FUNCTIONAL GENOMICS ANALYSIS OF THE *ARABIDOPSIS* ABI5 BZIP TRANSCRIPTION FACTOR

A Dissertation

by

JUNG-IM HUR

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Molecular and Environmental Plant Sciences

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Approved by:

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ABSTRACT

Functional Genomics Analysis of the *Arabidopsis* ABI5 bZIP
Transcription Factor. (December 2007)
Jung-Im Hur, B.S., YeungNam University;
M.S., YeungNam University
Chair of Advisory Committee: Dr. Terry L. Thomas

During embryogenesis, the architecture of the plant and the food reserves for seed germination are established. Abscisic acid (ABA) regulates seed development and dormancy. It controls genes involved in stress responses. ABA-responsive basic leucine zipper (bZIP) transcription factors are identified by interaction with ABA responsive *cis*-regulatory elements. The transcription factor ABI5 is one of these. It regulates gene expression during embryogenesis and in response to ABA. An ABA-insensitive mutant, *abi5-6,* exhibits no gross morphological defects other than the effect on seed germination in the presence of ABA. Thus, microarray analysis was employed to search for molecular phenotypes. We used cDNA microarrays to analyze ABA regulated gene expression and the role of ABI5 in seedlings. 310 genes were identified as ABI5/ABA regulated genes. 161 of these genes were regulated by ABI5, and 134 of ABI5-regulated genes were co-regulated by ABA. Only a small number of genes altered expression in both Pro35S:ABI5 and *abi5-6* genetic backgrounds indicating the preferential binding of the bZIP protein dimers to specific promoter sequences. To determine the optimal platform for identifying ABI5-regulated genes in seeds, a cDNA microarray, the Agilent *Arabidopsis* Oligo microarray, and the Affymetrix ATH1 arrays were tested. Cross platform comparisons utilized 4,518 genes present on all three platforms. The best correlation was between the Agilent and the Affymetrix results. Furthermore, the Affymetrix

results correlated best with qRT-PCR validation data for selected genes. A small number of genes including *AtCOR413 pm-1* showed a consistent expression pattern across the three platforms. A robust ABRE *cis*-regulatory element was identified in the promoter of *AtCOR413 pm-1*. Further studies showed binding of ABI5 to the promoter of *AtCOR413 pm-1* by Electrophoretic Mobility Shift Assays (EMSA) and validated the expression of *ABI5* and *AtCOR413 pm-1* in *abi5-6* seeds by qRT-PCR and RNA gel blot analysis. Transactivation assays using *AtCOR413 pm-1* promoter:GUS fusions in *Arabidopsis* dry seed and seedlings revealed ABI5 acts as a negative regulator for *AtCOR413 pm-1* in dry seeds, while other proteins may play major roles in regulating responses to ABA and low temperature (LT) in seedlings.

DEDICATION

I dedicate this dissertation to my parents and my husband, Daehoon, for their love and support during my graduate study.

ACKNOWLEDGEMENTS

I would like to express the greatest appreciation to my advisor, Dr. Terry Thomas, for providing me an opportunioty to work in his laboratory as a graduate student. His insightful advice, encouragement, support, and patience helped me through a tough period to accomplish my goal and also guided me throughout the course of my graduate study.

I also would like to express sincere gratitude to my committee members, Dr. Thomas McKnight, Dr. Page Morgan, and Dr. Alan Pepper, for their support and guidance during my graduate study.

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Last, but not least, I would like to express my sincere appreciation to all great teachers I have had for their encouragement and support that made me grow as a scientist.

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CHAPTER I
INTRODUCTION: ABSCISIC ACID (ABA) AND ABI5 BZIP TRANSCRIPTION FACTOR

Environmental stresses, such as drought, cold, high salinity and low temperature, have adverse effects on plant growth and seed production. Plants respond and adapt to these stresses through various biochemical and physiological processes, thereby acquiring stress tolerance. The phytohormone abscisic acid (ABA) integrates environmental signals that are linked to changes in water availability with metabolic and developmental programs. In developing seeds, ABA regulates several key processes including suppression of precocious germination prior to desiccation, the induction of dormancy, and the accumulation of seed reserves. Despite large amounts of physiological, molecular, genetic, and biochemical data implicating the involvement of ABA in stress response and developmental programs, ABA-mediated regulatory pathways are still largely unknown.

ABSCISIC ACID (ABA)

Plant hormones (phytohormones) are small organic molecules that are mediators of endogenous developmental programs and that integrate extracellular signals to regulate and optimize plant growth. ABA is ubiquitous in higher plants; it plays an important role during plant growth and development

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This dissertation follows the style and format of The Plant Cell.

and regulates plant development through complex signaling processes. ABA regulates many processes, including gene expression, closure of stomata to reduce water loss due to transpiration, photosynthesis, and adaptation to environmental stresses. It is also involved in signal transduction pathway(s) by regulating gene expression at specific developmental stages or in response to environmental stresses (Bray, 1993). The stomatal responses are relatively fast, occurring within minutes and involving changes in the activity of various signaling molecules and ion channels. Other ABA mediated responses, such as the induction of tolerance of water, salt, and cold stress, are slower and require changes in gene expression (Finkelstein et al., 2002).

In contrast to other plant hormones, ABA concentration changes dramatically in response to environmental stress or developmental cues (Bonetta and McCourt, 1998). In vegetative tissues, ABA accumulates in response to drought, salinity and cold stresses. High levels of ABA are accumulated during seed maturation and then the ABA content drastically decreases by completion of maturation (Karssen et al., 1983; Weatherwax et al., 1996).

EFFECTS OF ABA ON SEED DEVELOPMENT

ABA is more important in seeds than in other plant organs. In seeds, ABA plays an important role to improve seed survivability and to prevent precocious germination during seed development. In seeds, after the embryo pattern is completed, ABA concentrations rise while the embryo establishes dormancy and storage reserves increase. Early in development, the ABA content is low (Fig. 1-1). As development proceeds, ABA levels increase reaching a maximum at about 50% higher than the levels as the initiation of maturation; ABA levels then

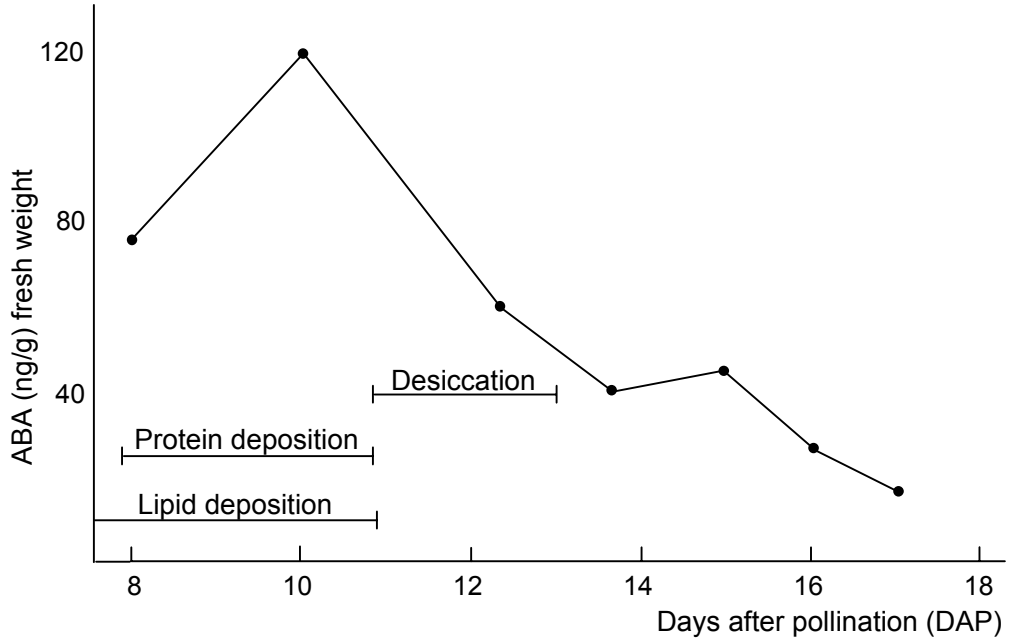


Figure 1-1. Changes in ABA concentration during *Arabidopsis* embryo development. (Koornneef and Karssen, 1994 and Arabidopsis Book, An Atlas of Morphology and Development, 1994).

decrease drastically (Fig. 1-1), either accompanying or preceding water loss (Karssen et al., 1983; Black, 1991). Embryonic ABA is necessary for the acquisition of desiccation tolerance and the induction of seed dormancy during seed maturation (Leung and Giraudat, 1998). At later phases of seed development, ABA concentration decreases to levels that are conducive to germination on rehydration. In addition, to break dormancy and promote germination, gibberellic acid (GA) is required (Skadsen, 1998).

During late maturation phase of seed development, ABA is involved in the induction of late embryogenesis-abundant (LEA) proteins which might protect cellular structures in the mature embryo during seed desiccation (Baker et al., 1988). Maize VP1, an orthologue of ABI3, is a transcriptional factor involved in the regulation of maturation-related genes expression including LEA (McCarty et al., 1991). Maize VP (*viviparous*) mutants germinate prematurely on the cob by blocking early steps in ABA biosynthesis or perception (Neill et al., 1987), and it has been shown that ABA is required to prevent precocious germination (Nambara et al., 1992; Ooms et al., 1993).

ABA RECEPTORS

Attempts to identify putative ABA receptors have been undertaken over past decades. ABA-binding proteins (Pedron et al., 1998; Zhang et al., 1999) and carrier-mediated uptake of ABA (Astle and Rubery, 1983; Bianco-Colomas et al., 1991; Perras et al., 1994; Windsor et al., 1994) have been reported, but there was no evidence to link these proteins to the physiological effects of ABA.

Studies using guard cells showed that exposure to ABA stimulated Ca^{2+} channel activity at the plasma membrane of guard cells (Hamilton et al., 2000). In other

studies, externally applied ABA repressed gibberellin-induced α-amylase expression in barley aleurone protoplasts (Gilroy and Jones, 1994). Furthermore, external application was sufficient for the induction of ABA-responsive gene expression and preventing stomatal opening (Assmann, 1994). These results suggest the existence of both intracellular and extracellular ABA receptors.

Recently, FCA (for flowering time control A), an RNA-binding protein was identified as a receptor involved in RNA metabolism and in controlling flowering time (Razem et al., 2006). Later, the ubiquitous protein ABAR (ABA-binding protein)/CHLH (H subunit of Mg-chelatase) was shown to bind ABA and positively regulated ABA signaling in seed germination, post-germination growth and stomatal closure (Shen et al., 2006).

Most recently, a plasma membrane ABA receptor, GCPR2 (G protein-coupled receptor) was identified in *Arabidopsis*. This GCPR was shown to genetically and physically interact with the G-protein α–subunit GPA1 to mediate all known ABA responses (Liu et al., 2007). Furthermore, it was shown that ABA binds GCPR2 and then dissociates from the receptor-GPA1 complex to induce ABA response (Liu et al., 2007). Also, GCR1, a candidate GPCR in *Arabidopsis*, shows significant sequence similarity to nonplant GPCRs (Pandey and Assmann, 2004). It was shown to physically interact with GPA1 *in vitro* and in planta (Chen et al., 2004; Pandey and Assmann, 2004). Additionally, the *gcr1* mutant exhibited higher levels of ABA regulated transcripts and enhanced drought tolerance (Pandey and Assmann, 2004).

GENETIC ANALYSIS OF ABA SIGNAL TRANSDUCTION

Regulation of seed development and ABA signaling have been analyzed by

genetic, molecular and biochemical approaches. Genetic screens for ABA response mutants have detected a number of loci that are required for seed maturation and/or ABA responses. These genes apparently act in multiple intersecting response pathways that likely involve a complex interplay of positive and negative regulators. Some act as transcription targets that specifically bind ABA responsive regulatory DNA sequences; while others, e.g. kinases, phosphatases and a farnesyl transferase, that may interact with these factors and probably others, could be located further upstream in the signaling pathway (Leung and Giraudat, 1998).

