing their ABA sensitivities or biosynthesis to compensate for altered growth factor sensitivities. To distinguish between these possibilities, analyses similar to the one that determined the EIN2 role in growth factor responses need to be carried out for these mutants.

Mutant screens using transgenic *Arabidopsis* plants have also been utilized in ABA mutant hunts. In these screens transgenic plants carrying constructs containing the ABA response promoter RD29, fused to a reporter gene, luciferase, have had success in isolating ABA response mutants in addition to stress signaling mutants (Ishitani et al., 1997). These type of screens can be powerful in targeting ABA signaling components involved in ABA induced gene expression. Since there appear to be multiple signaling pathways for ABA, use of specific promoters to branched ABA responses can further identify signaling components specific to each pathway.

G) The characterization of ABA signal transduction pathway(s) requires more ABA response mutants

The characterization of an ABA signal transduction pathway using genetic analysis has suffered from a shortage of ABA response
mutants. Seed ABA sensitivity screens have been successful in the identification of a number of mutants, however, the genes identified in these screens seem somewhat peripheral to the central core of what is expected for a signal transduction pathway. For example, although a farnesyltransferase and phosphatases have been identified, their ABA related signaling targets remain unidentified. Furthermore, an ABA receptor has not yet been recovered. In this study, I have embarked on a number of approaches to identify new genes affecting ABA responsiveness in Arabidopsis. One approach I took was to further characterize the ABI1 gene in Arabidopsis and to develop a new screen to identify genes that may interact in an ABI1 dependent pathway. In a parallel study, I characterized a new ABA response mutant that interacts with ABI1 through the ethylene response pathway. These results suggest ABI1 does play a role in ABA signaling; also ethylene and ABA interact to regulate growth and development in the plant.
CHAPTER 2

MATERIALS AND METHODS
Materials

All chemicals were reagent grade. Restriction endonucleases were obtained from New England BioLabs and Pharmacia. (α32P) dCTP (3000 CimM⁻¹) was obtained from Amersham.

Growth conditions

The abil mutation was in a Landsberg erecta (Ler) background. Ler was used as the parental strain for experiments in chapter 3 unless noted otherwise. Columbia (Col) was the parental background used in the noted crosses in chapter 3. Meyerowitz Columbia (MCol) was the ecotype for era3 mutants.

Seeds were surface sterilized by immersing in 95% ethanol for 15-20 minutes, removing the ethanol and vacuum drying for 10 minutes to remove the ethanol. Seeds were imbibed on Petri plates containing 0.8% agar supplemented with 1.1g/L MS basal culture salts buffered with 50 mM MES pH5.7 (for ABA assays).

Seedlings from plates or seeds in 0.2% agar were transferred to a standard autoclaved soil medium containing equal parts of vermiculite, perlite and sphagnum and saturated with 1g/L of a 20-20-20 standard nutrient solution in water. Plants were usually grown in continuous light. For abe mutants a long day cycle (16 hour) was implemented. In all cases illumination was at 200μE m⁻²s⁻¹ at 22°C and 50% relative humidity.
The **RAB18-promoter-GUS** plasmid was made by fusion of the binary vector pBI101 (Clontech) with a 600 base pair fragment of the upstream region of the **RAB18** gene. The **RAB18** upstream region was isolated by polymerase chain reaction (PCR) amplification of this region from chromosomal DNA using specifications listed in table 2.1. The product was treated with protease K as previously described (Sambrook et al., 1989). The product was then extracted with phenol/chloroform and precipitated with ethanol. Next, the pellet was digested with restriction enzymes *HindIII* and *BamH1* (Pharmacia) and fractionated by electrophoresis on low melting agarose. The DNA fragments corresponding to the 600 bp region were then isolated by phenol extraction (Sambrook et al., 1989). The binary vector pBI101 was also digested with the same enzymes, *HindIII* and *BamH1*. Upon completion of the reaction the enzymes were heat-inactivated as described by the manufacturer (Pharmacia). The **RAB18** promoter fragment was ligated to the vector using T4 DNA ligase under conditions described by the manufacturer. The reaction was transformed into *Escherichia coli* (JM109), as described in Pharmacia site directed mutagenesis kit manual, and plated on kanamycin selective media. The colonies were then screened for **RAB18-promoter-GUS** constructs, by restriction digests of plasmid
DNA isolated from the transformants. The plasmid DNAs from 2 positive clones were independently transformed by electroporation (BioRad) into Agrobacterium tumefaciens strain GV3101 (provided by Sean Cutler, Stanford University, California).

The transformants were then used in whole plant transformation of wild-type Arabidopsis Columbia ecotype as previously described by Clough and Bent (http://www.cropsci.uiuc.edu/~a-bent/protocol.html). The T1 seeds were screened on MS media, containing 20µg/ml of Kanamycin, for transformants. T2 transformants were tested for kanamycin resistance on 50µg/ml kanamycin concentrations. T3 seeds were tested for kanamycin segregation. Lines whose T3 seeds were resistant to kanamycin were back-crossed to wild-type plants and F2 kanamycin segregation was scored. The plants in which the kanamycin resistance segregated as a single dominant trait were used in experiments in this thesis.

To transfer the RAB18-promoter-GUS constructs to era1-2, era3-1 and abil-1 mutant lines, these mutant lines were crossed to a homozygous RAB18-promoter-GUS line. F2 seeds from these crosses were then screened on 0.4µM ABA for era1 and era3 non-germinators and on 10µM ABA concentration for abil germinators. These were then transferred to 50µg/ml kanamycin plates to select for kanamycin resistant plants. The F3 seeds from this screen were then
separately tested for kanamycin resistance and ABA sensitivity to isolate homozygous mutant lines carrying the homozygous \textit{RAB18}-promoter-\textit{GUS} construct.

\textbf{Preparation of transgenic lines expressing the Anti-sense \textit{ABI1} constructs}

The vector used to transform Columbia plants carrying the \textit{RAB18}-promoter-\textit{GUS} construct for over expression of \textit{ABI1} in the anti-sense orientation was constructed as follows; a \textit{BamH1/Xhol} fragment containing the \textit{ABI1} cDNA was ligated to the same site of the binary vector \textit{pEGAD} (courtesy of Sean Cutler, Stanford University, California). This vector contains the constitutive cauliflower mosaic virus promoter, \textit{CaMV35S}, which activates transcription of the green fluorescent protein gene (\textit{GFP}) and downstream DNA sequences. This vector was used to transform wild-type Columbia plants as mentioned above with the exception of the selection drug to recover plant transformants. Here, selection was on Gluphosinate ammonium (Crescent Chemical Co., Inc.), at 10\textmu g/ml concentrations.

\textbf{GUS staining}

\textit{GUS} staining was carried out as described by Jefferson (Jefferson, 1987).
Screen for enhancers of *abil*

The *abil* mutant seed used in this study were generated by suspending approximately 20000 *abil* seeds in 0.4% (v/V) ethyl methanesulfonate (EMS) for 24 hours at 4°C. This incubation was followed by extensive washing with water (15X) over a six hour period to remove residual EMS. These M1 seeds were sown immediately into 40 pots (approximately 500 plants per 6 inch pot). M1 plants were allowed to set seed, and the resulting M2 seed were harvested in 40 separate batches approximately 7 weeks later. These seed lots were kept separate from each other in order to minimize the re-sampling of the same mutant.

To isolate enhancers of *abil*, EMS mutagenized seed pools were screened for seeds displaying germination in the presence of 100μM ABA (a level which is inhibitory to the *abil* parent). 2000 seeds from each of the 40 pools of EMS mutagenized seed were surface sterilized and plated onto 0.8% agar/MS plates containing 100μM ABA. Seeds that germinated after 7 days were transferred to soil and grown to maturity. Offspring from these putative mutants were harvested and dried for at least one week. Dose response curves of putative mutants were generated by plating seeds of putative mutants on MS, 30, 60, 100 or 200μM ABA levels. Seeds were scored for germination after 7 days.
To remove background mutations, the abe mutants were backcrossed 3 times to abil. F2 seeds from these crosses were screened for ABA insensitivity at 40μM ABA for homozygous abe lines. For the abe6 lines, F2 seeds were isolated on 100μM ABA.

**Seed Fatty Acid Composition**

50 mg of seed were weighed into 50 mL screw cap Pyrex tubes. 1 ml, 1.5N HCl:1CH3OH was added. These were microwaved on power 5 for 2 min at which point the tubes were cooled briefly and vortexed. This was repeated twice more but microwaved for just 1 min. 100 μl 78nmol C-15, 0.5 ml ddH2O and 1ml hexane were added and the contents vortexed for 2-3 min. They were then centrifuged for 10 min at 2000 rpm. 0.5mL of the supernatant containing the seed fatty acids were placed in gas chromatography (GC) tubes and subjected to GC analysis. The raw data were then converted to percentage of total fatty acids.

**Water Loss Assay**

Water loss assays were performed as described by Koornneef et al. (1984). Plants of approximately the same size, with one primary stem, were sprayed with 1μM ABA and placed in the dark for one hour to induce stomatal closure. The plants were excised just below the rosette leaves and placed in pre-weighed buckets. The plants and
buckets were weighed every 20 min over a four hour period. Subsequently, to establish the dry weight and the total amount of water in the plants, they were placed in 37°C for approximately three days. Percent water loss over time was calculated. Five plants per genotype per bucket were used. The data points are averages of three replicates.

**Root growth sensitivity assays**

New root growth in the presence of growth factors was assayed as described by Hobbie (Hobbie and Estelle, 1995), with some slight modifications. Seeds were surfaced sterilized and plated on 0.8% agar (phytoagar) plates. Plates were placed vertically to prevent the roots from penetrating the agar. After 2 weeks, the seedlings were moved to MS media agar Petri plates supplemented with growth factor or other inhibitors. The length of the roots was marked and three days later, the new root growth was measured.

**Seedling ABA treatment for Northern hybridization analysis proline assays and histochemical GUS staining**

Seeds were surfaced sterilized and plated on 0.8% agar plates. Plates were placed vertically to prevent the roots from penetrating the agar. When the seedlings were approximately two weeks old, they were moved to MS plates supplemented with ABA. Twenty-four hours
later, RNA was isolated and analyzed for Northern hybridization (Sambrook et al., 1989; Verwoerd et al., 1989). Intact seedlings were used in staining for GUS activity. In other cases, seedlings were used for proline assays (Bates et al., 1973; Finkelstein and Somerville, 1990).

**Probe preparation**

All the probes used in Northern hybridization analysis in this thesis are listed in Table 2.2. The PCR conditions for probes that were isolated by PCR amplification are: 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds for 30 cycles. The products of PCR reaction or restriction digests were fractionated by electrophoresis on low melting agarose and used for the preparation of radioactive probe (Sambrook et al., 1989).

**Genetic Mapping analysis**

The ab1 enhancer lines were crossed to Columbia plants for SSLP (simple sequence length polymorphism) and caps (co-dominant cleaved amplified polymorphic sequences) mapping. The F2 seeds were screened for germination on 40 μM ABA. DNA was extracted from these seedlings and subsequently used in PCR reactions with SSLP or CAPS mapping primers (Bell and Ecker, 1994; Konieczny and Ausubel, 1993; McKinney et al., 1995). One new caps marker named EREBP3 was
generated in this process (at position 93.6 cM, clone MLN1 on the integrated chromosome V map). Based on the genomic sequence available from the Arabidopsis data banks, primers to this region were made and used to amplify DNA from Col and Ler ecotypes (94°C for 30 seconds, 64°C for 30 seconds and 72°C for 50 seconds for 40 cycles). The products were then digested with a number of restriction enzymes to identify an RFLP. The digestions with the restriction enzyme Dra1 showed a RFLP between these ecotypes (Table 2.3). Map distances were calculated based on the number of recombinants in the F2 population. For abe2 no recombinants in the 100 lines analyzed were observed for the C-20 marker (CAPS marker, personal communication E. Nambara). For abel, 2 recombinants in 100 lines analyzed, were observed for C-20 marker. The analysis of abe2 lines with the EREBP3 CAPS marker places this mutation north of C-20 marker.
Table 2.1  PCR specifications for amplification of RAB18 upstream region.