To date, at least six distinct mutations, designated *abi* (ABA insensitive), have been well characterized in *Arabidopsis*. The *abi1* and *abi2* mutations resulted in reduced sensitivity to ABA for seed germination, seedling growth, seed dormancy and stomatal control (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Leung et al., 1997). Both *ABI1* and *ABI2* encode members of the 2C class of protein serine/threonine phosphatases (PP2C) and are part of a negative control mechanism exerted by a postulated repressor of ABA-signal transfer (Merlot et al., 2001). The phenotypes of the other *abi* mutations appear to be restricted to late seed development. ABI3 is the orthologue of the maize VP1 (*viviparous 1*) and encodes the transcription factor VP1 (McCarty et al., 1991). The *abi3* mutant is defective in seed development, and its embryo shows precocious germination. Two additional mutants, *abi4* and *abi5*, were also selected for decreased sensitivity to ABA which is inhibitory for WT seed germination (Finkelstein, 1994). ABI4 encodes an *APETALA*2 domain transcription regulator, and ABI5 encodes a basic leucine zipper (bZIP) transcription factor. *ABI5* mutations resulted in decreased sensitivity to ABA during germination and inhibition of the expression of several known ABA-regulated genes during seed maturation (Finkelstein and Lynch, 2000; Unpublished data from our laboatory). It was shown that ABI5 is identical to

AtDPBF-1 (*Arabidopsis* Dc3-promoter binding factor) identified in our laboratory (Finkelstein and Lynch, 2000; Kim et al., 2002).

Recently, a severe ABA response mutant, ABA-insensitive 8 (*abi*8), was isolated. In addition to ABA resistance, *abi*8 mutants have pleiotropic growth defects resulting a severely dwarfed phenotype, defective stomatal regulation, delayed flowering, and male sterility (Brocard-Gifford et al., 2004). *ABI8* encodes a protein with unknown function but belongs to a small plant-specific protein family (Brocard-Gifford et al., 2004). Molecular identification of the ABI8 revealed that *abi*8 is allelic to two dwarf mutants, elongation defective 1 (*eld*1) and kobito1 (*kob*1) (Cheng et al., 2000; Pagant et al., 2002).

A second class of mutations affecting ABA signaling was identified. *ERA1* (ENHANCED RESPONSE TO ABA) encodes the β-subunit of farnesyl transferase. Mutations in the *ERA1* gene result in loss of enzyme activity and enhanced ABA sensitivity in the seed (Cutler et al., 1996).

Although molecular analysis of many of these ABA mutants is not yet completed, their phenotypes suggest that there are at least two partially redundant pathways for ABA signaling. One is specific to seed development, and the other is required for both seed and vegetative responses (Gampala et al., 2002).

ABA-REGULATED GENE EXPRESSION

ABA-mediated stress responses are partially accomplished by the transcriptional regulation of stress-responsive gene expression (Busk and Pagès, 1998). Many genes which are expressed during late embryogenesis can be induced in vegetative tissues by abiotic stress treatments (Gomez et al., 1988; Mundy and

Chua, 1988). Genes expressed during later stages of seed development have been well characterized. Seed storage proteins and late embryogenesis abundant (LEA) proteins are known to be transcriptionally active during seed maturation and are then repressed before dormancy (Goldberg et al., 1994). The accumulation of *LEA* mRNA and protein is associated with endogenous ABA levels. Many *LEA* genes are characterized by their ABA modulated expression in late maturation embryos and by their ABA-induced expression in response to various environmental cues, including desiccation (Skriver and Mundy, 1990). It is proposed that the products of *LEA* genes accumulate to protect cellular structures in the mature embryo during seed desiccation and in water-deficient vegetative tissues (Baker et al., 1988). The dissection of the *LEA* promoters has identified several *cis*-regulatory elements that regulate embryo-specific and ABA-induced gene expression. The carrot *LEA* gene, *Dc*3, is expressed preferentially in late maturation seeds, but can be induced in vegetative tissues by exogenous ABA and in response to various environmental cues (Wilde et al., 1988). The promoter of the *Dc*3 gene has been functionally dissected into two regulatory regions including a promoter proximal region (PPR) which contains the *cis*-regulatory elements that are responsible for the *Dc*3 expression in seeds and confers ABA-independent seed specific expression (Thomas, 1993; Kim et al., 1997) and the distal promoter region (DPR) which contains several repeats of the TCGT motifs that interact with the PPR to regulate expression (Chung et al., 2005).

***CIS*-REGULATORY ABA-RESPONSIVE ELEMENTS (ABRES)**

A number of *cis*-regulatory elements known as ABA-responsive elements (ABREs) that control ABA- and/or stress-responsive gene expression have been identified (Giraudat et al., 1994), and *trans*-acting factors that bind to ABREs

have been identified as ABRE binding transcription factors (Busk and Pagès, 1998). ABRE elements have been found in a number of ABA inducible promoters. These function as *cis*-regulatory DNA elements and many share the (T/G/C)ACGT(G/T)GC consensus with ACGT core sequence. The sequence flanking the ACGT core is important for function and for protein binding (Salinas et al., 1992). In general, a single ABRE is not sufficient for ABA response, but various combinations of multimerized ABREs can regulate ABA response (Busk and Pagès, 1998; Chung et al., 2005).

There are also other *cis*-regulatory elements that contain ACGT core sequence (Guiltinan et al., 1990). ACGT sequences are present in many promoters and regulate gene expression by light, UV-light (Weisshaar et al., 1991) and coumaric acid (Loake et al., 1992). Moreover, the strong ABRE, (C/T)ACGTGGC, is identical to the light responsive element G-box. Therefore, the ABRE is a subset of ACGT containing element that is defined by its function rather than by the flanking sequences (Busk and Pagès, 1998).

Another group of *cis*-acting DNA elements, known as a coupling element (CE), does not contain the ACGT core element, but rather contains the CGCGTG core sequence which is active in combination with an ACGT core sequence (Shen and Ho, 1995; Shen et al., 1996). The coupling elements CE1 (TGCCACCGG) and CE3 (ACGCGTGTCCTC) have partly different functions. ABA induction of ABRC (ABA-responsive complexes) containing CE3 is enhanced by the transcription factor VP1, but not in an ABRC containing CE1 implying the involvement of two distinct ABA signal transduction pathway for these CEs (Shen et al., 1996).

The stress-inducible Myb transcription factors and their recognition sequences are known to be involved in the regulation of ABA-induced gene expression

(Urao et al., 1993; Iturriaga et al., 1996). Myb (AAC(G/T)G) and Myc (CANNTG) binding sequences are present in an ABA-responsive 67 base pairs sequence of the *rd*22 gene, and MYC and MYB proteins function as transcriptional activators in the ABA and desiccation activation of the *rd*22 gene (Shinozaki and Yamaguchi-Shinozaki, 1996; Abe et al., 1997).

Other *cis*-elements involved in ABA-induced transcription include a Sph element-containing sequence CGTGTCGTCCATGCAT of the maize *C*1 gene (Hattori et al., 1992), and the DPBF motif (ACACNNG) from the *Dc*3 gene from carrot (Kim et al., 1997).

PLANT BASIC LEUCINE ZIPPER (BZIP) PROTEINS THAT INTERACT WITH ABRES

A number of *trans*-acting factors, including several basic leucine zipper (bZIP) factors, have been isolated as putative ABA-responsive transcription factors based on their interaction with documented ABREs. bZIP proteins bind to specific DNA sequence as dimers to regulate gene expression. The first identified ABRE-binding protein was the wheat *Em*-binding protein (EmBP-1). It interacts specifically with an 8-base pair sequence (CACGTGGC) in a defined ABRE and is a typical leucine zipper transcription factor (Guiltinan et al., 1990). It has been shown in rice that VP1 regulates gene expression through ABREs without directly binding to them. In rice, TRAB1 (transcription factor responsible for ABA regulation) bZIP protein physically interacts with both VP1 and an ABRE (Hobo et al., 1999). The basic region of the TRAB1 bZIP protein is very similar to that of the sunflower bZIP proteins DPBF-1 and DPBF-2 (Kim et al., 1997). It has been proposed that this reveals a novel molecular mechanism for the VP1 dependent, ABA-inducible transcription that controls seed dormancy and

maturation (Hobo et al., 1999). In a recent study, it was shown that TRAB1 is phosphorylated at a specific Ser residue rapidly after ABA treatment. Furthermore, this phosphorylation is necessary for ABA-induced activation of transcription (Kagaya et al., 2002).

An important group of bZIP proteins that interacts with ABREs are the DPBFs first identified in sunflower. The sunflower DPBFs bind to the proximal region of the *Dc*3 gene (Seffens et al., 1990), and the genes encoding them were cloned using a yeast one-hybrid system. The basic domains of DPBF-1 and DPBF-2 are 90% identical and are similar to the plant G-box binding factor GBF-4 (Menkens and Cashmore, 1994). Unlike other plant bZIP proteins, DPBF-1 and DPBF-2 appear to have a broader range of sequence specificity and bind to sequences containing the core ACACNNG (Kim et al., 1997). Five distinct *Arabidopsis* DPBF homologs were isolated in our laboratory from an immature *Arabidopsis* seed cDNA library (Kim et al., 2002). Sequence analysis showed that AtDPBF-1 is identical to the ABA-insensitive gene *ABI*5 (Finkelstein and Lynch, 2000). The basic leucine zipper (bZIP) region is highly conserved among *Arabidopsis* DPBFs and between *Arabidopsis* and sunflower DPBFs. Outside the bZIP regions of these proteins are very divergent from each other, although the phosphorylation sites are highly conserved (Kim et al., 2002).

Another class of ABA responsive bZIP transcription factors, ABRE binding factors (ABFs), was isolated from young *Arabidopsis* plants under stress conditions during vegetative growth (Choi et al., 2000). *In vitro* binding assays revealed that ABF1 and ABF3 interact with both G/ABRE, G-box-like ABRE and C/ABREs, CGCGTG-containing ABRE as do the DPBFs, although they have a higher affinity to the strong G/ABRE, CACGTGGC. Expression of the ABFs is inducible by ABA and abiotic stresses such as low temperature, drought and

salinity. However, ABFs display differential expression patterns that suggest their involvement in different stress-regulatory network (Choi et al., 2000).

ARABIDOPSIS ABI5 SUBFAMILY OF BZIP PROTEINS

The *Arabidopsis* genome encodes 75 putative bZIP proteins. Thirteen of these genes encode proteins belonging to the ABI5/ABF/AREB subfamily (Fig. 1-2) (Jakoby et al., 2002). Eight of these genes have been characterized and named *ABI5/AtDPBF-1, ABF1, ABF2/AREB1, ABF3/AtDPBF-5, ABF4/AREB2, AREB3/AtDPBF-3, GBF4, and EEL/AtDPBF-4* (Menkens and Cashmore, 1994; Choi et al., 2000; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Uno et al., 2000; Bensmihen et al., 2002; Kim et al., 2002). The five uncharacterized genes are *AtbZIP13, AtbZIP14, AtbZIP15, AtbZIP27*, and *AtbZIP67/AtDPBF-2* (Jakoby et al., 2002; Kim et al., 2002).

The amino acid sequence of the bZIP region for DNA binding is highly conserved among the 13 members of the ABI5 bZIP subfamily, and *in vitro* binding assays suggest that all members of this subfamily recognize similar *cis*-regulatory elements (Bensmihen et al., 2002; Kim et al., 2002). However, expression analysis of the ABI5 subfamily of bZIP proteins revealed that these genes exhibited variable inducibility by ABA, salt, cold or drought stresses (Choi et al., 2000; Kim et al., 2002). Several AtDPBF genes are expressed in developing seeds grown under optimal conditions. *ABI5* is also inducible by ABA in vegetative tissues (Kim et al., 2002). Studies of ABF3 and ABF4 transgenic lines revealed the involvement of these in stress-responsive ABA signaling (Kang et al., 2002). It was further shown that ABF2 is required for glucose response and is involved in the regulation of vegetative growth (Kim et al., 2004).

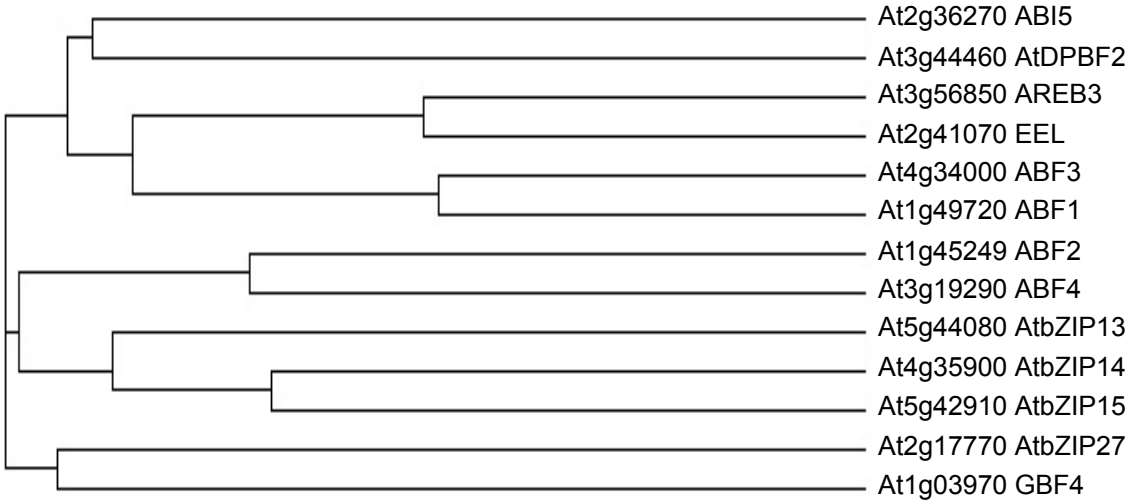


Figure 1-2. Phylogenetic analysis of ABI5 subfamily of bZIP transcription factors. Alignment of the protein sequences of the 13 members of the ABI5 subfamily of bZIP transcription factors was performed by ClustalW program at the European Bioinformatics Institute.