<table>
<thead>
<tr>
<th>Profile</th>
<th>Denaturing</th>
<th>Annealing</th>
<th>Extension</th>
<th># of cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>RAB18 forward (HindIII): GGCGTTTGGTAAAGC TTAGTAAATT</td>
<td>94°C</td>
<td>30 seconds</td>
<td>50°C</td>
<td>30 seconds</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>Primer 2 reverse (BamHI): GGTTCTGGTAGGA TCC CATGTTCTTC</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme</td>
<td>Vent DNA polymerase (New England Biolabs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Template</td>
<td>Chromosomal (Colombia)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

underlined bases are substitutions from wild-type sequence to create a new restriction enzyme recognition sequence

Table 2.2 List and origins of DNA probes used in Northern hybridization analysis

<table>
<thead>
<tr>
<th>probe</th>
<th>source</th>
<th>Preparation, Primer list or Restriction enzyme used in excision of the insert from the original clone</th>
</tr>
</thead>
</table>
| RAB18 | PCR from genomic DNA | For-GGCGTTTGGTAAAGC TTAGTAAATT  
Rev-CGACTGGTTACAAACCCTC |
| RAB21 | Plasmid pBluescript | pBluescript, EcoRI excises a 0.7 kb fragment |
| ADH   | PCR from Genomic DNA | For-TCC CCT TCT CGT TTT CTC TT  
Rev-ATC ATC TGC GAG AGA ATG GC |
| β-TUB | Plasmid Stock center | KpnI/BamHI excises a 1 kb fragment |
| CAB 140 | plasmid Stock center | SalI/NotI excises a 0.88 kb fragment |
| rRNA  | plasmid J. Coleman | EcoRI excises a 3.6 kb fragment |
| ABA1  | Plasmid pZipLox from Stock center | HindIII and EcoRI excises a 0.6 KB fragment |
Table 2.3 Specification of the new CAPS markers on chromosome V.

<table>
<thead>
<tr>
<th>CAPS Marker</th>
<th>PCR product (kb)</th>
<th>RESTRIC. enzyme</th>
<th>Product (kb)</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-20</td>
<td>0.128</td>
<td>Taq1</td>
<td>Col:</td>
<td>For:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.089</td>
<td>CATTACCGAGCAGATGTGAAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.027</td>
<td>Rev:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.012</td>
<td>AGGACACAAAGTAACCAAAGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ler:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.101</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>EREBP3</td>
<td>1.22</td>
<td>DraI</td>
<td>Col:</td>
<td>For:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.15</td>
<td>TTGACATGCCTCCATAGCATCCTCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.42</td>
<td>Rev:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.95</td>
<td>AACCGGACAAACCAGAAAGCTCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ler:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.95</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 3

ISOLATION AND CHARACTERIZATION OF ENHANCERS OF $abi1$

$(abe)$ MUTATION
INTRODUCTION

The plant growth factor ABA has been implicated in the establishment of seed dormancy and the induction of osmotic stress responses (Zeevaart and Creelman, 1988, McCourt, 1999). Recently, molecular biological, biochemical and physiological studies have been fruitful in the characterization of ABA responses in plants (Leung and Giraudat, 1998). However, the signal transduction pathway(s) leading to the induction of these responses remain mainly uncharacterized.

Genetic studies of ABA response mutants have provided some insight into ABA signaling in Arabidopsis (Bonetta and McCourt, 1998). ABA sensitivity screens have identified a number of mutants with reduced (abi) and enhanced (era) ABA responsiveness. Mutations in the ERA1 locus confer seed and guard cell ABA hypersensitivity (Cutler et al., 1996). ERA1 encodes the β-subunit of a protein farnesyl transferase and has been assigned roles in the negative regulation of ABA signaling.

The abi mutants were identified in screens for mutant seeds that were able to germinate on ABA concentrations that are inhibitory to wild-type seeds (Finkelstein, 1994; Koornneef et al., 1984). Two classes of abi mutants have been recovered. The first class is the semi-dominant mutants, abil and abi2, which, in addition to seed ABA insensitivity, also display reduced
sensitivities to ABA in their vegetative tissue. Unfortunately, because loss-of-function alleles for ABI1 or ABI2 do not exist, the semi-dominant nature of these mutations has made it difficult to assign ABA functions for the wild-type loci. However, since a number of ABA responses are defective in these mutants, ABA functions can be assigned to the mutant isoforms. ABI1 and ABI2 belong to the PP2C family of proteins (Leung and Giraudat, 1998). The genetic analysis of PP2C functions in Schizosaccharomyces pombe has suggested redundant roles for these proteins in yeast osmo-regulatory responses (Shiozaki and Russell, 1995). Redundant PP2C functions may also account for the shared ABA response defects, which have been characterized in abil and abi2 mutants and may also explain the dominant nature of the mutant alleles. In addition to the common pathways that abil and abi2 affect, there also appears to be distinct ABA signaling pathway(s) leading to mutant specific ABA defects in abil and abi2 (Leung et al., 1997). For example, the ABA inducibility of a number of downstream ABA markers are differentially affected in these mutant backgrounds. This suggests that abil and abi2 may impinge on both common and distinct ABA signaling pathways. Thus these mutants provide genetic and biochemical evidence for certain redundancies in ABA signaling pathways leading to gene expression.
The second class of abi mutants include the abi3, abi4 and abi5 mutants (Finkelstein, 1994; Koornneef et al., 1984). These are recessive mutations which share reduced ABA responsiveness in their seeds as a common defect. Genetic analysis has placed ABI3, ABI4 and ABI5 in separate seed ABA signaling pathways from abil and abi2 and suggests the presence of multiple seed ABA signaling pathways (Finkelstein, 1994; Finkelstein and Somerville, 1990).

Although a number of ABA response mutants have been isolated in Arabidopsis, mutations in the main ABA signaling components, such as the ABA receptor, remain unidentified. There are a number of possibilities why mutations in these components have not been isolated. First, the loss-of-function mutants in these components may be lethal. However, the viability of ABA auxotrophic mutants suggests that ABA functions are not essential to plants; hence loss-of-function alleles of ABA signaling components should be viable. However, if these components have essential functions outside ABA signaling, then mutations in these loci could be pleiotropic and be lethal. For example, severe alleles of abi3 are desiccation intolerant and ABI3 has been postulated to have functions in both ABA-dependent and ABA-independent seed maturation programs (Nambara et al., 1992). A second possibility is the presence of multiple ABA signaling pathways. In this scenario, loss-of-function mutations in ABA signaling components may only
result in weak or no ABA related phenotypes, due to compensatory effects of other ABA signaling pathways. A number of biochemical and genetic finding, support this model (Allan et al., 1994; Finkelstein, 1994; Finkelstein and Somerville, 1990; Pei et al., 1997). In addition, the ABA response mutant screens have failed to isolate simple recessive mutations that affect all aspects of ABA signaling, which again suggests the presence of redundancies in ABA signaling. In fact, the only mutants that alter most aspects of ABA responses are rare semi-dominant mutations in PP2C proteins that, as mentioned above, may have functions in multiple signaling pathways. To recover mutations in unidentified ABA signaling components may therefore require more sensitive ABA screens that take redundancy into account. For example, different genetic backgrounds can result in changes in ABA responsiveness of the weak abi mutants. The double mutant combinations of abi4/abil or abi5/abil, result in seeds that are approximately 20-60 times more ABA insensitive than the parental types (Finkelstein, 1994). If the effects of such weak ABA insensitive mutations can be amplified in an abil genetic background, then abil may also function as a sensitizing background for isolation of other weak ABA insensitive mutants that otherwise would not be detected by conventional abi screens.
With these concerns in mind, I used the semi-dominant *abil-1* genetic background to screen for enhancers of this mutation. The premise of this enhancer screen is that in the sensitized background of *abil*, mutations in redundant ABA signaling components will lead to increased ABA insensitivity. In principle, these enhancers are expected to carry mutations in positively acting genes in ABA signal transduction, which may or may not have weak ABA insensitive phenotypes in a wild-type *ABI1* genetic background.
RESULTS

Seed and root ABA responses of transgenic plants carrying the ABII antisense construct

Since loss-of-function alleles for ABII do not exist, it has been difficult to assign roles for ABII in ABA signaling. To examine the ABII functions in ABA signaling, ABII was expressed in an antisense orientation in an attempt to down regulate ABII wild-type expression. To examine seed ABA sensitivity in these lines, the seeds of 3 independent transgenic lines, carrying the antisense ABII construct, were tested for their germination on 3 and 5 μM ABA. The transgenic seeds showed insensitivity to 3 μM ABA, an ABA level that inhibits the germination of wild-type seeds (more than 50 seeds per line were tested). However, at 5 μM ABA, an ABA level permissive to abil germination, these seeds did not germinate, suggesting weak ABA insensitivity for seeds of these lines.

To examine ABA-induced gene expression in the ABII anti-sense transgenic lines, transgenic seedlings were exposed to 1 and 10 μM ABA and stained for GUS activity. The roots of wild-type seedlings showed ABA induction of RAB18 at these ABA levels (Figure 3.1). However, the roots of transgenic lines failed to induce RAB18 expression under similar conditions, demonstrating reduced ABA
sensitivity at the level of RAB18 gene induction in these lines. These findings suggest that loss-of-function alleles of ABII may result in reduced ABA sensitivity, suggesting a positive role for ABII in ABA signaling.

**Isolation of enhancers of abil**

Forty thousand M2 EMS-mutagenized abil-1 seeds were screened for germination on 100μM ABA, an ABA concentration that inhibits abil germination. After the initial identification of enhancers of abil (ena) mutant isolates, the enhancer lines were then plated on a gradient of concentrations of ABA to determine their degree of ABA responsiveness at germination. All ena lines were able to germinate on 30μM ABA, an ABA level that inhibits abil germination (Figure 3.2). However, ena16, ena20, ena23, ena34 and ena40 germination is more than 90% inhibited at 100μM ABA, an ABA concentration that does not effect ena30 and ena31 germination. Based on these results, the ena lines can be divided into two groups. In group one are the mutants that displayed weak enhancement of abil at the level of seed germination. These are ena16, ena20, ena23, ena34 and ena40. The second group are the mutants which show strong enhancement of abil at the level of seed germination. These include ena30 and ena31. Members of this group displayed ABA insensitivity to 200μM ABA at the level of seed germination.
Figure 3.1 ABA induction of RAB18 in a ABI1 antisense line. The seedlings from left to right are; 1) ABI1 antisense line exposed to 10μM ABA, 2) ABI1 antisense line exposed to 1μM ABA, 3) ABI1 antisense line, no ABA treatment, 4) wild-type seedling exposed to 10μM ABA, 5) wild-type seedling exposed to 1μM ABA, 6) wild-type seedling not exposed to ABA. All seedlings carry RAB18-GUS reporter constructs. More than 30 seedlings from 3 independent transgenic lines were tested for each ABA concentration in this assay. The GUS staining in the cotyledons is the result of embryonic GUS expression driven by the RAB18 promoter.
Figure 3.2  Seed ABA sensitivities of ena lines at the level of germination. Seeds were allowed to germinate for 7 days before scoring. Approximately 100 seeds in 3 independent experiments were assayed for each point on the graph.
Table 3.1 Genetic segregation of ABA resistance in ena lines. The ABA level used in most cases was 40μM (an ABA level inhibitory to ab1 germination). In experiments involving ena30 and ena31 crosses a second ABA concentration of 100μM was additionally used. (Number of seed germinated/total seeds. The ratios in the table are germination: no-germination).