Analysis of the genetic and molecular interactions between ABI5 and AtbZIP12/EEL indicated that these proteins compete for the same binding site within the *AtEm1* promoter. EEL forms a heterodimer with ABI5, and these two transcription factors play antagonistic roles to fine-tune LEA gene expression during *Arabidopsis* seed development (Bensmihen et al., 2002).

ABI5 encodes a transcription factor that belongs to a seed specific subfamily of bZIP factors (Finkelstein and Lynch, 2000). This gene has been characterized with respect to genetic, developmental, and environmental regulation. The *Arabidopsis abi5* mutants, like many other ABA-insensitive mutants, were selected on the basis of ABA-resistant germination and vegetative growth (Finkelstein, 1994; Unpublished data from our laboratory).

ABI5 transcript levels are highest in the later stages of embryogenesis, peaking in desiccating dry seed (Finkelstein and Lynch, 2000; Brocard-Gifford et al., 2004). Vegetative *ABI5* expression is induced by ABA, and ABA also regulates *ABI5* accumulation post-transcriptionally by increasing protein stability and induces ABI5 phosphorylation (Lopez-Molina et al., 2001). Furthermore, *ABI5* is induced by other stresses like glucose, cold, NaCl, and drought treatment in specific tissues (Brocard-Gifford et al., 2004).

HIGH-THROUGHPUT TECHNOLOGY

The *Arabidopsis thaliana* genome was the first plant genome to be fully sequenced (THE ARABIDOPSIS GENOME INITIATIVE, 2000). This sequence information, together with the large collection of expressed sequence tags (ESTs), has facilitated new approaches to study gene expression patterns in plants on a global scale (Schaffer et al., 2000). The advent of newer high-

throughput genomic technologies, such as quantitative reverse transcription-polymerase chain reaction (QRT-PCR) and DNA chips, or microarrays, has enabled rapid and simultaneous comparison of mRNA levels for thousands of genes in virtually any biological sample (Lobenhofer et al., 2001). Specifically, huge amounts of genetic information generated by DNA sequencing projects can be analyzed in a facile and economical manner by microarray analysis (Case-Green et al., 1998). Furthermore, DNA microarray technology allows genome-wide gene expression analyses across any biological sample. Because physiological responses involve changes in the complex regulatory networks that affect gene expression, DNA microarrays help to monitor a number of genes that may be regulated in a coordinate manner. In some cases, mutations do not exhibit any obvious phenotype. Thus, the application of DNA microarrays can define a molecular phenotype of mutants that may facilitate genetic, molecular or biochemical studies. DNA microarrays can also help to understand the mechanisms of action of compounds and can improve basic science research as well as biomedical research including disease diagnosis and new drug development (Lobenhofer et al., 2001).

Different microarray platforms are available for *Arabidopsis,* including spotted cDNA arrays, long oligonucleotide arrays and the Affymetrix Gene Chip arrays. Genomic-scale gene expression during development of *Arabidopsis* ranging from embryogenesis to senescence and diverse tissues were analyzed by employing high-throughput Affymetrix ATH1 microarray platform (Birnbaum et al., 2003; Schmid et al., 2005). Also, large quantities of expression profiling data have been generated using abiotic stress treated plants (Fowler and Thomashow, 2002; Hazen and Kay, 2003; Seki et al., 2004) and developing seeds (Ruuska et al., 2002). Combined with other available expression data, this may enable the identification of modules that regulate plant development and stress response.

RESEARCH PLAN

ABI5 is a key factor regulating gene expression during embryogenesis and in response to ABA. High-throughput genomic tools like DNA microarray technology were employed in a functional genomic analysis of the ABI5 bZIP transcription factor that contributes to the regulation of gene expression during seed development and in response to ABA.

Specific objectives include: 1) Genomic analysis of *Arabidopsis* gene expression in response to ABA and identification of ABA-responsive and ABI5-regulated genes; 2) Functional analysis of the role of ABI5 during embryogenesis utilizing Affymetrix ATH1 Chips, Agilent Oligoarray and cDNA microarray and 3) Analysis of the regulation of *AtCOR413 pm-1* gene by the ABI5 subfamily of bZIP transcription factor during seed development and in response to abiotic stresses.

CHAPTER II
PHENOTYPIC CHARACTERIZATION OF PRO35S:ABI5 AND *abi5-6*

INTRODUCTION

The phytohormone abscisic acid (ABA) regulates seed development and the initiation and maintenance of seed dormancy. It also plays an important role in vegetative tissues by regulating plant stress responses to drought, salinity and cold (Himmelbach et al., 1998; Finkelstein et al., 2002). Many of these developmental regulation and stress responses accompany the transcriptional changes of stress responsive genes, and a number of genes involved in ABA responses have been identified (Gomez et al., 1988; Mundy and Chua, 1988; Busk and Pagès, 1998). Furthermore, a large number of loci involved in ABA response have been identified in *Arabidopsis* by genetic studies and were named ABI (ABA insensitive) and ERA (enhanced response to ABA).

In the promoters of many ABA-responsive genes, ABA-responsive elements (ABREs) have been identified that control ABA- and/or stress-responsive gene expression (Giraudat et al., 1994). ABREs function as *cis*-regulatory elements, and many share the (T/G/C)ACGT(G/T)GC consensus with an ACGT core sequence. Another type of ABRE, known as a coupling element, does not contain the ACGT core element, but contains the CGCGTG core sequence which is active in combination with an ABRE but not alone (Shen and Ho, 1995; Shen et al., 1996).

Based on their interaction with demonstrated ABA responsive *cis*-regulatory elements, a number of basic leucine zipper (bZIP) transcription factors have

been identified as ABA-responsive transcription factors (Busk and Pagès, 1998). The wheat EmBP1 was first shown to encode a bZIP protein. In addition, *Arabidopsis* DPBFs (*Dc3* promoter binding factor) and the sunflower DPBFs bind to a motif without the ACGT core sequence and *Arabidopsis* ABFs, a family of ABA-responsive element binding factors were also identified. Rice TRAB1, an ortholog of ABI5, was shown to interact with VP1 and represents another class of transcription factors for ABA-regulated gene expression in seed tissues (Guiltinan et al., 1990; Nakagawa et al., 1996; Kim and Thomas, 1998; Hobo et al., 1999; Choi et al., 2000; Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001; Kim et al., 2002).

ABA-INSENSITIVE 5 (ABI5) is identical to *Arabidopsis* DPBF-1 and is one of the bZIP transcription factors that bind to an ABRE *cis*-regulatory element and regulates gene expression in seed and in response to ABA (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001; Kim et al., 2002). Like other ABA-insensitive mutants, the *Arabidopsis abi5* mutants were selected based on their insensitivity to ABA for germination and seedling growth (Finkelstein, 1994; Lopez-Molina and Chua, 2000; Lopez-Molina et al., 2001).

ABI5 expression in vegetative tissue is strongly induced by ABA, and also is induced by other stresses like glucose, cold, NaCl, and drought stress (Lopez-Molina et al., 2001; Brocard et al., 2002). Furthermore, *ABI5* over-expression results in hypersensitivity to ABA during germination and subsequent vegetative growth. It is also known that ABA plays an important role in the activation of ABI5 protein by phosphorylation and the stabilization of ABI5 protein by preventing degradation (Lopez-Molina et al., 2001).

In this study, we isolated and characterized an *Arabidopsis abi5-6* mutant in the Columbia background with respect to root growth and seed germination in the

presence of ABA. Additionally, the phenotype of an *ABI5* over-expressing line (Pro35S:ABI5) was characterized in the same way as *abi5-6*. We found that the *abi5-6* mutant was ABA insensitive in germination test. Furthermore, transgenic seeds and seedlings over-expressing *ABI5* were hypersensitive to ABA. Five μM ABA severely inhibited germination of the Pro35S:ABI5 line, but all Pro35S:ABI5 seeds germinated into green seedlings in the absence of ABA.

RESULTS

Identification of *abi5-6* T-DNA knockout line

The mutant, *abi5-1* [Wassilewskija (Ws) ecotype background], was germinated on high concentration of ABA and showed a decrease in seed-specific gene expression (Finkelstein, 1994). While other *abi5* mutants have been generated, none of them were publically available (Finkelstein, 1994). Therefore, we obtained an *Arabidopsis abi5* knockout mutant in the Columbia background from the Syngenta Arabidopsis Insertion Library (SAIL) T-DNA collection. A T-DNA insertion population was screened by PCR analysis with specific sets of primers derived from the *ABI5* gene and the T-DNA left border to obtain knockout lines. The genomic region of the *ABI5* locus was characterized by DNA sequence analysis to determine the location of the T-DNA insertion. As shown in Figure 2-1, the *ABI5* locus in this line contained a tandem T-DNA insertion joined right border to right border at nucleotide position 1117 in exon 2, before the basic leucine zipper (bZIP) region.

To determine the number of T-DNA copies in the identified *abi5-6* T-DNA knockout mutant, DNA from WT and *abi5-6* plants was hybridized on a DNA

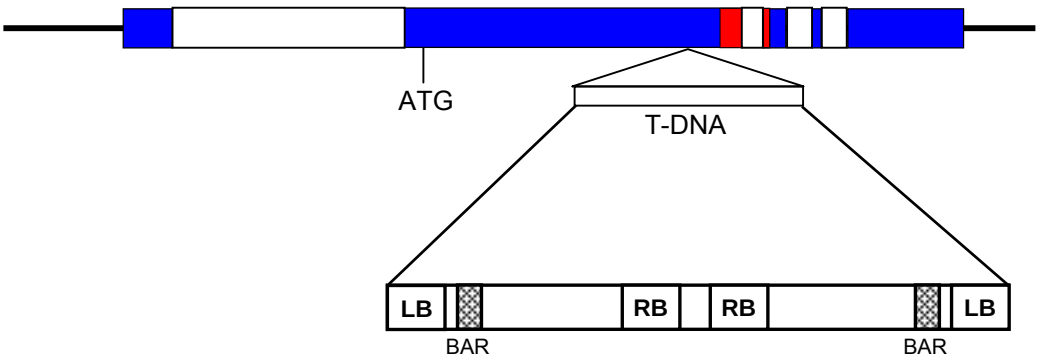


Figure 2-1. Localization of the T-DNA insert in the *Arabidopsis ABI5* locus. The T-DNA insertion site is in the predicted second exon of ABI5 adjacent to the bZIP region. Blue boxes represent exons; the red boxes represent the basic leucine zipper region; and the open boxes represent introns.

gel blot with a *BASTA* cDNA probe (Fig. 2-2). WT and *abi5-6* genomic DNA was digested with XmaI/XhoI, XbaI or DraIII/SpeI restriction enzymes which cut once within the T-DNA insert. A second set of digestions of WT and *abi5-6* genomic DNA was carried out using SpeI, XmaI or HpaI restriction enzymes, which do not cut the T-DNA insert. The DNA digests were then subjected to DNA gel blot analysis. Based on the known DNA sequence of the T-DNA insert and the *Arabidopsis ABI5* locus, a restriction profile was generated using MacVector software (MacVector Inc., Cary, NC). The program predicts two DNA fragments that hybridize with the probe in the XmaI/XhoI (2.8 and 1.9 kb), XbaI (2 and 1.5 kb) and DraIII/SpeI (3.5 and 2.9 kb) digestions. Each of the digestions made with enzymes cutting within T-DNA yielded hybridization bands of approximately the expected sizes (Fig. 2-2). Unexpectedly, an additional hybridization band (approximately 7 kb) was detected, that might have been caused by a mutation occurring when the two T-DNAs were joined and inserted into the plant genome. As expected, digestion with SpeI, XmaI or HpaI, restriction enzymes that do not cut the T-DNA insert, liberated a single DNA fragment and are consistent with the presence of a single-tandem insertion into the chromosome.