<table>
<thead>
<tr>
<th>Parental cross</th>
<th>F2 germ. on MS</th>
<th>F2 germ. 40μM ABA (100μM)</th>
<th>Observed ratio</th>
<th>Segregation pattern</th>
<th>χ² for 3:1 P&gt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>ena16 X ab1</td>
<td>87/87</td>
<td>30/95</td>
<td>1:2.16</td>
<td>recessive</td>
<td>2.19</td>
</tr>
<tr>
<td>ena20 X ab1</td>
<td>79/79</td>
<td>29/118</td>
<td>1:3.07</td>
<td>recessive</td>
<td>0.01</td>
</tr>
<tr>
<td>ena23 X ab1</td>
<td>98/98</td>
<td>22/81</td>
<td>1:2.68</td>
<td>recessive</td>
<td>0.2</td>
</tr>
<tr>
<td>ena30 X ab1</td>
<td>93/93</td>
<td>58/99, 18/80 (100)</td>
<td>2.3:1.7</td>
<td>recessive</td>
<td>59.9</td>
</tr>
<tr>
<td>ena31 X ab1</td>
<td>105/105</td>
<td>24/86, 0/98 (100)</td>
<td>1:2.58</td>
<td>recessive</td>
<td>0.266</td>
</tr>
<tr>
<td>ena34 X ab1</td>
<td>112/112</td>
<td>26/120</td>
<td>1:3.6</td>
<td>recessive</td>
<td>0.711</td>
</tr>
<tr>
<td>ena40 X ab1</td>
<td>90/90</td>
<td>22/95</td>
<td>1:3.6</td>
<td>recessive</td>
<td>0.711</td>
</tr>
</tbody>
</table>
Table 3.2  Genetic segregation of ABA sensitivities in ena lines, in the absence of abil mutation. The F2 seeds were tested on 3μM ABA, an ABA level inhibitory to wild type seed germination and not to seeds carrying the abil/ABII or abil/abil mutations. Only 3/16 classes of the F2 seeds are expected not to germinate if the seeds that carry the ena/ena mutations show ABA insensitivity to 3μM ABA at the level of seed germination. These classes are; 2/16 (ena/ENA ABI1/ABI1) and 1/16 (ENA/ENA ABI1/ABI1). However, if the 1/16 (ena/ena ABI1/ABI1) are responsive to 3μM ABA at the level of seed germination, a 4:12 (no-germination:germination) is expected. (Number of seed germinated/total seeds. The ratios in the table are no-germination:germination).

<table>
<thead>
<tr>
<th>Parental cross</th>
<th>F2 germ. (MS)</th>
<th>F2 germ. (3μM ABA)</th>
<th>Observed Ratio</th>
<th>χ² for 3:1 P&gt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ler</td>
<td>161/161</td>
<td>1/186</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>abil X Ler</td>
<td>122/122</td>
<td>196/256</td>
<td>0.9:3.1</td>
<td>3.692</td>
</tr>
<tr>
<td>ena16 X Ler</td>
<td>365/365</td>
<td>257/333</td>
<td>3.7:12.3</td>
<td>3.6</td>
</tr>
<tr>
<td>ena20 X Ler</td>
<td>247/248</td>
<td>218/289</td>
<td>3.9:12.1</td>
<td>6.34</td>
</tr>
<tr>
<td>ena23 X Ler</td>
<td>117/117</td>
<td>154/202</td>
<td>3.8:12.2</td>
<td>3.32</td>
</tr>
<tr>
<td>ena30 X Ler</td>
<td>164/164</td>
<td>267/329</td>
<td>3:13</td>
<td>0.2</td>
</tr>
<tr>
<td>ena31 X Ler</td>
<td>156/156</td>
<td>154/267</td>
<td>6.8:9.2</td>
<td>22.88</td>
</tr>
<tr>
<td>ena34 X Ler</td>
<td>112/112</td>
<td>196/245</td>
<td>3.2:12.8</td>
<td>0.255</td>
</tr>
<tr>
<td>ena40 X Ler</td>
<td>241/241</td>
<td>383/468</td>
<td>2.91:13.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Figure 3.3  Eicosenoic acid (20:1) content of ena seeds.

Three sets of samples were analyzed for each mutant line.
Genetics of ena mutants in abil background

To determine the genetic basis for reduced seed ABA responsiveness at the level of seed germination caused in the ena lines, they were crossed to the abil parent and F2 segregation analysis was carried out by seed germination tests on 40μM ABA (Table 3.1). The ena16, ena20, ena23, ena31, ena34 and ena40, lines, all show segregation patterns expected for a recessive mendelian trait (Table 3.1). This suggests the enhancement of the abil seed ABA responsiveness at the level of germination is controlled by single recessive mutations in these lines. However, the ena30 line which appears to be a single recessive mutation for the enhancement of the abil seed ABA responsiveness at the level of germination at 100μM ABA, segregates in a dominant manner for enhancement of abil at 40μM ABA levels (Table 3.1). This suggests that gene dosage may play a role in the enhancement of abil germination in the ena30 line. Interestingly, the seeds of ena31, which showed insensitivity to 100μM ABA in the previous germination assay (Figure 3.2), did not germinate when F2 seeds from the abil backcross were assayed for germination on 100μM ABA levels (Table 3.1).
Seed ABA sensitivities of ena lines in the absence of the abil mutation

In the absence of the abil mutation, the ena mutants are expected to have weak seed ABA responsiveness at germination. To test this, the ena lines were crossed to wild-type plants and the germination of F2 seeds was tested on 3μM ABA, an ABA level that is inhibitory to wild-type germination but permissive to weak ABA insensitive seeds like abi5 (Table 3.2). In this assay a germination ratio of 4:12 (no-germination:germination) is expected when ena/ena (ABI1/ABI1) lines are responsive to 3μM ABA concentrations. However, a germination ratio of 3:13 (no-germination:germination), would be an indication of ABA insensitivity for the homozygous ena/ena (ABI1/ABI1) lines. The enhancer lines ena30, ena34 and ena40, displayed germination ratios expected for recessive single gene mutations, that result in seeds that are insensitive to 3μM ABA concentrations at germination. However, ena16, ena20 and ena23 showed germination ratios expected for seeds that are sensitive to 3μM ABA concentrations (Table 3.2). This suggests that ena16, ena20 and ena23 homozygous mutations show wild-type seed ABA sensitivities at the level of germination, when the abil mutation is removed. The F2 progeny of the Ler and ena31 cross displayed an unexpected F2 germination ratio of 6.8:9.2 on 3μM ABA (Table 3.2). However, the same batch of seeds displayed a
2.8:12.8 germination ratio after an eight month drying period, a condition that has been shown to break seed dormancy.

abi3, abi4 and abi5: the 3 classes of predicted enhancers of abl1, are recovered in the abl1 enhancer screen

Mutations in at least three genetic loci (ABI3, ABI4 and ABI5) are predicted to result in the enhancement of abl1 seed ABA insensitivity at the level of germination. (Finkelstein, 1994; Finkelstein and Somerville, 1990). A number of seed defects that result from mutations in these loci can be used as phenotypic signatures. For example, mutations in the ABI3 locus, result in a reduction in eicosenoic acid (20:1) content of seeds; thus, the fatty acid content of ena seeds was measured (Finkelstein and Somerville, 1990). With the exception of ena40, the majority of ena mutants show a fatty acid profile similar to that of abl1 (Figure 3.3). The eicosenoic acid levels of ena40 are similar to abi3, suggesting ena40 is a new abi3 allele. To test this possibility, a genetic allelism test between ena40 and abi3-1 was carried out. The F2 seeds from this cross were assayed for germination at 5μM ABA concentrations, an ABA level inhibitory to wild-type seeds. All F2 seeds germinated indicating ena40 and abi3-1 are allelic.

Preliminary identification of ABI5 alleles was performed by following expression of the embryo abundant protein, ATEm6. Both
abi3 and abi5 mutant seeds show reduced accumulation of this transcript (Finkelstein, 1994; Parcy et al., 1994). As expected the ena40 line showed a large reduction in its AtEm6 transcripts (Figure 3.4). However, AtEm6 transcripts in ena30 were also reduced. This result together with the normal levels of eicosenoic acid suggested ena30 was possibly a new allele of abi5. An allelism test between ena30 and abi5 performed on F2 progeny from this cross indicated that ena30 was allelic to abi5.

The ena34 line displayed seed ABA insensitivity at the level of germination to 3μM ABA in the absence of the abi1 mutation (Table 3.2). However, this line appeared to have normal seed AtEm6 transcript accumulation and eicosenoic acid levels, suggesting it may be an abi4 allele. To examine whether these mutations are allelic they were crossed. At 3μM ABA concentrations, all F2 seeds of the ena34 and abi4 cross germinated, indicating that ena34 is allelic to abi4.

**ena mutants identify ctrl and 2 new genetic loci**

With the elimination of all predicted mutant classes, genetic complementation tests were carried out to determine the number of genetic loci for the remaining mutants (Table 3.3). These results show that the remaining ena mutants belong to 3 genetic complementation groups. These mutants were renamed abe, for abi...
enhancers and the numbering system was changed from the pool number to the complementation group number (Table 3.4).

Genetic mapping analysis of ABE1 and ABE2 loci showed a tight linkage between abe2 and C-20, a CAPS marker, at position ≈105cM on the integrated genetic map of chromosome V. abel also mapped to 2cM north of C-20 marker. Since no other growth factor response mutant has been linked to this region, ABE1 and ABE2 represent 2 new genetic loci with ABA response functions (Figure 3.5). Genetic mapping analysis of ABE3-1, determined a tight linkage between abe3-1 and the ATH-CTR1 locus, a small sequence length polymorphism (SSLP) marker at the tip of chromosome V. abe3-1 also displayed a dwarf phenotype typical of ctrl mutants. To examine the possibility that abe3-1 is allelic to ctrl-1, these lines where crossed in an allelism test. The F2 seeds from this cross all germinated on 3μM ABA concentrations showing that these mutations are allelic. Two other alleles of abe3 were isolated in subsequent ena screens and were named abe3-2 and abe3-3.3. Because these alleles identify new ctrl alleles, they have been renamed ctrl-6, ctrl-7 and ctrl-8. These lines all have low seed set and reduced post germination viability.
Table 3.3 Genetic complementation analysis of ena lines. The F1 and F2 seeds from the parental crosses listed in the table were assayed for ABA sensitivity at the level of germination on 40µM ABA. When the ena mutations are allelic (mutations do not complement), a 100% germination of the F1 and F2 seeds on 40µM ABA is expected. When the ena mutations are not allelic (a positive complementation) a 7:9 ratio of germination:no-germination on 40µM ABA is expected.

<table>
<thead>
<tr>
<th>Parental cross</th>
<th>F1 germination on 40µM ABA</th>
<th>F2 germination on MS</th>
<th>F2 germination on 40µM ABA</th>
<th>F2 ratio Ger:No-Ger</th>
<th>Complementation result</th>
</tr>
</thead>
<tbody>
<tr>
<td>ena16 × ena20</td>
<td>16/17</td>
<td>71/71</td>
<td>53/55</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ena16 × ena23</td>
<td>0/32</td>
<td>48/48</td>
<td>63/115</td>
<td>7.2:8.8</td>
<td>+</td>
</tr>
<tr>
<td>ena20 × ena23</td>
<td>0/58</td>
<td>64/64</td>
<td>70/129</td>
<td>7.3:8.7</td>
<td>+</td>
</tr>
<tr>
<td>ena16 × ena31</td>
<td>0/9</td>
<td>68/68</td>
<td>62/116</td>
<td>7.4:8.6</td>
<td>+</td>
</tr>
<tr>
<td>ena20 × ena31</td>
<td>0/7</td>
<td>75/75</td>
<td>50/91</td>
<td>7.2:8.8</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 3.4  A summary of the ena mutants. Renaming is based on the complementation groups.

<table>
<thead>
<tr>
<th>Complementation group</th>
<th>ena</th>
<th>Genetic Locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>abe1-1</td>
<td>ena20</td>
<td>Chromosome V</td>
</tr>
<tr>
<td>abe1-2</td>
<td>ena16</td>
<td>Tightly linked to C20 (position = 105cM)</td>
</tr>
<tr>
<td>abe2</td>
<td>ena23</td>
<td>Chromosome V</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.5cM North of C20 (position = 105cM)</td>
</tr>
<tr>
<td>abe3-1</td>
<td>ena31</td>
<td>ctr1-6</td>
</tr>
<tr>
<td>abe3-2</td>
<td>ena11</td>
<td>ctr1-7</td>
</tr>
<tr>
<td>abe3-3</td>
<td>ena18</td>
<td>ctr1-8</td>
</tr>
<tr>
<td>abe4</td>
<td>ena40</td>
<td>ABI3</td>
</tr>
<tr>
<td>abe5</td>
<td>ena34</td>
<td>ABI4</td>
</tr>
<tr>
<td>abe6</td>
<td>ena30</td>
<td>ABI5</td>
</tr>
</tbody>
</table>
Figure 3.4 AtEm6 and Rab18 transcript analysis of ena seeds. Lanes are: 1) Ler, 2) abi1-1, 3) abi3-1, 4) ena16, 5) ena20, 6) ena23, 7) ena30, 8) ena34, and 9) ena40. The blots were probed and stripped in the same order they appear in the Figure. From top to bottom. 5μg of total seed RNA was loaded per lane.
The enhancement of abil seed ABA insensitivity at the level of seed germination by ctrl is influenced by the maternal tissue

Germination of ctrl-6/abil double mutants displayed a dramatic reduction in seed ABA sensitivity (Figure 3.2). However, the F2 seeds from an ctrl-6/abil double mutant and abil cross failed to germinate on 100μM ABA (Table 3.1), an ABA level previously determined not to be inhibitory to ctrl-6/abil double mutant seed germination (Figure 3.2). However, when ABA concentrations were reduced to 40μM, a recessive germination pattern was observed in the F2 seeds. The only difference between the F2 seeds tested in Table 3.1 and the seeds tested in Figure 3.2 is the genotype of the parents of these seeds which are ctrl-6/CTR1-6 for plants tested in Table 3.1, and ctrl-6/ctrl-6 for plants tested in Figure 3.2. Since the seed coats have maternal origins in Arabidopsis, these results suggest that the seed coats contribute to the enhancement of abil seed ABA insensitivity at the level of seed germination by ctrl-6.
Figure 3.5 The chromosomal position of ABE1 and ABE2. This diagram represents a segment of chromosome V. The position of EREBP3 mapping marker created in this report is displayed in this diagram.
Chromosome V (96-107cM)
**abe mutants do not enhance guard cell and root growth insensitivity of abil seedlings**

To investigate the vegetative ABA responses in abe mutant lines, a number of physiological and molecular markers are available. The guard cells of abil plants are non-responsive to ABA (Pei et al., 1997). To examine the possible enhancement of this phenotype in the abe mutant background, the transpiration rates through the detached shoots of abe mutants was measured. The abe3 mutants were not examined in these experiments, due to their poor growth. The measurement of abe transpiration did not show any significant difference between these lines and the abil parental type (Figure 3.6A). This suggests that the abe mutants do not enhance guard cell ABA insensitivity of abil.

Roots of abil mutants show reduced sensitivity to ABA growth inhibition. To examine the possible enhancement of this phenotype in the abe mutant background, the roots of these mutants were tested for their ABA sensitivities. All examined abe mutants showed root ABA sensitivities similar to abil roots, suggesting that these mutations do not enhance the ABA resistance of abil with respect to root growth (Figure 3.6B).
abe mutants have altered responses from abil in the up-regulation of certain ABA induced gene markers.

Expression of a number of genes in Arabidopsis shows ABA inducibility. However, the ABA induction of these genes may not be the result of a universal ABA signal transduction pathway. For example, the ABA inducibility of alcohol dehydrogenase (ADH), a root specific ABA-upregulated gene, is defective in abi2 mutants but normal in an abil mutant background (de Bruxelles et al., 1996). By contrast, there are also common ABA signaling pathways affected in both abil and abi2 mutants. For example, the ABA induction of RAB18 and RAB21 shows defects in both abil and abi2 mutants (Gosti et al., 1995; Lang and Palva, 1993). To examine the ABA signal transduction pathways leading to gene expression in abe mutants, the expression of ABA up-regulated genes RAB18, RAB21 and ADH was examined.

The ABA induction of RAB18 has been shown to be severely defective in the abil mutant (Gosti et al., 1995; Lang and Palva, 1993). However, RAB18 appears to be inducible by ABA in abil (Figure 3.7). All abe mutants with the exception of abel-1 and abe4 (abi3) show normal ABA induction of this marker when compared to abil. The abel-1 displays a reduction in ABA inducibility of this marker, suggesting an enhancement of the abil mutation by abel-1 at the level of RAB18 expression. Interestingly, abe4 (abi3) also
shows a reduced ABA inducibility when compared to abil. abe4 carries an uncharacterized mutation in the ABI3 locus, and since ABA defects in the abi3 mutation have only been described in embryonic tissue, it is surprising that ABA responses in abi3 are altered in seedlings.

The ABA induction of RAB21, a drought-inducible marker, is not affected in an abil background (Gosti et al., 1995). This suggests that the ABA signaling pathway that leads to the induction of this marker is independent of the RAB18 pathway, which is affected by abil. As was observed for RAB18 expression in abe4 (abi3), the RAB21 accumulation is decreased in this mutant (Figure 3.7). Since the signaling pathways leading to RAB18 and RAB21 expression appear different, it is surprising that mutations in ABI3 might affect both signaling pathways. However, since ABI3 encodes a transcription activator protein, it is possible that these signaling pathways may merge at ABI3 for transcriptional induction of genes.

The root specific ABA inducible gene, ADH, shows normal ABA inducibility in the abil mutant background. The abil, abel-1, and abe2-1 mutants showed normal ABA-upregulation of this marker, however, the abe4 (abi3), abe5 (abi4), and abe6 (abi5) mutant lines showed a reduced ABA inducibility of this marker (Figure 3.7).
ABA induced repression of β-tubulin and chlorophyll binding protein is shown defects in abe2 mutant

ABA is involved in the suppression of a number of plant genes such as β-tubulin (Giani et al., 1998). To examine the ABA repression responses in abe mutants the transcripts for β-TUB were examined (Figure 3.7). As expected, β-TUB transcripts are ABA-down regulated in most abe lines. However, β-TUB does not appear to be down-regulated by ABA in the abe2 line, suggesting a defect in ABA signaling leading to repression of β-tubulin expression by ABA. To examine other ABA repression responses in the abe2 mutant the transcripts for the chlorophyll binding protein (CAB) were analyzed. Similar to β-tubulin transcripts, ABA-down-regulation of CAB transcript was defective in the abe2 mutant (Figure 3.8). The above results together with the recessive nature of the abe2 mutation, suggest that the ABE2 gene may function as a positive regulator of ABA pathway leading to vegetative gene repression.

Cross growth factor sensitivities in abe mutants

Although many growth factor mutants were originally identified by altered sensitivities to a single growth factor, subsequent analysis revealed that many of these mutants were defective in other hormonal responses (McCourt, 1999). To examine possible changes in a broader range of growth factor sensitivities in abe mutants, these lines were tested for root growth on auxin, the
ethylene precursor, ACC and kinetin, a cytokinin. The abe mutants did not show any alterations from abil in their sensitivities to the tested growth factors (data not shown). In addition, these lines were assayed for ethylene induced triple responses in etiolated seedlings. With the exception of abe3-1 (ctrl-6), which displayed triple response in the absence of ACC, all other lines displayed similar triple response to wild-type and abil seedlings. At this cursory level it appears that with the exception of abe3-1 (ctrl-6), the abel and abe2 mutants only have ABA-specific defects.
Figure 3.6 A) Kinetics of water loss in excised 4 week old plants during a 4 hour water loss assay of wild-type and abe mutants. Plants of approximately the same size, with one primary stem, were sprayed with 1μM ABA and placed in the dark for one hour to induce stomatal closure. The plants were excised just below the rosette leaves and placed in pre-weighed buckets. The plants and buckets were weighed every 20 min over a four hour period. Subsequently, to establish the dry weight and the total amount of water in the plants, they were placed in 37°C for approximately three days. Percent water loss over time was calculated. Five plants per genotype per bucket were used. The data points are averages of three replicates. B) Root growth sensitivities to ABA in abe mutants. Each value represents the mean of measurements of at least 10 seedlings (2 week old). Relative growth is relative to mean root elongation of the same genotype on medium without ABA.
Figure 3.7 Transcript analysis of genes induced or repressed by ABA in wild-type and abe mutant background. The ABA-upregulated genes tested are RAB18, RAB21 and ADH. The ABA-down-regulated gene tested is β-tubulin. Two week old seedlings were exposed to agar media supplemented with 0 or 10μM ABA for a 24 hour period. Approximately 5μg of total RNA was loaded per lane. The order of the probing and the stripping of blots were; ADH, RAB21, RAB18, β-TUB, and rRNA.
Figure 3.8 Transcript analysis of ABA-down-regulated CAB gene. Two week old seedlings were exposed to agar media supplemented with 0 (lane 1) or 10μM ABA (lane 2) or 100μM ABA (lane 3) for a 24 hour period. Approximately 5μg of total RNA was loaded per lane. The order of probes used were, CAB and rRNA.
DISCUSSION

Phenotypes of loss-of-function mutations that alter ABA signaling may be masked by to genetic redundancies in ABA signal transduction pathways. Here, it was shown that the abi1 mutant may act as a sensitized background to isolate abi mutants that would otherwise have weak or undetectable ABA insensitivity. In all, mutations in six genetic loci were isolated, including three known ABA response genes, ABI3, ABI4 and ABI5. The transcript analysis of vegetative ABA-inducible genes in these backgrounds suggests that ABI3 may have an important role in ABA signal transduction outside the seed. This screen also resulted in isolation of mutations in the CTR1 locus. CTR1 is a known component of the ethylene signal transduction pathway and this is the first time this locus has been implicated in ABA signaling (Kieber et al., 1993). In addition to already characterized genetic loci, two new loci were identified. These mutations do not appear to cause detectable ABA insensitive seed germination phenotypes in the absence of the abi1 mutation. The ABA-induced transcript analysis of abel suggests roles for ABE1 in ABA pathways leading to RAB18 induction. ABE2 appears to function in ABA signal transduction pathways leading to ABA-down-regulation of CAB and β-tubulin. In addition, antisense expression of ABI1 resulted in reduced ABA sensitivity at the level of RAB18
gene expression and germination, suggesting a role for ABII in ABA signaling leading to these responses.

PP2Cs have been assigned roles in the negative regulation of eukaryotic signaling pathways (Hanada et al., 1998; Maeda et al., 1993; Maeda et al., 1994; Shiozaki and Russell, 1995). However, the ABII antisense plants displayed reduced ABA sensitivity at the level of RAB18 gene expression and seed germination, suggesting a positive role for ABII in ABA responses. The manner in which a negative regulator may act to increase the flux of the ABA signaling pathway may be the result of ABII action on negative regulator(s) of ABA signaling. Evidence to support this model is from the electrophysiological studies of abil guard cell ABA responses (Pei et al., 1997). In this study, the application of a protein kinase inhibitor resulted in the restoration of ABA responses in abil guard cells. This suggests that a protein kinase may be acting to negatively regulate ABA responses downstream of abil action (Figure 1.4). It appears that similar protein kinases, which may be the potential targets for ABII enzymatic function, may also act in seeds in addition to vegetative tissues.

Genetic and molecular analysis of the Arabidopsis ABI3 locus have assigned seed specific ABA functions for ABI3 (Finkelstein and Somerville, 1990; Koornneef et al., 1984; Parcy et al., 1994). However, a more careful transcript analysis of ABI3 has detected
its transcripts in the vegetative tissue, which may suggest roles for ABI3 outside the embryo (Rohde et al., 1999). In addition, mutant screens for suppressors of the eral mutation have yielded a number of abi3 mutations (Sarkar, 1999). These mutations not only suppress eral embryonic ABA hyper-sensitivities, they also suppressed some vegetative eral defects, providing genetic evidence for vegetative ABI3 functions outside the embryo. The transcript analysis of a number of ABA inducible genes in abe4, which carries an uncharacterized mutation in the ABI3 locus, has shown a reduced sensitivity to ABA for the induction of these genes. Therefore, it appears that ABI3 may have functions in vegetative ABA signal transduction pathways leading to gene activation. Interestingly, the ABA-induced markers in these assays, which were chosen based on their differential inducibility by different ABA signal transduction pathways all showed ABA insensitivity in the abe4 background. This suggests that ABI3, which encodes a transcriptional activator, may play a role in merging the ABA signaling pathways leading to ABA-up-regulated gene expression (Figure 3.9). Since independent abi3 mutants do not have defects in ABA gene induction, it appears that this central role for ABI3 could be redundant in ABA signaling in vegetative tissues.

Molecular analysis of the ABA inducible RAB18 had previously shown a complete lack of ABA inducibility for this gene in an abil
mutant background (Gosti et al., 1995; Lang and Palva, 1993). However, it appears that RAB18 maintains some ABA inducibility in \textit{abil}. The reduced ABA inducibility may be the result of leakiness in the ABA signaling pathway which \textit{abil} impinges on. This could result from residual PP2C activity of the \textit{abil} isoform. In fact, biochemical studies have demonstrated a 30% PP2C activity in \textit{abil}. Alternatively, a separate ABA signaling pathway may lead to RAB18 ABA expression in the \textit{abil} background. Mutations in the \textit{abel}-l locus greatly reduce the ABA sensitivity of this marker in the \textit{abil} mutant background. This suggests a role for ABE1 in ABA signaling pathway(s) leading to ABA induction of RAB18 (Figure 3.9).