Disruption of ABI5 mRNA expression

The expression of *ABI5* was examined in WT and *abi5-6* vegetative tissues and developing embryos by northern blot analysis. Previously, we used an *ABI5* promoter:GUS reporter to show that *ABI5* expression is observed starting at the heart stage embryo and reaches its maximum in dry seeds (unpublished data from Thomas Lab.). To determine *ABI5* transcript levels in seedlings and developing embryo, an RNA gel blot was incubated with the ^{32}P-labeled 3′ region of an *ABI5* cDNA probe. Consistent with our previous observation, *ABI5* transcript levels increased during embryogenesis and reached a maximum in dry

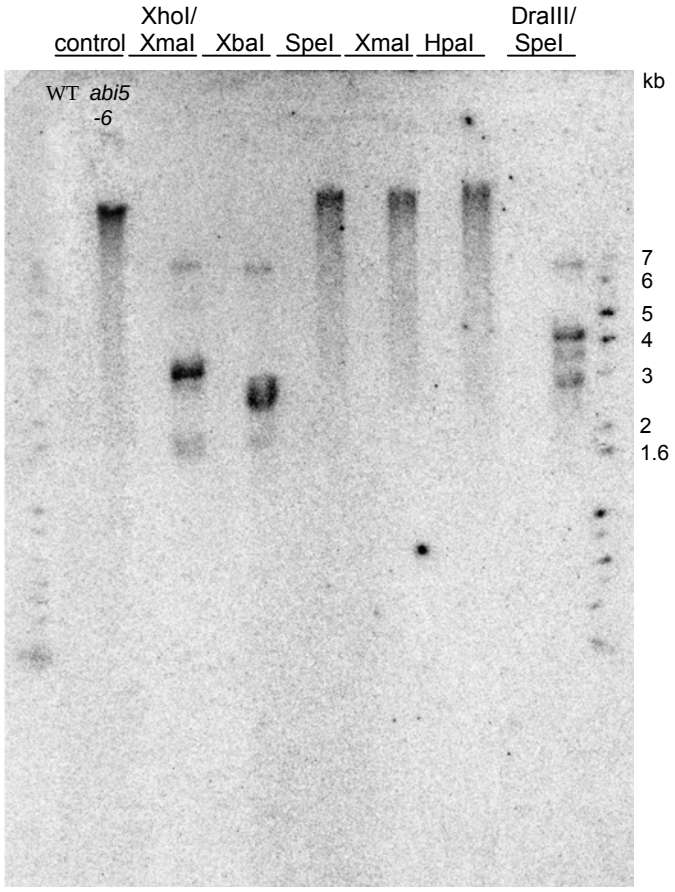


Figure 2-2. DNA gel blot analysis of genomic DNA from *abi5-6* and WT. WT and *abi5-6* genomic DNA was digested with the enzymes as indicated and subjected to DNA gel blot analysis using a ^{32}P-labeled PCR fragment generated by amplification of the *BASTA* cDNA. DNA marker is loaded at left and right. Undigested WT and *abi5-6* genomic DNA was added as control (lane 1 and 2)

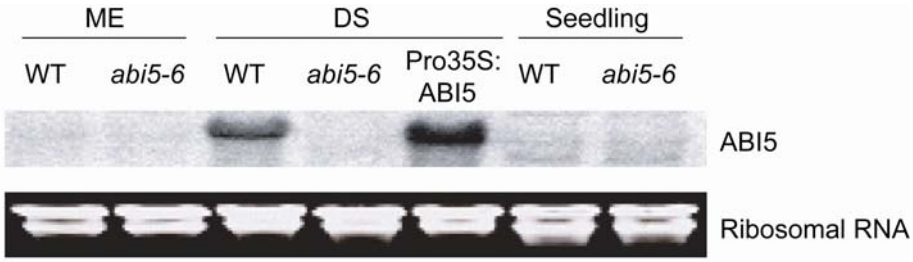


Figure 2-3. Expression of *ABI5* in the developing embryo and seedlings of WT, Pro35S:ABI5 and *abi5-6*. RNA-gel blot analysis was performed using 15 µg of total RNA and a ^{32}P-labeled 3´ region of the *ABI5* cDNA.

seeds of WT. No detectable hybridization product was identified in WT and *abi5-6* seedlings (Fig.2-3).

Root growth inhibition

To examine whether ABA enhances or inhibits root growth in the Pro35S:ABI5 and *abi5-6* lines, seeds from these transgenic lines were germinated and grown for 5 days on MS medium without ABA. After 5 days, seedlings were transferred to plates containing different amounts of ABA or without ABA, and subsequent root growth was scored after 5 days (Table 2-1).

Transgenic Pro35S:ABI5 seedlings supplemented with 5 μM ABA exhibited reduced root growth compare to WT. Similar results were obtained with seedlings supplemented with 10 μM ABA, which exhibited a further reduction in root growth than Pro35S:ABI5 seedlings supplemented with 5 μM ABA. The Student's *t*-test was used to calculate the significance of observed differences between the means of WT and Pro35S:ABI5 seedlings. Roots of Pro35S:ABI5 treated with 5 μM and 10 μM ABA showed significant reduction compared to WT seedlings treated with the same amount of ABA ($P < 0.05$). However, *abi5-6* mutant seedlings supplemented with 5 μM and 10 μM ABA displayed moderately decreased sensitivity to ABA compared to WT (Table 2-1) and did not show significant differences compared to WT seedlings treated with same amount of ABA. Our results are similar to the results described by Lopez-Molina and Brocard's group indicating that over-expression of *ABI5* was sufficient to confer hypersensitivity to ABA for inhibition of root growth (Lopez-Molina et al., 2001; Brocard et al., 2002).

Table 2-1. Inhibition of root growth of WT and the ABI5 transgenic lines.

	ABA concentration (μM)	
	5	10
WT	59.8 ± 5.3	55.6 ± 1.8
Pro35S:ABI5	48.2 ± 1.8	46.2 ± 4.7
abi5-6	62.7 ± 1.8	61.7 ± 3.8

Five-day-old seedlings grown in the absence of ABA were transferred to MS media containing 0, 5, and 10 μM ABA for 5 days. Root length of ABA-treated seedlings was presented as percentage of non-treated seedlings.

Table 2-2. Drought tolerance of WT and the ABI5 transgenic lines.

	Dehydration time (hr)				
	1	6	12	24	72
WT	85.6 ± 2.2	45.7 ± 1.4	22.9 ± 4.4	14.2 ± 6.4	13.3 ± 6.6
Pro35S:ABI5	87.4 ± 3	52.6 ± 0.1	29 ± 2.9	23.7 ± 5.5	17.7 ± 9.4
abi5-6	83.7 ± 3.9	43.3 ± 2.2	26.2 ± 3.5	24.4 ± 3.8	16.8 ± 8.7

Five-day-old seedlings were transferred to plates lined with 3M paper and weighed at different time points indicated. Seedling weights were presented as percentage of non-treated seedlings. Data are means ± SD (n = 20). FW, fresh weight.

Drought tolerance test

We also examined water loss to examine the sensitivity of the Pro35S:ABI5 line to the dehydration stress. Seedlings grown on MS media for 5 days were removed from plates and incubated under the same conditions used for seedling growth. The seedlings were weighed at the time points indicated. *ABI5* over-expressing seedlings retained water slightly better than WT and *abi5-6* over time, but no significant differences were observed among these lines ($P > 0.05$) (Table 2-2). Also, there was no significant difference among WT, Pro35S:ABI5 and *abi5-6* after 12 hr of treatment. However, Lopez-Molina's group reported that *ABI5* over-expressing plants were hypersensitive to ABA and retained water more efficiently (Lopez-Molina et al., 2001).

Germination

We analyzed the effect of ABA on germination of WT, ABI5-OE and *abi5-6* by germinating seeds on MS medium supplemented with different concentrations of ABA or without ABA (Fig. 2-4). Addition of 5 μM ABA severely inhibited germination of the Pro35S:ABI5 line, but had almost no effect on germination of the *abi5-6* mutant line. Most of the Pro35S:ABI5 seeds supplemented with 0.5 μM ABA germinated and had a growth pattern similar to wild type seeds supplemented with 5 μM ABA. Furthermore, treatment of the *abi5-6* mutant line with 0.5 μM ABA showed the same growth pattern as *abi5-6* without ABA. When WT seeds were germinated on MS media supplemented with 5 μM ABA, most of the seeds could germinate yielding seedlings with etiolated cotyledons and short hypocotyls.

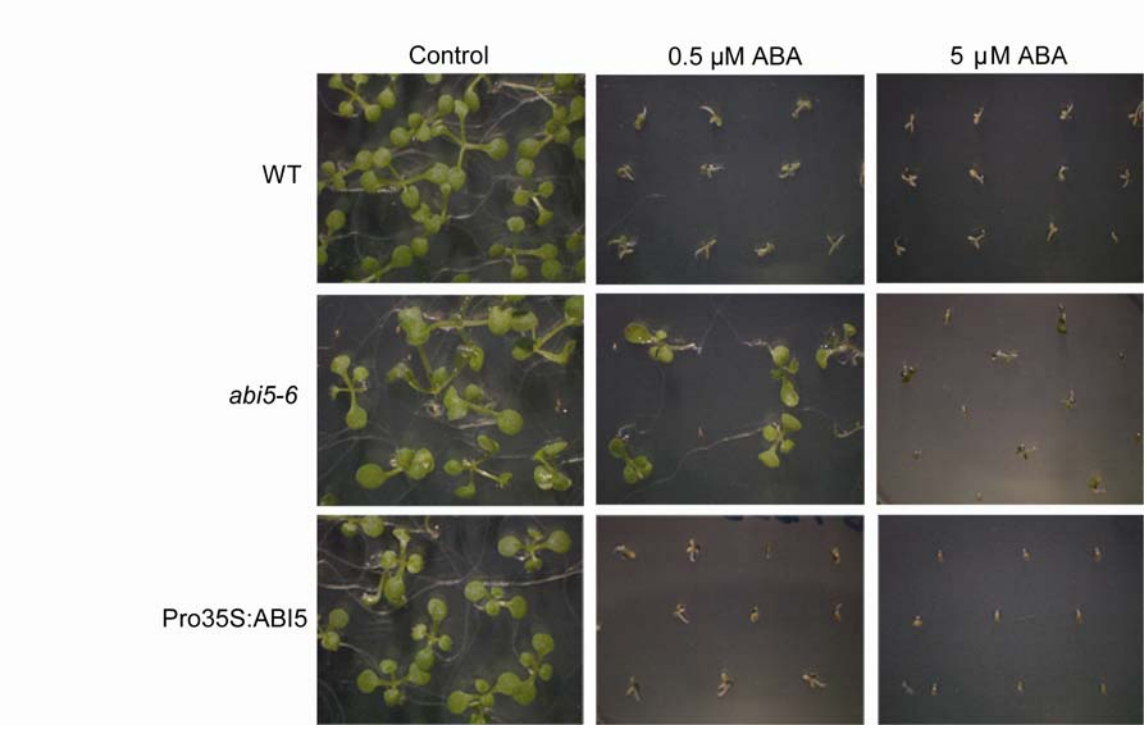


Figure 2-4. Seed germination of ABI5 transgenic lines. WT, Pro35S:ABI5 and *abi5-6* mutant line were germinated and grown for 5 days on MS medium supplemented with the indicated concentrations of ABA. MS plates containing seeds were chilled for 3 days at 4°C in darkness, and then incubated for 5 days at 22°C under continuous light.

WT, ABI5-OE and *abi5-6* seeds germinated normally into seedlings with green cotyledons and long roots on the medium without ABA (Fig. 2-4). However, about five percent of *abi5-6* mutant seeds plated on MS medium failed to germinate in 2 days, and some of the germinated seedlings exhibited retarded growth. All of WT and Pro35S:ABI5 seeds plated on MS medium could germinate and grow into seedlings within 1 day of incubation on MS plates.

Lopez-Molina's group reported that the treatment of *ABI5* over-expressing lines with 0.5 μM ABA showed similar response to WT plants treated with 5 μM ABA, and the treatment of *ABI5* over-expressing lines with 5 μM ABA severely delayed germination (Lopez-Molina et al., 2001).

DISCUSSION

ABI5 expression is induced by ABA and other abiotic stresses in specific tissues throughout plant development. ABI5 is highly expressed in the later stages of embryogenesis peaking in desiccating seed (Finkelstein and Lynch, 2000; Brocard et al., 2002; Brocard-Gifford et al., 2004). Despite its expression throughout plant development, *abi5-6* plants did not show obvious gross morphological changes implying that the loss of ABI5 might be complemented by other functionally redundant proteins during plant development.