Under extended drought periods, ABA acts to inhibit general plant growth (Davis and Zhang, 1991). This inhibition of growth is in part due to suppression of a number of genes such as \textit{β-Tubulin} (Giani et al., 1998). The ABA signaling pathway leading to these responses are uncharacterized. The \textit{abil} mutant does not display defects in the repression of \textit{CAB} or \textit{β-TUB}. The \textit{abe2} mutant, however, appears to have lost this ABA-induced function, since the transcripts of \textit{CAB} or \textit{β-tubulin} do not show ABA down-regulation even at high ABA concentrations. This suggests that ABE2 may function in the vegetative ABA signal transduction pathway leading to ABA induced \textit{CAB} and \textit{β-TUB} repression (Figure 3.9). This pathway appears to be independent of other ABA signaling pathways, since no
other ABA signaling mutant shows defects in this pathway. The ABA repression signaling pathway may also function in seeds. For example, ABA has been implicated in the transcriptional repression of germination-promoting enzymes such as α-amylase in monocot seeds (Hoecker et al., 1999; Hooley et al., 1990). In Arabidopsis, ABA also plays a similar repressive role in the inhibition of seed germination through the repression of proteolytic enzymes required for this process (Garcia-Rubio et al., 1997). The ABE2 gene product may have functions in seed ABA-induced repression pathways since abe2 mutation further reduce the ABA sensitivity of abil mutant at the level of seed germination.

CTR1 plays a central role in ethylene signal transduction (Kieber et al., 1993). Mutations at the Arabidopsis CTR1 locus, result in a constitutive response to ethylene suggesting a negative regulatory role for CTR1 in ethylene signaling. Here, it was shown that mutations in the CTR1 locus result in strong reduction in ABA sensitivity of abil mutant at the level of seed germination. This implicates CTR1 in ABA signaling in the seed. The involvement of CTR1 in ABA signaling could either be through ethylene and the ethylene signal transduction pathway, or it may be independent of this pathway. Physiological findings have implicated ethylene as a positive factor in the germination of a number of seeds (Abeles et al., 1992). In addition, the ethylene insensitive mutants of
Arabidopsis have been reported to have poor germination rates, supporting a role for ethylene and ethylene signal transduction in seed germination (Chang et al., 1993). However, since sensitivities of seeds in CTR1 mutants is altered to ABA, it is possible that ethylene's positive influence on germination is through the negative regulation of ABA action in seeds. These results open interesting possibilities as to the interaction of these two growth factors in seed germination.

The genetic interactions between ctrl and abil mutants are similar to those reported when abi3, abi4 or abi5 are introduced into an abil genetic background (Finkelstein, 1994; Finkelstein and Somerville, 1990). One interpretation of these synergies has been that these genes define separate ABA pathways from abil in the seed (Finkelstein, 1994; Finkelstein and Somerville, 1990). However, if ethylene and other signaling pathways negatively regulate ABA responsiveness in the seed, it is possible that some mutants identified through ABA sensitivity screens may identify genes in response pathways peripheral to ABA signaling.
Figure 3.9 A schematic diagram summarizing genetic pathways leading to ABA-regulated gene expression in the vegetative tissue. The model is based on the information gathered from transcript analysis of ABA-inducible genes RAB18 (Gosti et al., 1995; Lang and Palva, 1993), ADH (de Bruxelles et al., 1996), and ABA-repressible gene β-TUB (Giani et al., 1998), and CAB, in ABA response mutant background. The mutations that affect the expression of the listed markers are drawn on the hypothetical pathway. RAB21 shows normal ABA-inducibility in abil background and has been placed in a separate ABA signaling pathway from the one that leads to RAB18 gene expression. X is an uncharacterized transcriptional regulator that has redundant functions to ABI3.
CHAPTER 4

INTERACTIONS OF ABA AND ETHYLENE SIGNAL TRANSDUCTION PATHWAYS IN ARABIDOPSIS SEED GERMINATION AND ROOT GROWTH

A number of experiments in this section were carried out in collaboration with Dr. Eiji Nambara.
INTRODUCTION

A number of physiological findings described the antagonistic and synergistic interactions of ethylene and ABA in plant growth and development. During seed development, ABA functions to establish seed dormancy, an effect that is antagonized by germination promoting functions of ethylene (Abeles et al., 1992; Ketring and Morgan, 1972). In Arabidopsis roots, however, ABA and ethylene appear to work synergistically to inhibit growth (Jackson, 1979; Leung et al., 1994). Although little is known about the specificity of these interactions, there are well-characterized examples of ethylene interactions with other growth factors. For example, Arabidopsis root growth inhibition appears to be an ethylene dependent process that is also influenced by cytokinin. The manner in which cytokinin influences this process is through increased ethylene biosynthesis, by transcriptional induction of the ACC synthase gene, the first committed step in ethylene biosynthesis (Cary et al., 1995).

Alternatively, one set of growth factors may directly influence the sensitivity to a second set of growth factors for communication. For instance, jasmonates and ethylene can both induce similar pathogen defense responses in Arabidopsis (Penninckx et al., 1998). Loss-of-function mutations in the EIN2 locus, a
characterized component of the ethylene signal transduction pathway, results in loss of jasmonate and ethylene-dependent pathogen defense responses. This suggests that the shared jasmonate and ethylene responses are induced by jasmonic acid through the EIN2 segment of the ethylene signaling transduction pathway (Alonso et al., 1999). ein2 mutants have also been recovered in mutant screens for plants resistant to auxin transport inhibitors and cytokinins (Fujita and Syono, 1996; Su and Howell, 1992), suggesting that EIN2 may mediate cross-talk between many growth factor signaling pathways.

Mutations in the Arabidopsis EIN2 locus are recessive mutations that confer insensitivity to both exogenous and endogenous ethylene (Chen and Bleecker, 1995; Roman et al., 1995). These mutants show complete insensitivity to ethylene at the morphological, physiological and molecular levels. In fact, ein2 mutants are the only recessive mutants that effect all aspects of ethylene signaling, and thus, this gene is thought to play a central role in this pathway. The EIN2 gene product does not show any significant similarity to known proteins. However, its N-terminus shows some sequence identity to the integral membrane domain of the disease-related Nramp family of metal transporter proteins (Alonso et al., 1999). However, yeast cells expressing EIN2 failed in the transport of divalent cations, suggesting
functions other than cation transport for this protein. The ectopic expression of the *EIN2* carboxyl-terminus in an *ein2* mutant background results in the restoration of jasmonic acid and paraquat-induced oxygen radical responses. This suggests that the C-terminus of *EIN2* is involved in cross-talk with jasmonate and oxidative stress signaling pathways in *Arabidopsis*. Despite the well-defined role of *EIN2* in ethylene signaling pathway, the mechanism by which this protein influences ethylene signaling remains to be determined. Genetic epistatic analysis of ethylene mutants have placed *EIN2* downstream of the ethylene receptor, *ETR1* (Johnson et al., 1998).

The *ETR1* locus has been characterized by genetically dominant mutations that result in whole plant ethylene insensitivity (Bleecker et al., 1988). *ETR1* encodes a protein with similarity to bacterial two component sensing systems (Chang et al., 1993). Two component systems are the main route by which bacteria sense and respond to external stimuli (Parkinson, 1993). This system is characterized by linking of a sensor domain with an associated response regulator. The carboxyl end of *ETR1* is similar to both the histidine kinase sensor component and the response regulator domain of the two component system. Analysis of genetic epistasis placed *ETR1* early in the ethylene signal transduction pathway (Kieber et al., 1993; Roman et al., 1995), and a substantial body of evidence
indicates that ETR1 is an ethylene receptor. The most compelling
evidence comes from a study of yeast cells expressing wild-type or
mutant ETR1 protein. Cells expressing wild-type ETR1 protein bound
ethylene with a high affinity (Schaller and Bleecker, 1995). ETR1
is present as a small gene family in Arabidopsis, that includes
ETR2, ERS1, EIN4 and ERS2 (Hua et al., 1995; Hua et al., 1998;
Sakai et al., 1998). ERS1, which is almost identical to ETR1 at the
amino acid level, is missing the receiver domain of its response
element. ERS1 has some functional redundancy with ETR1, since the
mutations that confer dominance to ETR1 also resulted in dominant
ethylene-insensitivity to ERS1 when expressed in Arabidopsis (Hua
et al., 1995). ETR2, ERS2 and EIN4 are closely related at their
amino acid levels. The histidine kinase domain of these proteins
lacks major motifs required for its activity (Bleecker et al.,
1998). However, similar to etr1-1 mutation, the ers2 mutations
confer dominant ethylene insensitivity, suggesting that this domain
might not be crucial for ethylene responses in these proteins
(Gamble et al., 1998).

Upon ethylene binding, the ethylene receptor family relays the
signal through CTR1 (Clark et al., 1998). The CTR1 locus has been
classified by recessive mutations that confer constitutive
ethylene response in Arabidopsis. This suggests that CTR1 functions
as a negative regulator of ethylene signaling (Kieber et al.,
1993). The CTR1 gene encodes a serine/threonine protein kinase, most similar to the Raf protein kinase family. Raf is a part of conserved cascade of protein kinases that are central to the signal transduction of a diverse group of external regulatory signals ranging from growth hormones to mitogens and developmental signals (Paul et al., 1997).

In the process of characterizing Arabidopsis mutants with enhanced sensitivity to ABA (era) at the level of germination, it was discovered that two independent alleles of era3 were new loss-of-function alleles of EIN2. Although these alleles are supersensitive to ABA at the level of seed germination they are strongly insensitive to ABA at the level of root growth. Furthermore, a number of other ethylene response mutants also show this developmental dependent alteration in ABA sensitivity. Although functional ethylene signaling molecules are required for a correct ABA response, ethylene biosynthesis is not necessary. Aside from indicating that the ethylene signaling pathway can influence ABA responses in Arabidopsis, the tissue specific alterations in ABA sensitivities observed in ein2 mutants suggest the developmental and physiological history of the tissue profoundly affects how a particular cell will respond to a hormone. In this case it appears roots and embryonic cells have entirely different outputs in response to the ABA signal. I discuss the EIN2 ABA
responses in context of how ABA and ethylene may intersect to regulate seed dormancy and root growth.
RESULTS

Genetic analysis of era3 mutants

The era3-1 mutation was previously identified by screening for seeds that failed to germinate on ABA concentrations not inhibitory to wild-type germination (Cutler et al., 1996). To determine the genetic basis for ABA hypersensitivity in era3-1 seeds, this line was back-crossed to wild-type parental types and its seed ABA sensitivity at the level of germination was scored in the F1 and F2 generations (Table 4.1). ABA hypersensitivity in era3-1 segregates as a simple recessive Mendelian trait. Following this analysis, a second fast-neutron allele, era3-3, was identified based on genetic complementation tests with era3-1 (Table 4.1). Also, during a collaborative project with Dr. Giraudat’s group, a third allele, era3-2, was isolated through a screen for suppressors of the abil mutation, at the level of seed germination. Genetic mapping analysis of the ERA3 locus determined a tight linkage between era3 and ATHCTR1, a small sequence length polymorphism (SSLP) marker at the tip of chromosome V.

The germination of era3-1 and era3-3 seeds displayed ABA hypersensitivity. To determine the levels of seed ABA sensitivity in era3 mutants, seed ABA dose responses for each mutant were measured. Unlike the era1-2 null alleles, seeds of both era3 mutant lines that were older than three months were able to germinate on
0.3μM ABA, but were severely inhibited at concentrations above 0.6μM (Figure 4.1A). era3-1 however, displays a more robust ABA sensitivity at the level of seed germination when compared to era3-3, which may reflect on the severity of lesions in the ERA3 locus in these lines. era1 mutants and aba mutants of Arabidopsis are hyper-dormant and non dormant respectively. To determine whether era3 mutants displayed dormancy phenotypes, seed dormancy was measured in era3-1 (Figure 4.1B). Wild-type seeds, displayed high germination efficiencies within 5 days of imbibition at room temperature. However, the same treatment of era3-1 seeds resulted in less than 5% germination. A chilling period of 4 days alleviated dormancy in these mutants and resulted in germination rates similar to wild type seeds (Figure 4.1B). Similar to era1 mutants, cold rescue of the dormancy phenotype in era3-1 is dose dependent. The recessive nature of the era3 mutations, together with their increased ABA sensitivity and dormancy suggested that the ERA3 gene might play an important role in the negative regulation of ABA dependent seed dormancy.

**era3 vegetative tissues have increased ABA levels**

To investigate the vegetative ABA responses in era3 mutant lines, a number of physiological and molecular markers exist which allow the investigation of various aspects of ABA responses. In
Table 4.1 Genetic segregation of ABA hypersensitivity in *era3-1*. Hypersensitivity is scored as no germination on 0.3μM ABA. Wild-type sensitivity is scored as germination on 0.3μM ABA. Seeds were imbibed for 4 days at 4°C.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Generation</th>
<th>Number of seeds</th>
<th></th>
<th></th>
<th>X² (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>era3-1 x wild type</em></td>
<td>F1</td>
<td>0</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>141</td>
<td>382</td>
<td></td>
<td>1.07</td>
</tr>
<tr>
<td><em>era3-1 x era3-3</em></td>
<td>F1</td>
<td>11</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>era3-3 x era3-1</em></td>
<td>F1</td>
<td>11</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)X² was calculated based on an expected ratio of three germination (wt ABA sensitivity) to one no-germination (ABA hypersensitive at seed germination). P > 0.05
Figure 4.1. A) Seed ABA sensitivity of era3 lines. Seeds were imbibed for 4 days at 4°C. B) Seed dormancy in era3-1. Days chilling is the imbibing time in which the seeds were stored at 4°C. All seeds for this experiment were harvested at the same time from the same age plants. After a 30 day drying period, the seeds were sterilized and plated for dormancy assay. wt is the wild-type ecotype which is Meyerowitz Columbia (MC) for these seeds.
A

Days Post imbibition

[ABA] micromoles

% Germination

B

Days Post imbibition

% Germination

MC

era3-1 (0 day chilling)

era3-1 (2 days chilling)

era3-1 (4 days chilling)
Figure 4.2. ABA-induced proline accumulation in era3-1. Two week old seedlings were floated on liquid media containing 0, 0.3, 3 and 30 µM ABA for 24 hour period before extractions. Each data point represents a mean of 3 independent measurements and the standard deviation from the mean value.
Figure 4.3. Transcript analysis of zeaxanthin epoxidase (ABA1) in era3-1. Two week old seedlings were used in this experiment. The Northern blots were first hybridized with the ABA1 probe and then stripped for β-tubulin hybridization. 5µg of total RNA was loaded per lane.
Figure 4.4 ABA-inducible RAB18 expression in era3-1. A) Wild-type transgenic (RAB18-promoter-GUS) seedlings, from left to right treated with 0, 1, 10, 100μM ABA over a 24 hour period. B) era3-1 transgenic with similar ABA treatment as above. The lines were stained for GUS activity for 12 hours at 37°C.
**Figure 4.5** Root elongation of wild-type, era1-2 and era3-1 seedlings in the presence of plant of growth factors. Two week old seedlings were placed on MS media (agar) Petri plates supplemented with growth factors. The length of the roots were marked and after 3 days of vertical growth, the new root growth was measured. Relative growth is relative to mean root elongation of the same genotype on medium without growth factors. Each value represents the mean and the standard deviation of measurements of at least 10 seedlings.
Arabidopsis, the amino acid proline accumulates under osmotic stress responses and ABA application. Proline functions as a compatible solute to protect cells from osmotic stress damage. Under non-induced conditions, the levels of free proline in era3-1 seedling are slightly higher than the wild-type and era1-2 seedlings (Figure 4.2). However, upon 0.3μM ABA application the proline levels in era3-1 increase 2 fold more than in wild-type and era1-2 samples. ABA accumulation of proline in wild-type and era1-2 mutants was observed at concentrations of 3μM ABA, but even at these concentrations the levels do not reach those observed in era3 seedlings, which might reflect defects in ABA metabolism or signal transduction. To differentiate these possibilities, ABA levels in era3 seedlings were measured. The era3-1 seedlings have on average a 2 fold increase in their fresh weight ABA content when compared to either wild-type or same age era1-2 seedlings (Personal communication from E. Nambara). The increase in ABA accumulation is either the result of increased ABA biosynthesis or decreased catabolism. The transcript analysis of zeaxanthin epoxidase accumulation in era3-1 seedlings, increased in its abundance suggesting increased ABA biosynthesis might be the cause of increased ABA accumulation in these mutants (Figure 4.3). This increase in ABA accumulation is the likely reason for the increased proline accumulation in era3 seedling.
era3 is normal for ABA-induced RAB18 expression in roots and guard cell ABA responses, but shows ABA insensitivity during root growth.

The enhanced ABA-induced accumulation of proline in era3-1 suggests that other ABA regulated processes, such as ABA induced gene expression, stomatal closure and inhibition of root growth, may also be altered in this mutant. To examine ABA-upregulated gene expression in era3-1, the accumulation of transcripts for ABA-upregulated gene RAB18, was monitored in these lines through histochemical staining for β-glucuronidase activity in transgenic era3-1 lines carrying the RAB18 promoter region fused to a GUS reporter gene. Upon ABA induction, the GUS activity was localized to the roots of wild-type and era3 transgenic plants with similar intensities. These findings suggest that the ABA induction of this marker is not altered in era3-1 mutants (Figure 4.4 A,B). To determine the ABA sensitivity of era3 guard cells, stomatal conductance for these plants was measured in soil dehydration assays and by measurements of transpiration rates in detached era3 stems. The stomatal conductance of era3 plants was found to be similar to wild-type plants in these assays (data not shown), suggesting that era3 mutations do not alter guard cell ABA responses. Surprisingly, examination of era3-1 ABA root growth responses showed a reduced ABA sensitivity (Figure 4.5 A). The
variability of tissue specific ABA responses in era3 mutants suggests that the influence of ERA3 on ABA responses is dependent on specific stages of development.

A number of growth factor response mutants display changes in sensitivities to additional growth factors (McCourt, 1999). To determine if era3 mutations result in alterations in other growth factor sensitivities, root growth on cytokinins, auxin and ethylene were measured. The roots of era3-1 displayed a reduced sensitivity to the cytokinin, kinetin, and the ethylene precursors ACC, whereas root growth on auxin was not altered (Figure 4.5 B,C).

In fact, these patterns of growth factor sensitivities were similar to that reported for ein2 mutants. Genetic mapping of EIN2 has positioned this gene in the close vicinity of the ATHCTR1 marker. Therefore, genetic allelism tests between these mutants were carried out. era3-2 does not complement ein2 and hence is allelic (personal communication with J. Giraudat). Southern blot analysis of both fast neutron era3 alleles identifies a number of molecular polymorphism at this locus. Therefore, we believe era3-1 and era3-3 are new alleles of ein2 and have designated our alleles ein2-41 and ein2-43 respectively.
Inhibition of root growth by ABA requires functional ethylene signal transduction

EIN2 serves as an important converging point in the ethylene and jasmonate signal transduction pathways (Alonso et al., 1999). To determine whether EIN2 performs the same function in ABA signaling, etrl-4 roots were tested for growth sensitivity to ABA. At 10μM ABA, where wild-type root growth is inhibited by 50%, etrl-4 roots showed 100% relative growth. This shows that, similar to ein2, etrl-1 also displays ABA insensitivity at the level of root growth. This suggests that EIN2 is not the point of cross talk for ABA signaling and this point is at or before the ETR1 receptor (Figure 4.6). In addition, it appears that functional ethylene signal transduction is required for ABA inhibition of root growth.

Ethylene and ethylene signaling have been shown to be required for cytokinin inhibition of root growth. Cytokinin appears to influence this process through stimulation of ethylene biosynthesis (Cary et al., 1995), which may be inhibited through the application of ethylene biosynthetic inhibitors such as aminoethoxyvinylglycine (AVG) or ethylene signal propagation inhibitors, such as silver ions (Sisler, 1990; Yang and Hoffman, 1984). To examine the ABA mode of action on ethylene induced root growth, ABA responsiveness of wild-type and mutant roots were measured in the presence of the ethylene biosynthetic inhibitor AVG and the ethylene signal
propagation inhibitor, Ag⁺ (Figure 4.7). Similar to the etrl-4 mutation, Ag⁺ suppresses the inhibitory effects of 10 μM ABA on wild-type root growth. However, AVG, an ethylene biosynthetic inhibitor, elicits the opposite effect, making roots more sensitive to ABA inhibition. This suggests that ABA inhibition of root growth is independent of ethylene. Thus, unlike cytokinin-induced ethylene inhibition of root growth, ABA inhibition is not mediated via ABA induced ethylene synthesis. Similar results to AVG were observed when AVG was substituted with α-aminoisobutyric acid (AIB), an alternative ethylene biosynthetic inhibitor (data not shown) (Satoh and Esashi, 1983).

**Ethylene is a negative regulator of ABA action in Arabidopsis seeds**

Both genetic and chemical inhibition of ethylene action result in altered root ABA responsiveness suggesting that ethylene signal transduction is closely tied to ABA responses. ein2 mutations confer an increased sensitivity to ABA at the level of seed germination, thus, other ethylene response mutants should be perturbed in germination responses. For example, in many cases etrl alleles show poor germination in the absence of exogenous GA (Bleecker et al., 1988). To test this hypothesis, we examined the sensitivity of germination to exogenous ABA in etrl and ctr1 seeds. In contrast to wild-type seed plated on 0.3μM ABA and etrl-1 seed
plated on minimal media, etrl seed plated on 0.3μM ABA did not germinate even after five days of incubation at room temperature (more than 100 seeds tested, 3 independent times). Therefore, as observed with ein2 seed, etrl mutants have an enhanced response to exogenous ABA at the level of germination. Unlike ethylene insensitive mutants, ctrl is a loss-of-function mutation that confers a constitutive ethylene response to plants (Kieber et al., 1993). In principle, this mutant should have a decreased sensitivity to ABA at the level of germination. As expected, ctrl seeds germinate on 3μM ABA, a concentration that inhibits wild-type germination (Figure 4.8). Furthermore, the ctrl response can be phenocopied in wild-type seed by addition of ACC.

The germination phenotypes of individual ethylene response mutants in the presence of various concentrations of ABA predict that mutations that alter ethylene action should interact with mutations that perturb ABA responses. In fact, the screens for suppressors of abil at the level of germination have yielded ein2 mutants and screens for enhancers of abil mutations have yielded ctrl mutants.
Figure 4.6 Root elongation in wild-type, era3-1 and etrl-4 seedlings in the presence of ABA. Two week old seedlings were placed on MS media (agar) Petri plates supplemented with ABA. The length of the roots were marked and after 3 days of vertical growth, the new root growth was measured. Relative growth is relative to mean root elongation of the same genotype on medium without growth factors. Each value represents the mean and the standard deviation of measurements of at least 10 seedlings.
[ABA] microMolar

Relative root growth

---

- **wt**
- **era3-1**
- **etr1-4**
**Figure 4.7.** Root elongation of wild-type seedlings in the presence of AVG and AgNO₃. Two week old seedlings were placed on MS media (agar) Petri plates supplemented with inhibitors. The length of the roots were marked and after 3 days of vertical growth, the new root growth was measured. Relative growth is relative to mean root elongation of the same genotype on medium without growth factors. Each value represents the mean and the standard deviation of measurements of at least 10 seedlings.
wt + 10 microMolar AgNO3
wt + 2 microMolar AVG

[ABA] MicroMolar

Relative root growth
Figure 4.8. Germination of Wild-type and ctrl-1 mutant seeds in the presence of ethylene precursor, ACC. Each point is the mean value of 3 independent experiments, more than 50 seed per each experiment, per genotype were scored.
Days post imbibition on 3 microMolar ABA
DISCUSSION

The original enhanced response to ABA (era) screen was devised to identify negative regulators of ABA signal transduction (Cutler et al., 1996). One mutant class, designated era3, has an increased sensitivity to ABA at the level of germination and produces dormant seed that requires extended chilling or over-ripening periods to germinate efficiently. Surprisingly, some vegetative ABA responses in era3 mutants have reduced sensitivity to ABA and other growth factors. Subsequent genetic analysis has shown that era3 mutations are actually new loss-of-function alleles of EIN2, a gene that was originally identified by screening dark grown Arabidopsis seedlings for a reduced ethylene induced triple response (Chen and Bleecker, 1995; Roman et al., 1995). The ein2 mutation that confers an ethylene insensitive phenotype is recessive, suggesting the EIN2 gene product encodes a positive regulator of ethylene action. Other genetic screens including resistance to auxin inhibitors, and insensitivity to cytokinins, however, have also recovered recessive ein2 alleles (Fujita and Syono, 1996; Su and Howell, 1992). Furthermore, ein2 mutants show cross-resistance to the growth factor jasmonate, and the photosynthetic inhibitor paraquat (Penninckx et al., 1998). The multitude of response defects seen in ein2 mutants may mean that the EIN2 gene product is a signal intersection point for a number of response pathways in
Arabidopsis. Recent molecular dissection of the EIN2 gene has shown that this gene encodes separable functions for ethylene and jasmonate responses (Alonso et al., 1999). Alternatively, ethylene itself may play a pivotal role in the regulation of a number of these response pathways. For example, inhibition of root growth in Arabidopsis by cytokinins is directly due to the production of ethylene (Cary et al., 1995).