Furthermore, we have analyzed the effect of ABA on root growth inhibition and water loss control in WT, *ABI5* over-expressing line and an *abi5-6* insensitive mutant line. Seedlings of transgenic lines over-expressing *ABI5* showed enhanced responses to ABA, but it was not significantly different from WT and *abi5-6*. This suggested that ABI5 might not be a main factor regulating growth

after a certain point of seedling development. Alternatively, translation of over-expressed *ABI5* transcripts might not be properly conducted in the root tissue. Our data is consistent with the finding from Lopes-Molina's group in the Wassilewskija (WS) ecotype, showing ABA applied 5 days post-stratification failed to arrest growth and prevent greening (Lopez-Molina et al., 2001).

We have shown that all Pro35S:ABI5 seeds tested on MS plates in the absence of ABA could germinate and grow into green seedlings with long roots. However, addition of 5 μM ABA severely inhibited germination of Pro35S:ABI5 seeds. These results suggested that ABI5 protein might need to be further activated by interacting with other protein(s) or by modifications like phosphorylation or conformational changes. Previously, it was shown that ABA induces phosphorylation of ABI5 and regulates ABI5 accumulation post-transcriptionally by preventing ABI5 degradation (Lopez-Molina et al., 2001).

Even though WT and Pro35S:ABI5 seeds were supplemented with high concentrations (5 μM) of ABA, all the WT seeds and some of Pro35S:ABI5 seeds could germinate in 5 days. However, the growth of germinated seeds into green seedlings was arrested. This suggests that ABA and ABI5 might play a major role in growth inhibition following germination rather than in controlling seed germination. Interestingly, it was shown that ABI5 blocks the growth of germinated embryos to protect plants from unfavorable conditions (Lopez-Molina et al., 2001). However, the molecular mechanism underlying growth arrest by ABI5 in combination with ABA was not clearly revealed. This growth inhibition might be caused by blocking progression of cell cycle which is mainly regulated by cyclin-dependent kinases along with cyclins. It was shown that the *Arabidopsis* cyclin-dependent kinase (CDK) inhibitor, ICK1, expression is induced by abiotic stress conditions such as ABA and low temperature

treatments (Wang et al., 1998; Wang et al., 2000). ABI5 might control the expression of ICK1 to arrest the growth in the presence of ABA.

MATERIALS AND METHODS

Plant materials and growth

Arabidopsis thaliana seeds were surface-sterilized by treatment with 70% ethanol for 1 min followed by 50% household bleach for 7 min with rotation. Subsequently, seeds were washed 4 times with sterile water and plated on MS (Murashige and Skoog, 1962) medium. The plates containing sterilized seeds were incubated 3 days at 4°C to break dormancy. Thereafter, seeds were germinated and grown on MS medium containing 1% sugar and 0.25% phytoagar under continuous light at 22°C.

Plasmid construction and plant transformation

The plant transformation binary plasmid was constructed by Dr. Jinzhong Ma previously in Thomas Lab. Binary plasmid was constructed by cloning a *Eco*RI/*Xho*I fragment of ABI5 cDNA into the p12EB35 vector, a modified version of the pCam1201 vector (Cambia, Canberra, Australia). The transformant seeds were selected by plating *Agrobacterium* transformed seeds on MS plates containing 20 $\mu g \cdot mL^{-1}$ hygromycin and 500 $\mu g \cdot mL^{-1}$ carbenicillin. A second round of selection for transformant seeds was carried out on MS plates containing 20 $\mu g \cdot mL^{-1}$ hygromycin.

Genomic DNA isolation and T-DNA insertion line screening

A T-DNA insertion in the ABI5 coding region was identified in the Syngenta Arabidopsis Insertion Library (SAIL) T-DNA collection. *abi5-6* genomic DNA was isolated from two week old seedlings sprayed with BASTA (240mg·ml^{-1} in 0.005% Silwet L-77) as described (cited from Meyerowitz laboratory protocol). The screening of homozygous T-DNA insertion line was performed using following PCR primers. T-DNA left border primer (LB3) 5′-TAG CAT CTG AAT TTC ATA ACC AAT CTC GAT ACA C-3′, T-DNA right border primers (QRB3) 5′-CGC CAT GGC ATA TGC TAG CAT GCA TAA TTC-3′, ABI5-F 5’-GTT GTG CCC TTG ACT TCA AA-3′, ABI5-R 5′-AGG GAT AGC GAA CGA GTC TA-3′. PCR reactions (50 µl) consisted of 2.5 mM dNTP mix, 1 O.D. primer mix (LB3, ABI5-F, ABI5-R) and 1 µl of extracted genomic DNA. Amplifications were carried out using 1.25 units of ExTaq polymerase (Takera, Madison, WI) in 10X reaction buffer. The PCR amplification was performed with the following cycling conditions: an initial 4 min denaturation at 94°C, 40 cycles (94°C for 1 min, 56°C for 40 sec, 72°C for 1 min), followed by a single cycle of 10 min at 72°C. One-fifth of the PCR product was analyzed on a 1% agarose gel containing Etidium bromide.

DNA gel blot analysis

DNA gel blot analysis was performed to determine T-DNA copy number in the *abi5-6* mutant. The pCSA110 vector was analyzed with MacVector software (MacVector Inc., Cary, NC) to identify restriction enzymes that cut and did not cut the T-DNA. Approximately 200 ng of genomic DNA was digested with XmaI/XhoI, XbaI, DraIII/SpeI which cut T-DNA and SpeI, XmaI and HpaI which do not cut the T-DNA. After separation on a 0.75% agarose gel, the DNA was

transferred to Hybond-N+ TM membrane (Amersham, Arlington Height, IL) with 0.4M NaOH. Hybridization was performed with caseine base solution containing 1% Casein, 7% SDS, 1 mM EDTA and 0.25 M Na_2HPO_4 following standard procedures. *BASTA* coding region DNA probes were labeled with [α-^{32}P]-dATP by random primer extension using Klenow Fragment (3´→5´ exo–) (New England BioLab., Ipswich, MA). The hybridization was performed at 65°C for 12-18 hr. After hybridization, filters were washed with 2X SSC (1.5 M NaCl/0.15 M sodium citrate), 0.5% SDS for 5 min at room temperature, 2X SSC, 0.5% SDS for 15 min at 65°C, two times with 0.2X SSC, 0.1% SDS for 20 min at 65°C and 2XSSC for 5 min at room temperature. Signal detection was carried out using the Fujifilm BAS-5000 phosphoimaging system (Fuji Photo Film Co., Ltd, Japan).

RNA isolation and gel blot analysis

Whole *Arabidopsis* seedling tissues were collected and stored at –90°C. RNA extraction was performed from frozen tissues using the RNeasy Plant Kit (Qiagen, Valenci, GA) or LiCl precipitation protocol (Ausubel et al., 1994) following the manufacturer's instructions or standard procedures. Contaminating DNA was removed from each RNA preparations by using the DNA-*free*™ kit (Ambion, Austin, TX). The isolated total RNA (15 μg) was denatured at 65°C for 15 min with a mixture of 2 μl 10X MOPS, 3.5 μl formaldehyde and 10 μl deionized formamide, and resolved in 1% agarose/MOPS-formaldehyde gels. Subsequently, the RNA was capillary transferred to nylon membranes (Hybond N^+ TM, Amersham Pharmacia Biotech, Arlington, IL) using 20X SSC and fixed using a Stratagene UV crosslinker as described (Maniatis et al., 1982). The membrane was hybridized with ^{32}P-labelled 3′ region of an *ABI5* cDNA probe. Hybridization was performed at 42°C in 5X SSC, 5X Denhardt's solution, 1% SDS, 100 μg salmon sperm DNA, 5% Dextran Sulfate, and 50% formamide for

14-16 hr. After hybridization, filters were washed with 2X SSS, 0.1% SDS for 10 min and 0.2X SSC, 0.1% SDS for 5 min at 65°C respectively.

Root growth inhibition test

The surface sterilized WT, Pro35S:ABI5 and *abi5-6* seeds were germinated and grown for 5 days on MS pates without ABA as previously described. Thereafter, seedlings were transferred to plates containing 5 µM and 10 µM ABA, and root growth was examined after 5 days incubation. Values were obtained from three replicates of 15 seedlings for WT, Pro35S:ABI5 and *abi5-6.* The root length of ABA treated seedling was expressed as a percentage of non-treated seedlings. The data were analyzed by a two-tailed Student's *t*-test for equal variance to test if two means are different from each other.

Germination assay

Germination assays were performed with surface sterilized seeds as previously described by plating seeds on MS plates supplemented with 0.5 µM, 5 µM and 50 µM ABA or without ABA. Seeds were then stratified at 4°C for 3 days, and then transferred to 22°C in continuous light. The radicle emergence or the presence of green cotyledons was scored after 5 days.

Drought tolerance test

For leaf water loss measurements, WT and transgenic seedlings were germinated and grown on MS plates for 5 days, and then transferred to Petri

dishes, incubated under the same conditions used for seedling growth. Each sample was weighed at the specific times indicated in the text and figure. All experiments were repeated at least three times, and >20 seedlings were used in each comparison. The data were analyzed by a two-tailed Student's *t*-test for equal variance to test if two means are different from each other.

CHAPTER III
GENOMIC ANALYSIS OF *ARABIDOPSIS* GENE EXPRESSION IN RESPONSE TO ABA AND IDENTIFICATION OF ABA/ABI5-REGULATED GENES IN VEGETATIVE TISSUES

INTRODUCTION

The phytohormone abscisic acid (ABA) plays an important role in allowing vegetative tissues to adapt to abiotic stress, particularly water deprivation by regulating the opening of the stomatal aperture (Himmelbach et al., 1998). ABA also plays an important role in many physiological processes including the initiation and maintenance of seed dormancy and the accumulation of nutrient reserves. The physiological responses to ABA are largely achieved by regulating gene expression (Bohnert et al., 1995). While considerable insights have been gained on ABA-mediated transcriptional and post-transcriptional control, only a few of the regulatory components have been identified. The complexity of the ABA signaling network and the potential crosstalk with other signaling networks adds to the difficulty of determining the fundamental mechanisms of ABA regulated gene expression.

Many ABA-responsive *cis*-regulatory elements (ABREs) have been identified in the promoters of ABA-regulated genes. Most share the consensus sequence (T/G/C)ACGT(G/T)GC. The sequences flanking the ACGT core are important for adequate function and for protein binding (Salinas et al., 1992). In addition, other *cis*-regulatory elements called coupling elements have been identified. These coupling elements in combination with ABREs constitute ABA-responsive *cis*-

element complexes (ABRCs), which respond to ABA by inducing gene expression (Shen and Ho, 1995; Shen et al., 1996).

Several basic leucine zipper (bZIP) factors have been isolated as possible ABA-responsive transcription factors based on their interaction with documented ABREs (Busk and Pagès, 1998). These include wheat *EmBP1,* Rice TRAB1, rice OSZB8, sunflower DPBFs; tobacco TAF-1, *Arabidopsis* DPBFs and *Arabidopsis* ABFs (Guiltinan et al., 1990; Oeda et al., 1991, Guiltinan et al., 1990; Nakagawa et al., 1996; Kim and Thomas, 1998; Hobo et al., 1999; Choi et al., 2000; Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001; Kim et al., 2002).

Genetic and biochemical studies on ABA-INSENSITIVE 5 (ABI5) (also known as AtDPBF1) have confirmed that it is an ABA-responsive transcription factor (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001; Kim et al., 2002). ABA both induces *ABI5* expression in vegetative tissues and stabilizes the ABI5 protein by preventing its degradation (Lopez-Molina et al., 2001; Brocard et al., 2002). It has been shown that *ABI5* over-expression results in hypersensitivity to ABA during seed germination and subsequent vegetative growth. In addition to its role in stabilizing the ABI5 protein, it is known that ABA also plays an important role in activating the ABI5 protein by phosphorylation (Lopez-Molina et al., 2001).

Through phenotypic analysis of plants carrying the overexpression construct Pro35S:ABI5 and plants with the knockout mutation *abi5-6,* we showed that ABA and ABI5 play a major role in the growth inhibition that occurs following seed germination (Chapter II). However, the molecular mechanism of ABI5/ABA regulation of growth arrest following germination was not clearly revealed. In this study, we analyzed the global effects of ABI5 on ABA regulated gene expression

and also studied ABA-mediated gene expression in vegetative tissues on a genomic scale. Employing a cDNA microarray containing 7060 EST clones, we monitored the response of thousands of genes simultaneously and identified ABA responsive genes and ABA/ABI5 regulated genes. The microarray analysis revealed that there is a strong connection between ABA and ABI5.