**Interactions between ethylene and ABA seed responses**

Molecular genetic analysis of ethylene action in plants has identified a family of two-component receptor kinases (ETR1, ERS2, ETR2, EIN4) that bind ethylene (Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998). Perception of ethylene subsequently leads to the activation of the CTR1 protein kinase, which in turn negatively regulates EIN2 (Johnson and Ecker, 1998). Genetic screens to identify ABA response genes in Arabidopsis have identified two protein phosphatases (ABI1, ABI2), a protein farnesyltransferase (ERA1) and two transcription factors (ABI3, ABI4). However, in contrast to epistatic studies involving ethylene response mutants, genetic relationship between ABA response mutants has been difficult to discern. Mostly, this stems from a lack of multiple mutant allelic forms of these genes (McCourt, 1999). For example, only one dominant allele of ABI1 exists which confers an
ABA insensitive phenotype. Because no loss-of-function alleles exist for this gene it is difficult to assign wild-type ABII functions in ABA responses.

Physiological studies have indicated a role for ethylene in the promotion of seed germination and some evidence suggests ethylene influences this process by antagonizing the inhibitory effects of ABA on germination (Ketring and Morgan, 1972). Ethylene could accomplish this by negatively regulating seed ABA synthesis as has been reported for ethylene function in deep water rice (Kende et al., 1998). The increased ABA levels measured in ein2 adult plants indicate that ethylene sensitivity and ABA synthesis are tightly regulated. However, the fact that the abil mutation is suppressed by ein2 (personal communications J. Giraudat) and enhanced by ctrl suggests ethylene response mutants can influence ABA sensitivity more directly. The abil mutants are insensitive to ABA at the level of germination and the small increase in ABA concentration resulting from the ein2 mutation would not be expected to overcome the strong ABA insensitivity of abil seeds. Furthermore, although the ctrl mutation is a synergistic enhancer of seed ABA insensitivity in abil, the ctrl mutation by itself only results in a weak insensitivity to exogenous ABA. During wild-type seed development the embryo is exposed to endogenous ABA resulting in a state of dormancy that persists after the ABA levels decline.
Possibly, the activation of the ethylene pathway in immature ctrl embryos decreases the ability of ABA to establish dormancy. Consistent with this, ethylene insensitive mutants require prolonged ripening or chilling to germinate because they are in a deeper state of ABA-induced dormancy. Similar models have been proposed to explain the opposing influences of GA and ABA on setting levels of seed dormancy (Karssen and Lacka, 1985). Furthermore, GA application does rescue the hyper dormant phenotype of etrl mutants suggesting these three hormones may act in concert to establish the overall state of dormancy in the seed (Figure4.9 A).

**Interactions between ethylene and ABA vegetative responses**

The kinetics of vegetative ABA responses such as guard cell closure, gene expression or growth inhibition suggest ABA signal outputs vary in different tissues. In ein2 mutants, guard cell responses and RAB18 gene expression appear to be normal whereas root growth is less sensitive to ABA. Perhaps ein2 mutants define separate response pathways that are differentially affected by ethylene. For example, ABA may act directly on guard cells, but interact with ethylene signaling in the regulation of root growth. In wild-type plants, for example, root growth is responsive to direct ethylene application (Abeles et al., 1992). Possibly, ABA
induces ethylene synthesis, which in turn inhibits root growth. If true, compounds that inhibit ethylene synthesis or action should confer an ABA insensitive phenotype. Although the ethylene action inhibitor AgNO₃ does confer a root ABA insensitive phenotype, inhibition of ethylene synthesis using AVG or AIB does not. These results suggest ABA does not function to stimulate ethylene synthesis but ABA does require ethylene signaling components to elicit some responses. Interestingly, studies using ethylene biosynthetic and response inhibitors to understand the interaction of ethylene in IAA-induced elongation in light grown Arabidopsis hypocotyls gave similar conflicting results (Smalle et al., 1997). It must be noted, however, that the addition of hormone inhibitors in these experiments and ours, were preformed at saturating concentrations and, therefore, may not be physiologically relevant.

Nevertheless, it is possible that ABA inhibition of root growth and auxin promotion of hypocotyl elongation may be modulated by ethylene-signaling components in an ethylene-independent manner. Aside from the ethylene-binding domain, protein sequence analysis of the ethylene receptor family has defined a GAF domain that is thought to be important for cGMP binding in other systems (Bleecker et al., 1998). In addition, structural comparisons of ethylene receptors has raised the possibility that the histidine kinase activity associated with ETR1 may have roles outside of ethylene
signaling or may allow a subset of receptors to participate in additional pathways (Gamble et al., 1998) (Figure 4.9 B).

The observation that ein2 mutants, which were identified by an ABA supersensitive screen at the level of germination, are insensitive to ABA at the level of root growth is intriguing. Mutations that alter IAA and cytokinin sensitivities in opposite directions in different tissues have also been reported (McCourt, 1999). Developmentally different output responses to ABA may simply reflect different mechanisms for hormone responses in different cell types. In some tissues, ABA may repress events whereas in others it may have an inductive role. In these cases, the signal state determines whether loss-of-function mutations will be translated into inhibition or activation of the plant process. For example, the root and seed ABA response phenotypes of ein2 mutants can be explained if ABA activates a positive regulator of dormancy establishment but activates a negative regulator of root growth. In both cases activation leads to opposite sensitivities. Because the molecular nature of any ABA response is not understood, it is difficult to interpret developmentally dependent alterations in hormone sensitivity. However, ethylene response mutants can now be used as tools to study the interactions of ethylene with ABA.
response and should provide a clearer picture of how these two hormones intersect in signal transduction.
Figure 4.9 Models for ABA and ethylene signal transduction pathways interactions in seeds and roots of Arabidopsis. A) In seeds, ABA (purple) acts to inhibit germination (blunt arrow). Ethylene and/or ethylene signal transduction components (orange) act as negative regulator of seed ABA signaling for germination. B) ABA inhibits root growth through ethylene signal transduction pathway. First arrow (top) represents positive action of ABA signaling at ETR1, the point of intersection of ABA signaling with ethylene signaling components (orange). Second arrow represents other root ABA signaling pathway that may involve the axr2 and AXR4 (Hobbie and Estelle, 1995) gene product.
A

Embryo maturation → ABA → Ethylene

B

ABA

Root growth
GENERAL CONCLUSIONS AND FUTURE PROSPECTS

Our poor understanding of ABA signaling stems from the shortage of ABA response mutants that exist. This may be due to lethality or possibly because of redundancies in ABA signaling. These redundancies have created a need for new screens to accommodate these realities. One way to overcome these drawbacks is by screening in a sensitized genetic background. Here an abil background was used. In addition, the characterization of ein2/era3 ABA responses have provided insights regarding the biological interactions of ethylene and ABA in seeds. These studies have also suggested a role for the ethylene signal transduction pathway in root ABA signaling and have provided more evidence for ABA signaling redundancies (Figure GC1.1). For example, it was shown that ABA may signal root growth inhibition through the ethylene signaling pathway. However, it should be noted that root growth is not altogether insensitive to ABA when ethylene signaling is inhibited and only show reduced sensitivities in the tested mutants. This suggests the presence of other signaling pathways for ABA inhibition of root growth. Since auxin inhibition of root growth is not affected in ethylene insensitive mutants, another signaling pathway that could lead to root growth inhibition is that of the growth factor auxin. In addition, the auxin resistant
mutants, axr2 and axr4 (Hobbie and Estelle, 1995), are also insensitive to ABA inhibition of root growth, suggesting that ABA may use auxin signaling or even other unidentified signaling pathways for its inhibition of root growth. It will be interesting to test the root ABA responsiveness of ein2/axr4 double mutants to see if ABA responsiveness is further reduced since this would be predicted for two independent pathways.

With the completion of the Arabidopsis genome sequencing project in the near future, the identification of mutant loci identified by mutant screens will facilitate the cloning of relevant genes. However, it is my opinion that mutant screens are still the most effective manner for assigning gene function. For this reason, I propose a number of new screens that may result in the isolation of new ABA response mutants. The mutant screen for enhancers of abi1 have shown promise in isolating mutations in genes which may play redundant roles in the induction of ABA responses. Future screens for enhancers of the abi2 mutation may allow for the identification of different ABA related loci, since abi2 affects a number of abi1-independent genes. Screens for enhancers of abi3 mutations may reveal a different spectrum of mutants with ABA responses, since ABI3 functions appear to be redundant in vegetative ABA responses.
ABA hypersensitive screens are also useful in identifying redundancies in ABA signaling. The problem with this type of screen is the role that ABA performs in the establishment of seed dormancy. This ABA function may cause a strong ABA hypersensitive mutant to become severely hyperdormant and hence not recoverable in ABA hypersensitivity screens. An approach that may overcome this problem, is using an ABA auxotrophic background in new ABA hypersensitivity screens. This background could allow the isolation of new era mutants not recoverable by the original era screen.

*abel* and *abe2* mutations both map to sequenced regions in chromosome V. With the potential roles that *ABE1* and *ABE2* may play in ABA signaling, the cloning of these genes may be helpful in identifying new components which influence ABA responses.
Figure GC1.1 A general model for ABA pathways in Arabidopsis. The wild type and/or mutant loci that influence the pathway(s) are drawn on the pathway(s). Arrows represent pathways with positive influences and blunt end lines represent pathways with negative influences. The information for this model comes from integration of known ABA pathways with the information obtained from this thesis.
Seed Germination

ETR1-CTR1-EIN2

ABA

Stomatal Closure

Root Growth

β-TUB CAB

ADH RAB18 RAB21

Vegetative osmo-acclimation responses
APPENDIX 1

CHARACTERIZATION OF TRANSGENIC Arabidopsis LINES CARRYING A CONSTRUCT OF RAB18 PROMOTER FUSED TO THE GUS CODING REGION
ABA is involved in the induction of cellular acclimation responses to osmotic stress (Leung and Giraudat, 1998; Shinozaki et al., 1998). These responses include the transcriptional induction of a number genes which code for compatible solute biosynthetic enzymes, cellular repair components and dehydrin type proteins. In addition to ABA, a number of other stress signaling pathways may lead to transcriptional induction of these genes (Shinozaki et al., 1998). Due to redundancies in the transcriptional up-regulation of these genes, it has been difficult to isolate markers that have an ABA-dependency for their up-regulation. In Arabidopsis however, the drought inducible marker RAB18, represents one of the few markers that appear to be specifically ABA-dependent for vegetative expression. In an ABA biosynthetic mutant background, or by using ABA biosynthesis inhibitors, RAB18 transcripts fail to accumulate under a number of stress treatments such as dehydration, high salinity, or high temperatures (Lang and Palva, 1993).

The rapid detection of RAB18 transcripts may serve as a useful tool to examine ABA responses in Arabidopsis. To facilitate the detection of expression of a number of genes in Arabidopsis, GUS (β-glucoronidase) reporter constructs have been used. The expression of GUS protein in these lines, which is readily detectable by various methods, represents the expression of the
marker gene. Similar to this method, an Arabidopsis transgenic line, carrying a construct with the RAB18 promoter regions fused to the GUS coding region was created and assayed for GUS activity as an indication of RAB18 expression.

**RAB18 expression in embryonic tissue and young seedling**

RAB18 transcripts have been shown to accumulate in embryonic tissue (Parcy et al., 1994). To test this, transgenic wild-type embryos were stained for GUS activity (Figure APN. 1.1). These lines showed strong GUS activity in the embryo and young seedlings. However, 12 days after germination, the GUS expression was only localized to the cotyledons.

**The ABA induction of RAB18**

The transcriptional induction of RAB18 in vegetative tissue has been shown to be ABA dependent (Lang and Palva, 1993). To test the ABA inducibility of RAB18-GUS lines, they were exposed to 0, 1, 10 and 100µM ABA (Figure APN. 1.2). The roots of these plants showed increased GUS activity with increasing ABA concentrations. The ABA insensitive mutant, abil, displayed reduced GUS activity under the same conditions, suggesting that ABA application does not result in RAB18 transcript accumulation (Figure APN. 1.3). However, the ABA hypersensitive mutant era1 displayed wild-type RAB18 driven
GUS activity, suggesting that the signaling pathway(s) leading to RAB18 expression is not hypersensitive to ABA in eral (Figure APN. 1.4). The eral and abil embryos showed similar GUS activity as wild-type embryos (data not shown), suggesting that embryonic expression of RAB18 is not defective in eral or abil.

Application of ABA to mature leaves of the wild-type transgenic plants resulted in the detection of GUS activity in guard cells and weaker presence in the other cells (Figure APN. 1.5A). When leaves of the same transgenic plants were detached and allowed to dry at room temperature, a treatment that has been shown to increase endogenous ABA levels, a similar GUS activity was detected in the guard cells (Zeevaart and Creelman, 1988) (Figure APN. 1.5B). These suggests that guard cells are either more ABA responsive than other leaf cells, or there might be a rapid ABA transport mechanism to the guard cells resulting in increased RAB18 transcript accumulation in guard cells.

The preliminary analysis of the transgenic RAB18-GUS lines, demonstrates that the induced GUS activity in the vegetative tissue, shows ABA-responsiveness and hence, can be useful to examine vegetative ABA responses in Arabidopsis.
Figure APN. 1.1  The expression of RAB18 in embryos and young seedlings of wild-type RAB18-GUS transgenic lines. From left to right are; mature embryo (seed coat is seen below the embryo), and, 2, 4, 6, 8, 10, and 12 days old seedlings. The seedlings were incubated with the GUS staining solution for a period of 12 hours at 37°C. More than 20 seedlings and embryos for each time point were examined.
**Figure APN. 1.2**  
*RAB18* ABA-induction in wild-type-*RAB18*-GUS transgenic lines. 2 week old seedlings were treated from left to right with 0, 1, 10, and 100μM ABA for a 24 hour period. The seedlings were incubated with the GUS staining solution for a period of 12 hours at 37°C. More than 20 seedlings for each ABA treatment were examined.
Figure APN. 1.3  RAB18 ABA-induction in abi1-RAB18-GUS transgenic lines. 2 week old seedlings were treated from left to right with 0, 1, 10, and 100μM ABA for a 24 hour period. The seedlings were incubated with the GUS staining solution for a period of 12 hours at 37°C. More than 20 seedlings for each ABA treatment were examined.
Figure APN. 1.4  RAB18 ABA-induction in era1-2-RAB18-GUS transgenic lines. 2 week old seedlings were treated from left to right with 0, 1, 10, and 100μM ABA for a 24 hour period. The seedlings were incubated with the GUS staining solution for a period of 12 hours at 37°C. More than 20 seedlings for each ABA treatment were examined.
Figure APN. 1.5  

*RAB18* induction by ABA application and dehydration treatment in mature leaves of wild-type-*RAB18*-GUS transgenic line. A) The section of a mature leaf shown in this picture was treated with 10\(\mu\)M ABA for a 3 hour period before GUS staining. B) A mature leaf was detached from the mother plant and allowed to dehydrate for a 3 hour period at room temperature before staining. Both pictures represent the areas on the leaves that showed GUS staining. The leaves were incubated with the GUS staining solution for a period of 12 hours at 37°C.
APPENDIX 2

ISOLATION OF PROTEIN INTERACTORS OF Abi1 IN A

YEAST TWO-HYBRID SYSTEM
Protein-protein interactions are essential events in many signal transduction pathways. These interactions may serve as the basis for screens to identify signaling components of a specific signal transduction pathway. Such screens for protein interactors of ABI1 however, have not resulted in the isolation of components of ABA signaling (personal communications with J. Leung). This may be the result of the weak interactions that take place between ABI1 and its substrates. The abil mutation however, may serve as a better substrate in protein interaction screens. The abil carries a point mutation that causes an amino acid residue substitution in its active site. This change has been hypothesized to disrupt the conformation of the metal-coordinating residues that are critical for its enzymatic activity, resulting in reduced PP2C activity (Das et al., 1996). In agreement with this hypothesis, in vitro analyses of the Abil mutant isoform has shown a \( \approx 70\% \) reduction in its protein phosphatase activity (Bertauche et al., 1996). This loss of protein phosphatase activity in abil, and its semi-dominant phenotypes suggests that, abil may function as a dominant-negative type mutation. The dominant negative effects may be the result of the mutant enzyme forming complexes with its substrate(s) that may be more stable than the wild-type ABI1-substrate(s) complexes. Therefore, the Abil mutant isoform may provide a more suitable bait in screens for its protein interactors.
Yeast interaction traps provide an in vivo assay for interaction between two specifically constructed proteins. Protein interaction is assayed in yeast by using transcription of yeast reporter genes to measure the protein interaction. In the system used in this study, \textit{abi1} (amino acids 123-434) was fused to a bacterial DNA binding domain (LexA in pEG202) to create a bait protein and library cDNA encoded interacting protein was expressed as fusion to a transcription activation domain in (pJG4-5). Proteins that interact, activate transcription of specially designed reporter genes that carry the binding sites for LexA protein. Interaction trap cloning in yeast was performed essentially as described in Finley and Brent (Finley and Brent, 1995). All positive ABI1 mutant isoform interactors are listed in table APN. 2.1.

A number of yeast protein interactive screens for plant proteins repeatedly isolate Rubisco and thioredoxin (personal communication with J. Leung). However, a new \textit{Abi} mutant isoform interactor, from 4 independent pools was recovered. This was named, \textit{interactor of Abi} (ABI1 mutant isoform) clone 1 (INA1). The INA1 sequence shows no identity to known proteins.

ABI1 AND ABI2 transcripts increase with ABA applications, suggesting a role for these proteins in ABA mediated responses. To examine the ABA inducibility of INA1 transcripts, wild-type

135
seedling were treated with 0, 10 and 100 μM ABA for a 24 hour period, and examined for INAl transcript accumulation. The INAl transcripts were present in low abundance in 2 weak old seedlings. In addition INAl transcripts accumulated in response to ABA treatment (Figure APN. 2.1). This suggests that INAl may play a role in ABA influenced processes.

To determine the specificity of the interaction between INAl and ABI1 mutant isoform, a bait plasmid containing the wild-type ABI1 gene was constructed and tested for its interaction with INAl in a yeast two-hybrid assay. The wild-type ABI1 protein does not appear to activate the transcription of the β-galactosidase gene in the presence of INAl (Figure APN. 2.2 A and B). This suggests that ABI1 and INAl do not interact in a yeast two-hybrid assay. With the exception of the single base substitution in abil, the baits in these assay were identical, suggesting that the mutation in abil could be the factor causing the interaction of Abil mutant isoform and INAl. There are two possibilities that may explain why the wild-type ABI1 did not interact with INAl in this system. One possibility is that the interactions of ABI1 protein and INAl are generally weak and undetectable in a yeast interaction assay. Alternatively, the abil mutation could be neomorphic and cause the recognition of new targets, in this case INAl. Since abil was shown
not to be neomorphic, the first possibility is a more likely one (chapter 3).

The lack of interaction between ABI1 with INA1 may be the result of efficient dephosphorylation of INA1 protein by ABI1. To test this possibility, ABI1, Abil mutant isoform and INA1, were expressed and isolated as GST fusion proteins in *E. coli*. The INA1 was then phosphorylated with a cAMP dependent protein kinase (NEBiolabs) according to the manufacturers instructions. The resulting substrate was then used in a protein dephosphorylation assay using ABI1 and ABI1 mutant isoform (Figure APN. 2.3 A,B) (Bertauche et al., 1996). The products of the reactions were fractionated by electrophoreses on SDS-PAGE gels. The ABI1 protein was able to catalyze the removal of the radioactive phosphates from INA1. However, the ABI1 mutant isoform, which has been reported to have approximately 30% of PP2C activity of wild-type ABI1, showed a dramatic reduction in PP2C activity in this assay. This suggests that the specificity of the ABI1 mutant isoform interaction with INA1 may be the result of its defective PP2C active site.
Table APN2.1 List of all positive interactors of Abil mutant isoform. Each clone is from an independent pool of transformants. The insert size was determined by EcoR1/XhoI excision of the insert from the pJG4-5 vector. Genbank accession numbers were identified from the Arabidopsis data bank BLAST search engine. Clones were retested by isolating plasmids from the yeast strain using E.coli transformation and transformation of the original yeast test strain with the recovered plasmid. Some positive clones had a ACACACA repeat sequences at the junction of cDNA and the activation domain. These are listed as cloning artifacts.

<table>
<thead>
<tr>
<th>POOL#</th>
<th>size of insert</th>
<th>GENE BANK #</th>
<th>Similarity from BLAST search</th>
<th>comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>450bp</td>
<td>ATF9D1</td>
<td>putative protein</td>
<td>Re-tested</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AL035394</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AI997178</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>450bp</td>
<td>Same as clone 10 (INA1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>450bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>450bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>700bp</td>
<td>T22228</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>650bp</td>
<td>T22228</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>450bp</td>
<td>X13611</td>
<td>RUBISCO (S)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>450bp</td>
<td>X13611</td>
<td>RUBISCO (S)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>300bp</td>
<td>Z35474</td>
<td>THIOREDOXIN</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>600bp</td>
<td>?</td>
<td>SHORT POLYPEPTIDE</td>
<td>cloning artifact</td>
</tr>
<tr>
<td>14</td>
<td>350bp</td>
<td>?</td>
<td>SHORT POLYPEPTIDE</td>
<td>cloning artifact</td>
</tr>
<tr>
<td>42</td>
<td>900bp</td>
<td>?</td>
<td>SHORT POLYPEPTIDE</td>
<td>cloning artifact</td>
</tr>
</tbody>
</table>
Figure APN2.1 Transcript analysis of INAl. 2 week old seedlings were treated with 0, 10, and 100μM ABA for 24 hours. Approximately 5μg of total RNA was loaded per lane. The order of probes used was, INAl, RAB18, and rRNA. A weak signal for INAl transcripts is detectable in the 0 and 10μM ABA treated samples but cannot be seen in this figure.
Figure APN2.2  ABI1 and INA1 protein interactions in a yeast two-hybrid system. A) First two colonies from left are two independent yeast clones carrying the Abil-LEXA bait and the INA1-transcriptional activation domain. The second set of colonies are two independent yeast clones carrying the ABI1-LexA bait with the INA1-(transcriptional activation domain). These colonies were stained for β-galactosidase activity on the same filter for the same period of time (3 hours, RT). B) Western analysis using LEXA antiserum detect the LEXA protein in the clones tested above. Lane 1 is the control yeast strain carrying the bait vector with no inserts (pEG202). Lane 2 contains extracts from the yeast clone tested above carrying the ABI1-LEXA bait. Lane 3 contains extracts from the yeast clone tested above carrying the Abil-LEXA bait.
Figure APN2.3  Protein phosphatase assay for the interaction of Abil mutant isoform and INA1. A) INA1-GST protein was \(^{32}\)P rATP radio-labeled and added to all reactions except for lane 9 and 10 (MW marker). All lanes contain 1μg BSA. Lane 7 contains 1μg of unlabeled INA1-GST protein. The ABII or Abil-GST proteins were added as below:

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abi</td>
<td>100ng</td>
<td>Abi</td>
<td>50ng</td>
<td>Abi</td>
<td>25ng</td>
<td>Abi</td>
<td>25ng</td>
<td>Abi</td>
<td>100ng</td>
</tr>
</tbody>
</table>

B) Film exposed for 20 minutes to the dried gel above. The INA1-GST fusion was isolated with 2 other proteins, GST, and a second GST fusion protein that may be the result of protein degradation or premature translational termination of the GST-INAI transcript in *E.coli*.
BIBLIOGRAPHY


Arabidopsis is linked with competence for cell division. Plant Cell 5, 1711-23.


Schroeder, J. I., Schmidt, R. C., and Scheaffer, J. (1993). Identification of high-affinity slow anion channel blockers and