RESULTS

Transcription profiling of genes responsive to ABA

Identification of ABA responsive genes in *Arabidopsis*

cDNA microarray analyses were performed to identify *Arabidopsis* genes that are regulated in response to ABA. To get a broad analysis of gene regulation, 7060 *Arabidopsis* non-redundant EST clones, representing 5,333 unique *Arabidopsis* genes, were used in this study. RNA was obtained from three independent biological samples taken from seedlings harvested just before they were treated with ABA and at 2, 12, 24, and 48 hr after ABA treatment. Distinct fluorescent tags (Cy3 and Cy5) were used to label the transcription products from the untreated and treated seedlings. Two separate hybridizations were performed with independent microarray slides using identical RNA samples, but reversing the labeled tags. Fold changes in transcript levels were calculated for the three independent samples treated with ABA at each time point relative to the levels in the non-treated samples by comparing the intensities of the fluorescent Cy3 and Cy5 labeled cDNAs that hybridized to the cDNAs on the array.

The software, Genespring (Agilent Technologies, Inc., Santa Clara, CA) Gene Expression Analysis program, was used to eliminate highly variable expression data and to identify differently expressed genes. As expected, the majority of genes analyzed in these cDNA microarray experiments showed less than a 2-fold difference in signal intensity between ABA treated and untreated control (-ABA). Genes were designated as being up-regulated at a given time point if the ratio of treated to untreated transcript hybridizing to it was greater than 2 for four of the six microarray hybridizations carried out. Similarly, genes were designated as being down-regulated at a given time point if the hybridization intensity ratio was less than 0.5 for four of six microarray hybridizations.

Using these criteria, a total of 175 genes were found to represent ABA-responsive genes, corresponding to 2.5% of the total genes on the cDNA microarray (Figure on page 58). These genes showed an ABA response at a minimum of one time point during the course of experiment. The number of ABA-responsive genes increased to a maximum of 111 at 24 hr and then decreased to 103 at 48 hr. 122 genes were scored as being up-regulated in response to ABA, and another 53 were scored as being down-regulated at sometime after treatment (Fig. 3-1).

Identification of long-term up-regulated genes in response to ABA

Hierarchical clustering of the 122 up-regulated genes revealed that groups of genes showed different patterns of induction or repression over time (Fig. 3-2). It was evident that some genes were up-regulated transiently, whereas others were up-regulated over the entire period of the experiments. Of the 122 genes that were determined to be up-regulated in response to ABA, 16 genes showed

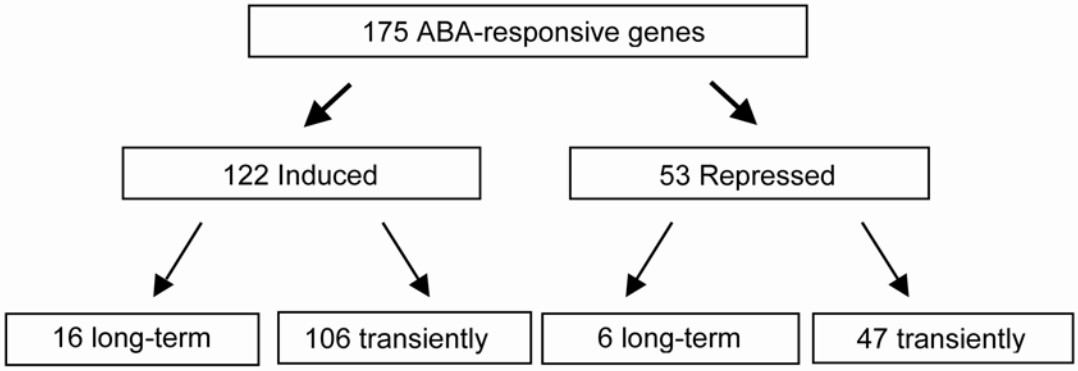


Figure 3-1. Summary of ABA responsive genes. Induced genes are defined as those that show a ratio of hybridization of 2 or more for treated transcript relative to untreated. Repressed are defined as those that show a ratio of hybridization of 0.5 or less for treated transcript relative to untreated.

more than a 2-fold increase at every time point during the course of experiment (Table 3-1 and Fig. 3-1). Three cDNAs on the array that display a long-term ABA response encode the ABRE binding factor ABF3 (At4g34000) (Kang et al., 2002). ABF3 mediates stress-responsive ABA signaling and is essential for ABA/stress response. Seven other long-term regulated genes encode proteins that function in stress response; these include dehydrin ERD14 (At1g76180), dehydrin ERD10 (At1g20450), cold regulated protein COR47 (At1g23120), cold pm-1 (At2g15970) (Table 3-1)**.** The protease inhibitor/seed storage protein/LTP family protein (At3g22600) was also induced at various time points during the course of the experiment suggesting that it might have a pathogenesis-defense role and might be involved in cuticle biosynthesis. One of the long-term ABA induced genes encoded no apical meristem (NAM) protein (At1g77450). Other long term ABA up-regulated genes, such as TRAF domain containing protein and rubber elongation factor, have not previously reported to be ABA regulated in *Arabidopsis*.

Identification of transiently up-regulated genes in response to ABA

Of the 122 ABA inducible genes, 106 genes were up-regulated transiently in response to ABA (Figs. 3-1 and 3-2). These genes encoded proteins with diverse known or proposed functions (Table 3-2). At each time point, new genes were up-regulated, in most cases, and they remained induced for only one or two of the time points. Of the 106 transiently ABA-induced genes, 5 corresponded to known transcription factors including the bZIP transcription factor ABI5 (At2g36270), homeobox-leucine zipper protein 6 (At2g22430), zinc finger (B-box type) protein (At5g24930), and homeobox-leucine zipper protein 5 (At3g01470). In addition, 13 genes involved in stress response including glutathione S- transferases (At2g02390), dehydrin RAB18 (At5g66400),

Table 3-1. Long-term ABA induced and repressed genes

Induced

Genbank	AGI	Description
t21007	at2g23120	expressed protein
n96955	at1g20440	dehydrin (COR47)
r30150	at1g77450	no apical meristem (NAM) family protein
t44357	at1g58270	meprin and TRAF homology domain-containing protein
t76794	at3g22600	protease inhibitor/seed storage/LTP family domain
t21841	at5g15970	cold-regulated protein COR6.6 (KIN2)
t42012	at3g56275	expressed pseudogene
w43270	at2g15970	cold acclimation protein WCOR413
aa042387	at3g01420	pathogen-responsive alpha-dioxygenase
aa042534	at1g76180	dehydrin ERD14
aa040976	at2g47780	rubber elongation factor (REF) protein-related
aa042369	at1g76180	dehydrin ERD14
n65644	at1g20450	dehydrin ERD10
af093546	at4g34000	ABA-responsive elements-binding factor (ABF3)
af334210	at4g34000	ABA-responsive elements-binding factor (ABF3)
n96800	at4g34000	abscisic acid responsive elements-binding factor(ABF3)

Repressed

Genbank	AGI	Description
aa042670	at3g15450	auxin down-regulated protein ARG10
n38558	at5g19120	extracellular dermal glycoprotein EDGP precursor
r87010	at3g15450	auxin down-regulated protein ARG10
aa042266	at3g15450	auxin down-regulated protein ARG10
aa042342	at1g03870	fasciclin-like arabinogalactan-protein (FLA9)
aa042353	at5g13240	expressed protein

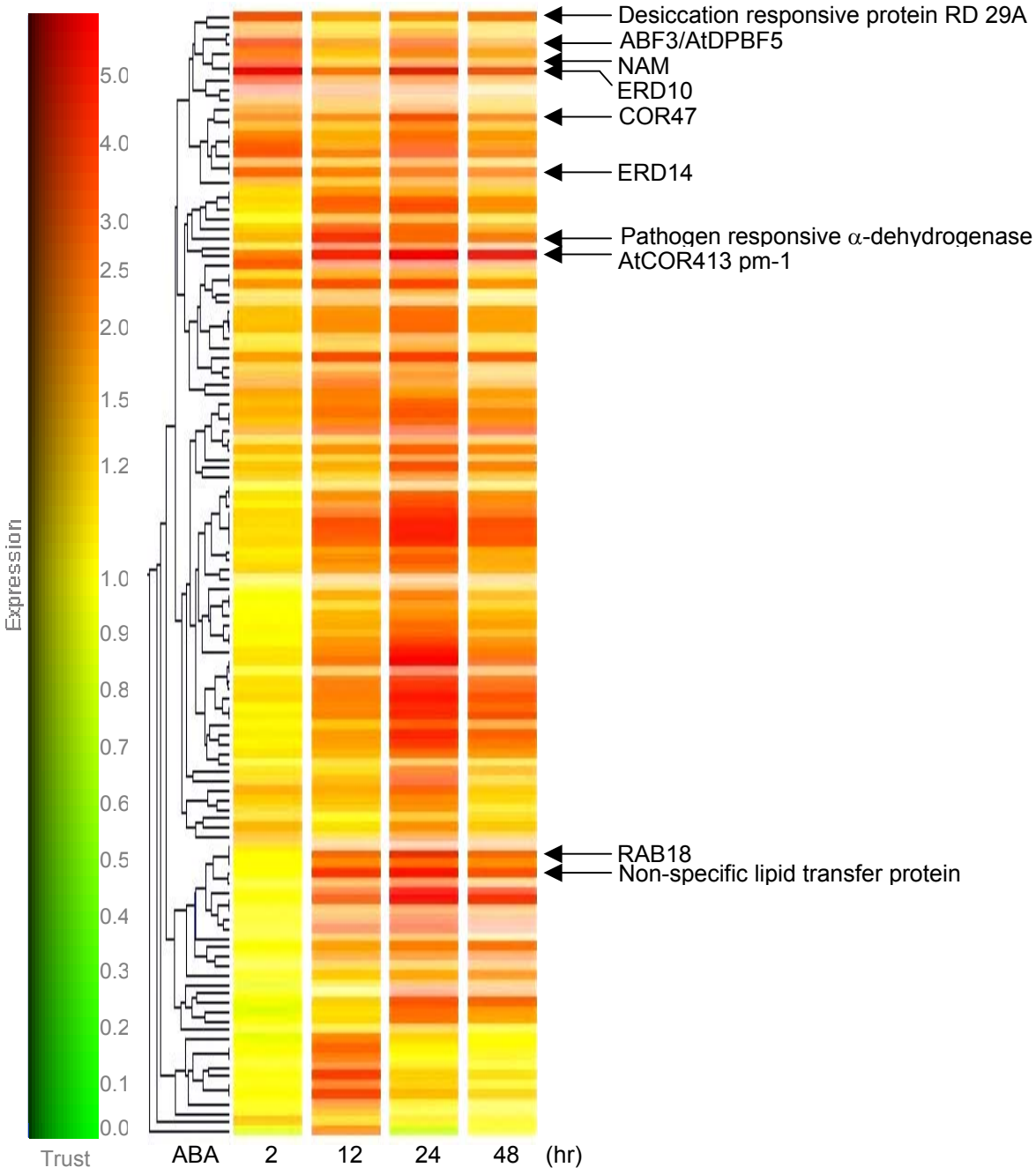


Figure 3-2. Classification of ABA-induced genes. 122 genes showing more than a 2 fold difference at each time points were selected and then were hierarchically clustered into groups using a Pearson correlation. A scale indicating the color assigned to each fold change is shown to the left of the cluster. The higher the absolute value of a fold difference, the brighter the color. The vertical dendrogram indicates the relationship among the induced genes by ABA treatment.

Table 3-2. Functional classification of transient ABA regulated genes

Activated genes

Functional category	No.	Description
Transcription factor	6	ABI5/AtDPBF1, homeobox-leucine zipper protein 6, zinc finger (B-box type) protein, homeobox-leucine zipper protein 5, CONSTANS protein 1
Stress response	21	osmotin-like protein OSM34, senescence/dehydration protein ERD7, drought-induced Di21, major latex proteindehydrin RAB18, peroxidase,
Hormone	1	gibberellin-regulated protein 1
Kinase	2	calcium/calmodulin-dependent protein kinase CaMK3, CBL-interacting protein kinase 6
Photosynthesis	8	light responsive protein, thioredoxin M-type 4
Ionic Homeostasis	2	metallothionein protein, ferritin 1 (FER1)
Transport	14	nonspecific lipid transfer protein 2, amino acid transporter, lipid transfer protein
Metabolism	31	beta-fructosidase BFRUCT3, glutathione reductase, succinate dehydrogenase, strictosidine synthase, alpha-dioxygenase, aquaporin, major latex protein, lactoylglutathione lyase, aldehyde dehydrogenase, glutathione S-transferase, cysteine proteinase, cytochrome P450
Unknown	14	pentatricopeptide (PPR) repeat-containing protein, glycine-rich protein , expressed protein
Unclassified	7	delta-adaptin, extracellular dermal glycoprotein

Repressed genes

Fnctional category	No.	Description
Transcription factor	4	bZIP transcription factor, zinc finger (B-box type) family protein, zinc finger (C2H2 type) protein
Stress response	8	1-aminocyclopropane-1-carboxylate oxidase, peroxidase
Hormone	5	gibberellin-regulated protein 4, auxin-regulated protein
Photosynthesis	1	photosystem I reaction center subunit PSI-N
Transport	2	lipid transfer protein , FAD-binding domain-containing protein
Metabolism	17	RuBisCO (ribulose bisphosphate caeboxylase), glutathione S-transferase, 60S ribosomal protein
Unknown	5	brix domain-containing protein, expressed protein
Unclassified	5	germin-like protein GER3, guanylate-binding family protein

osmotin-like protein OSM34 (At4g11650), and drought-induced protein Di21 (At4g15910) were induced transiently during the course of experiment. These results suggested that ABA treatment induced expression of large number of genes involved in stress response regulatory networks. A large number of metabolism related genes were also induced by ABA treatment, and several enzymes involved in secondary metabolism such as glutathione reductase (At3g24170), glutathione s-transferase (At2g02390), pathogen-inducible alpha-dioxygenase (At3g01420) and cytochrome p450 (At3g03470) were induced. These results suggest that metabolic reprogramming is initiated by ABA. Of the transiently up-regulated genes, 16 genes (13%) are of unknown function.

Cluster classification of ABA up-regulated genes

K-means clustering provides a way to further classify the regulated genes. This analysis was carried out using the GeneSpring software (Agilent Technologies, Inc., CA) and revealed that exposing seedlings to ABA induces a series of changes. The presentation of gene clusters showed that sets of genes were induced at each time point of ABA treatment, and these ABA-inducible genes were classified into 4 different groups based on their expression patterns during the course of experiment (Fig. 3-3).

The first group (Class I) containing ABF3/AtDPBF5 (At4g34000) was rapidly and highly induced reaching maximum expression at 2hr of ABA treatment, and in most case their expression remained induced for one or two additional time points during the course of the experiment. These expression patterns corresponded to northern blot analysis using ^{32}P-labelled cDNA probes corresponding to selected cDNA clones on the microarray slide (Fig. 3-3). Quantitative RT-PCR analysis indicated that ABF3/AtDPBF-5 (At4g34000) was

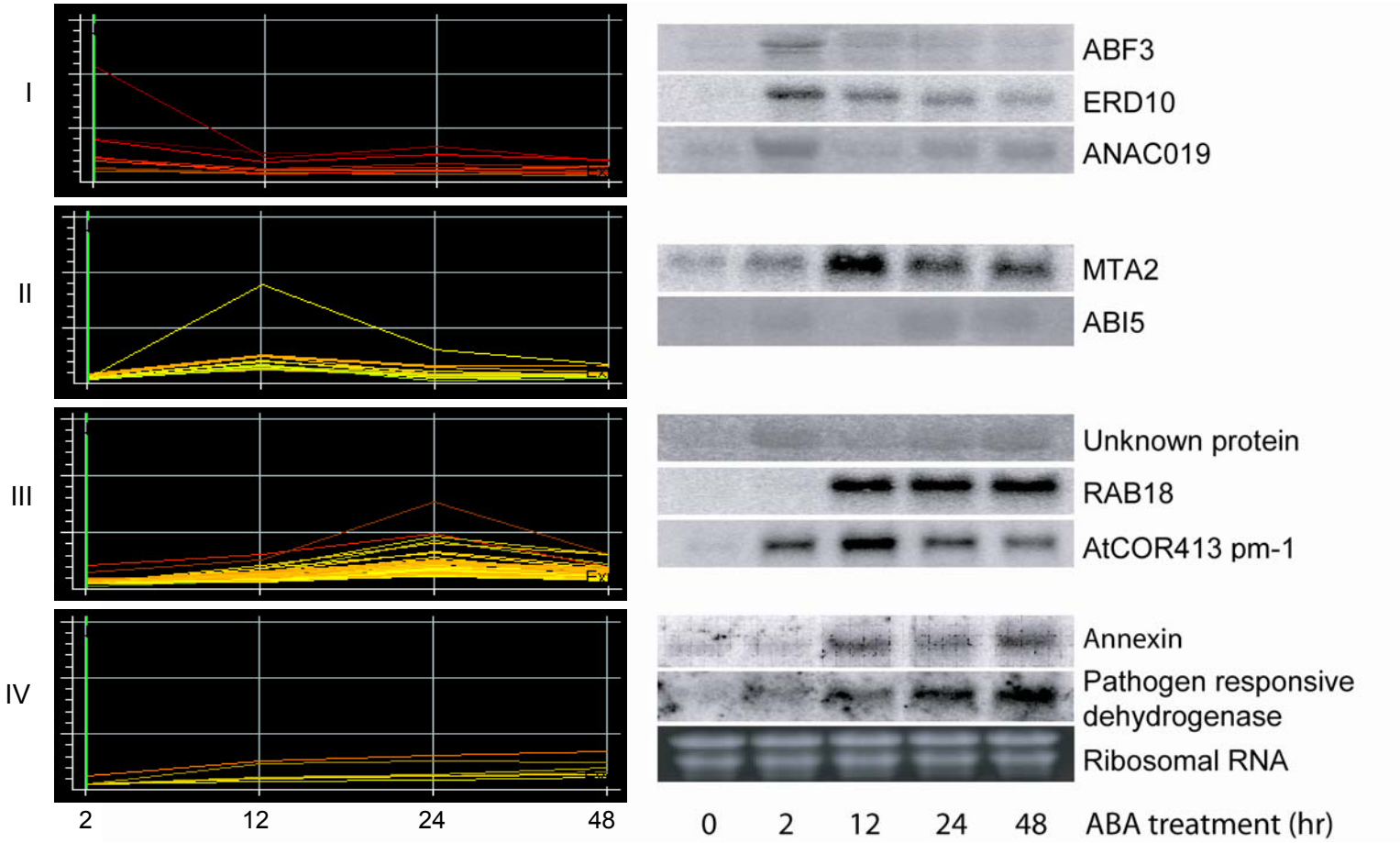


Figure 3-3. Classification of ABA-induced genes and confirmation of microarray data with northern blot analysis. K-means clustering of ABA induced genes and northern blot analysis. The genes showing more that 2-fold difference at each time points were selected and then were clustered into groups. Northern blot analysis was performed with identical RNA samples used for cDNA microarray analysis.

highly induced at 2hr of ABA treatment, but also highly induced at 48 hr of ABA treatment (Fig. 3-4). Ten of the 122 ABA induced genes were found to be classified into this group including ABRE binding factor ABF3 (At4g34000), no apical apical meristem and (NAM) protein (At1g77450), desiccation -responsive protein 29A (RD29A) (At5g52310) and senescence-associated protein 12 (At2g17840).

Class II showed maximum induction at 12 hr of ABA treatment and then decreased. This class contained ABI5/AtDPBF-1 bZIP transcription factor (At2g36270) and light regulated protein (At3g26740). This expression pattern was confirmed by northern blot analysis for MTA2 (At2g09390) which distinct roles in metal ion homeostasis and development, but neither northern blot analysis nor quantitative RT-PCR analysis of ABI5/AtDPBF-1 agreed with the cDNA microarray data (Fig. 3-3 and 3-4).

Class III included 91 (75%) of 122 ABA inducible genes and showed high induction at 24 hr of ABA treatment. The genes in this group encode a wide range of functions including the homeodomain transcription factor AtHB-6 (At2g22430), homeobox-leucine zipper protein ATHB-12 (At3g61890), homeobox-leucine zipper protein HAT5 (At3g01470), dehydrin RAB18 (At5g66400), cold-regulated protein AtCOR413 pm-1 (At2g15970), cold-regulated protein COR 6.6 (At5g15970), Lipid transfer protein (At1g48750) and cysteine proteinase RD21A (At1g47128).

Class IV genes showed a gradual increase with the highest induction at 48hr of ABA treatment. This class includes pathogen-responsive alpha-dioxygenase (At3g01420), strictosidine synthase (At1g74020) and glycosyl hydrolase (At3g57520). The expression pattern determined by microarray analysis in this group was consistent with northern blot analysis (Fig. 3-3)

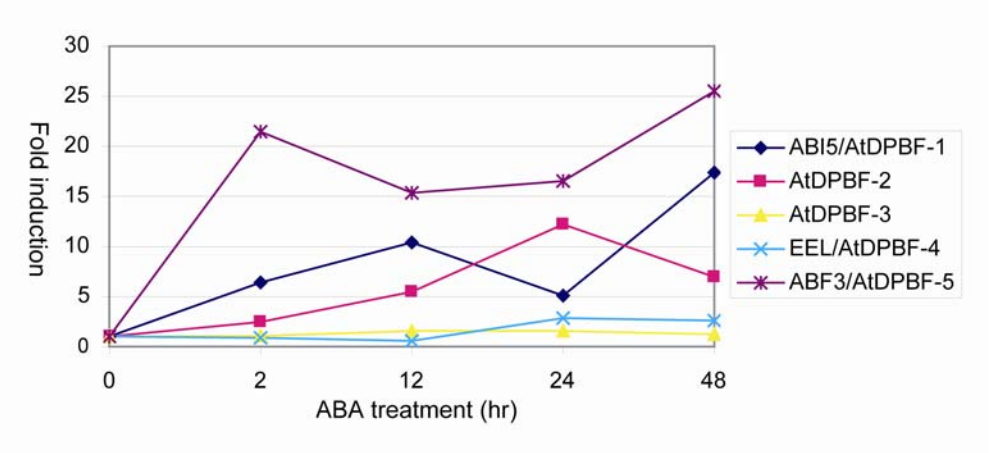


Figure 3-4. Confirmation of microarray expression data with quantitative RT-PCR (qRT-PCR). Transcript levels were normalized against actin levels and then the fold induction at each treatment was calculated relative to the control sample.

Identification of down-regulated genes in response to ABA

Of the 175 genes that were scored as being ABA responsive, 53 (30%) corresponded to genes that were down-regulated. 47 of which were affected transiently and 6 of which were affected over the long-term. Like the genes that were up-regulated in response to ABA, the down-regulated genes encoded proteins with a wide range of functions, including transcription regulation, stress response, signaling and cell maintenance/development.

Three of the long-term down-regulated genes encoded proteins involved in hormone signaling such as auxin down-regulated protein ARG10 (At3g15450) (Table 3-1). Furthermore, four transiently down-regulated genes encoded proteins involved in hormone signaling including gibberellin-regulated protein 4 (GASA4) (At5g15230) (Table 3-2). It is known that ABA and GA act antagonistically to regulate germination (Swain et al., 1997). Additionally, several genes encoding RubisCO (ribulose bisphosphate carboxylase) (At5g38410, At5g38420 and At5g38430) were found to be down-regulated.

Identification of ABA regulated genes via ABI5 regulatory network

The findings described above suggest that there might be two or more possible regulatory pathways, including the ABI5 regulatory pathway, which are activated in response to ABA. In order to determine which of the ABA responsive genes were also regulated by ABI5, transcriptome analysis was carried out on transgenic plants that either overexpresses the *ABI5* gene by the addition of Pro35S:ABI5 construct or had the *ABI5* gene knockout by a T-DNA insertion (*abi5-6*). The genes that were up- or down-regulated in these plants relative to wild-type plants were then compared to those that were ABA responsive. Three

independent lines of Pro35S:ABI5 seedlings and *abi5-6* mutant seedlings were used.

Analysis of these transgenic lines revealed that 10 genes showed changes in Pro35S:ABI5 seedlings and 44 genes showed changes in *abi5-6* seedlings. Among these regulated genes, in ABI5 over-expressing seedling, 4 up-regulated genes were found to be regulated in response to ABA in WT seedlings. Those include ABI5 bZIP transcription factor (At2g36270), pathogen-responsive alpha-dioxygenase (At3g01420) and peroxidase (At3g49120).

In *abi5-6* mutant seedlings, 17 up-regulated genes and 6 down-regulated genes were found to be also regulated in response to ABA in WT seedlings. These included cold acclimation protein AtCOR413 pm-1 (At2g15970), cold-regulated protein COR6.6 (At5g15970), nonspecific lipid-transfer proteins (At5g59310 and At5g59320) and peroxidase 21 (At2g37130). These 26 ABA-responsive genes that were also regulated in Pro35S:ABI5 seedlings and *abi5-6* seedlings could be considered members of the ABI5 regulon.

85% of ABA-regulated genes identified from this cDNA microarray experiments were not regulated either in Pro35S:ABI5 seedlings or *abi5-6* mutant seedlings. Therefore, these genes could be designated as being independent of the ABI5 regulon. It suggests the existence of regulons other than the ABI5 regulon in the ABA response regulatory network.

Promoter analysis of ABA responsive genes

To analyze ABA-regulated promoter sequences, sequences 1.5 kb upstream of known or predicted translation start sites of ABA regulated genes were obtained

from the Genbank sequence database. It was possible to identify upstream sequences for 135 of the 175 ABA-regulated genes identified by cDNA microarray analysis.

To identify *cis*-acting regulatory elements, PLACE (Plant *cis*-acting regulatory DNA elements) motif search analysis was performed. Figure 3-5 shows the occurrence of known ABA-related *cis*-regulatory motif ABRE (ABA-Responsive Element) and DRE (Dehydration Responsive Element)-like element in the promoter sequences. The analyses showed that G-box containing ABRE motifs and DRE regulatory motifs occur at significant frequencies in the promoters of ABA-induced genes. In the set of ABA-repressed genes, both ABRE and DRE regulatory motifs were detected in most of these genes and were presented at a moderate frequency in the promoter sequences.

In addition to searching for known motifs, we used the motif-finding program Weeder (Pavesi et al., 2004), which carries out an algorithm for the automatic discovery of conserved motifs in a set of related regulatory DNA sequences to identify over-represented motifs that occurred with statistical significance in the promoters of the ABA responsive genes. A number of motifs were identified in the promoters of ABA induced and repressed genes. Then the C++ program, Clover (*Cis*-eLement Over-representation) was used to determine *P*-values for motifs that Weeder identified as statistically over-presented in the promoter sequences.

When Weeder was run on the promoters of the set of ABA induced genes, ABRE-like motifs with the consensus sequence ACGT, DPBF core-like elements (Kim et al., 2002) with consensus sequence ACACNNG and CGCGBOX containing motif (Fig. 3-5 and Table 3-3) were over-represented. One of the identified ABRE-like motifs and a DPBF core-like element were significantly

present in the majority of the ABA induced genes. The ABRE motif sequences identified by this promoter analysis represent a subset of the classical G- box containing ABRE motif (C/G/T)ACGT(G/T)(A/C) (Yamaguchi-Shinozaki and Shinozaki, 2005) that is present in the promoters of large number of known ABA regulated genes. Subsequently, we applied the Clover program to determine if the identified motifs are statistically over-represented in the set of promoter sequences. The small *p*-values obtained for the given motifs suggest that the observed motifs were unlikely to have occurred by chance.

Running Weeder on the promoters of the ABA repressed genes showed two novel motifs and a CAATBOX containing motif were significantly over-represented in the majority of genes. Small *p*-values for the identified motifs also suggest that the observed motifs were statistically significant (Table 3-3).

Finally, we searched for highly conserved sequences in the promoters of ABA regulated genes by combining the sets of ABA induced and repressed genes in the same analysis (Table 3-3). ABRE-like motifs were statistically over-represented in most of ABA regulated genes, and a DPBF core-like element and CGCGBOX containing motif were over-represented in the promoters of some ABA regulated genes. The identified ABRE motif sequences represent a subset of the classical G-box containing ABRE motif (Yamaguchi-Shinozaki and Shinozaki, 2005).

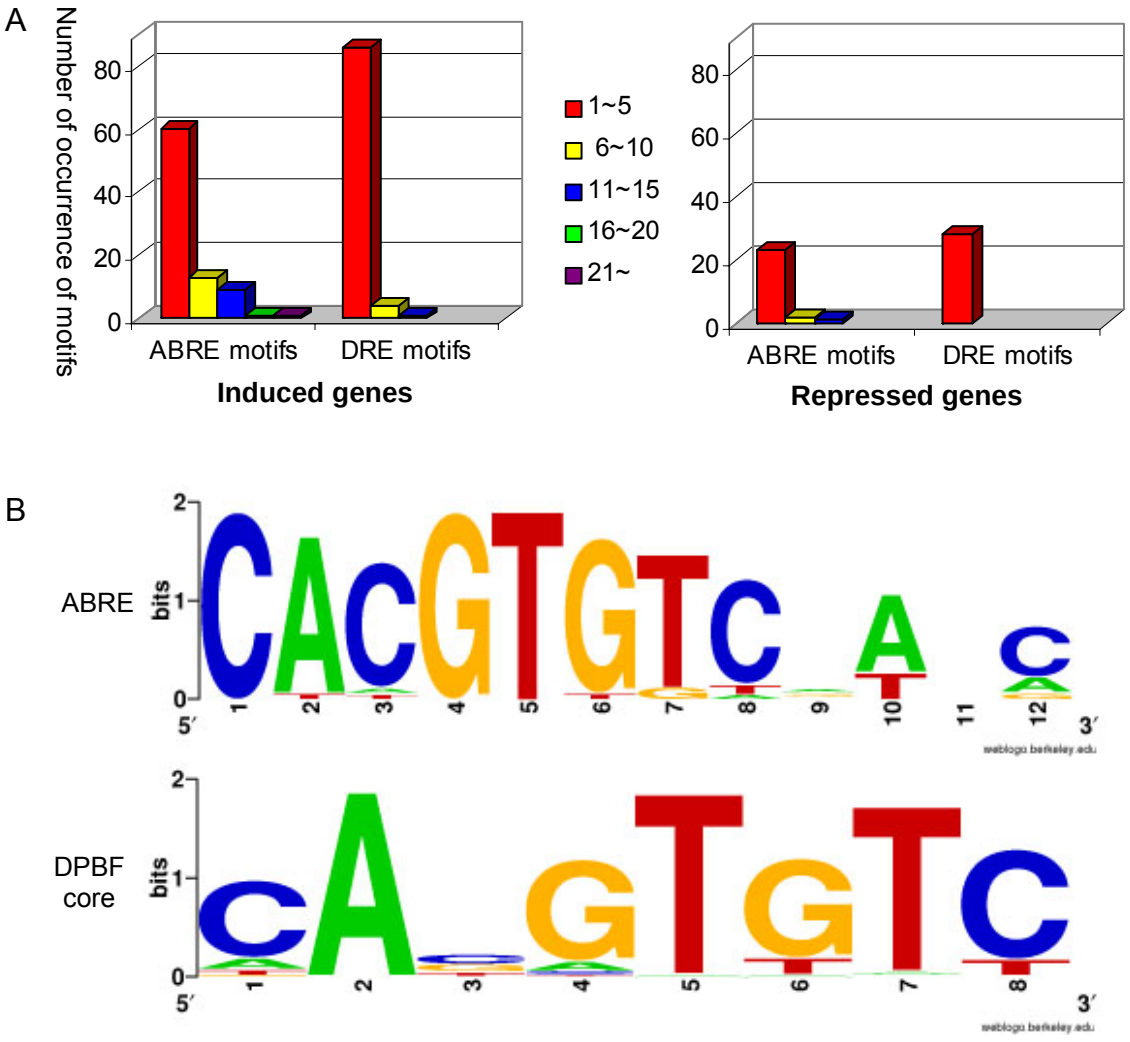


Figure 3-5. Organization of putative motifs in ABA-responsive genes. (A) Occurrence of putative ABRE and DRE motifs in the promoter of induced genes by ABA treatment. The promoter sequences were analyzed using PLACE motifs database to identify *cis*-regulatory elements. (B) Sequence Logo view of consensus ABRE and DPBF core motif sequences identified in the promoters of ABA induced genes using Weeder, an algorithm for the automatic discovery of conserved motifs in a set of related regulatory DNA sequences. The height of the letter at each position represents the degree of conservation.

Table 3-3. Statistically enriched motifs from ABA regulated genes

Coregulated Class	Sequence	Motifs ($P<10^{-8}$)	Genes with the motif
ABA Induced	CCACGT	ABRELATERD1	99%
	CAGGTGTC	DPBFCOREDCDC3	76%
		EBOXBNNAPA	
	CCGCGCTGGC	CGCGBOXAT	9%
	CACGTGTCGAGC	ABRELATERD1	19%
		CACGTGMOTIF	
		DPBFCOREDCDC3	
		MYCCONSENSUSAT	
ABA Repressed	CTACCA	N/A	100%
	TCTGGTAG	N/A	97%
	GCAATTAACGAC	CAATBOX1	77%
ABA Regulated	CCACGT	ABRELATERD1	100%
		ACGTATERD1	
	TAACGTGG	ABRELATERD1	91%
		ABRERATCAL	
		ACGTATERD1	
	CCGCGCTGGC	CGCGBOXAT	7%
	GAGGGACACGGG	DPBFCOREDCDC3	23%

Transcription profiling of Pro35S:ABI5 and *abi5-6* mutant seedlings in response to ABA

Identification of ABI5/ABA regulated genes in *Arabidopsis*

cDNA microarray analyses were performed as previously described to further dissect the pattern of ABI5- and ABA-mediated gene regulation in *Arabidopsis*. RNA was obtained from three independent biological samples taken from seedlings harvested just before Pro35S:ABI5 seedlings were treated with ABA and then at 2, 12, 24, and 48 hr after ABA treatment, and just before *abi5-6* seedlings were treated with ABA and then at 24hr after ABA treatment. Distinct fluorescent tags (Cy3 and Cy5) were used to label the transcription products from the untreated and treated seedlings. Two separate hybridizations were performed with independent microarray slides using identical RNA samples, but reversing the labeled tags. Fold changes in transcript levels were calculated for the three independent samples treated with ABA at each time point relative to the levels in the non-treated samples by comparing the intensities of the fluorescent Cy3 and Cy5 that hybridized to the cDNAs on the array.

A total of 310 genes were identified as ABI5/ABA regulated genes and were grouped into different classes (Figs. 3-6 and 3-7). This set of gene includes the genes previously identified as ABA responsive in WT. These ABA responsive genes were found to contain two classes. Class I was made up of 149 genes that responded to ABA, but were unaffected by *ABI5* expression levels. Class II was made up of 26 genes that responded to ABA and also were regulated by *ABI5* expression level. Among the 310 ABI5/ABA regulated genes was another class designated Class III (Fig. 3-6 and 3-7). This class was made up of 27 genes that were regulated by *ABI5* expression level, but that were not responsive to ABA in WT background. Finally, a fourth class was found to exist

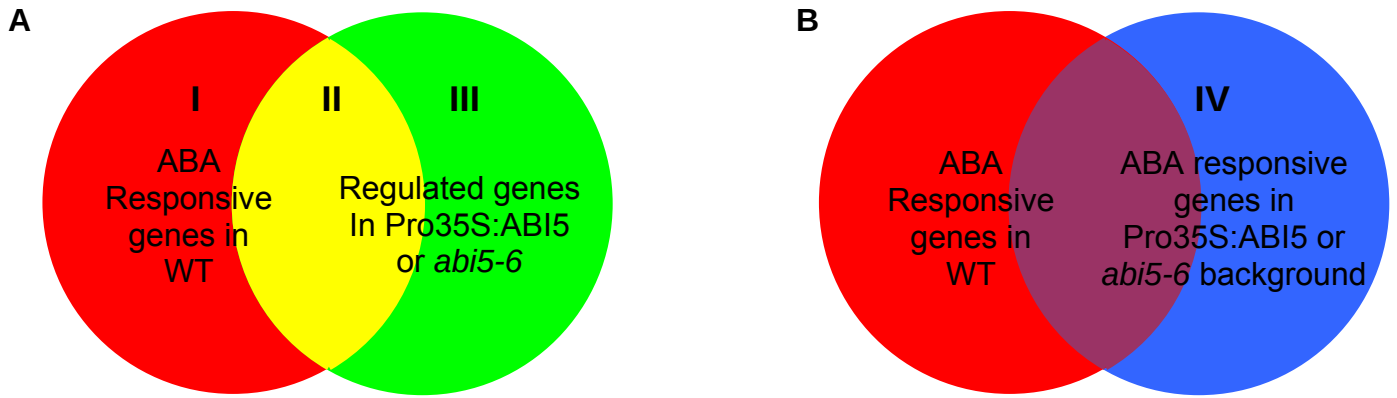


Figure 3-6. Venn diagrams analysis of ABI5/ABA regulated genes. (A) Analysis of genes regulated by ABA in WT and by *ABI5* expression level. The ABA responsive genes are red and yellow, and the genes regulated by *ABI5* expression level are green and yellow. The red colored genes are designated as Class I. The overlapping yellow colored genes are designated as Class II. The green colored genes are designated as Class III. (B) Analysis of genes regulated by ABA in WT as compared to genes regulated by ABA in Pro35S:ABI5 or *abi5-6*. Genes that respond to ABA in WT are colored red and purple. Those that respond to ABA in Pro35S:ABI5 or *abi5-6* are colored blue and purple. The blue colored genes were designated as Class IV genes. These genes are only regulated by ABA if the *ABI5* gene is either overexpressed or knocked down.

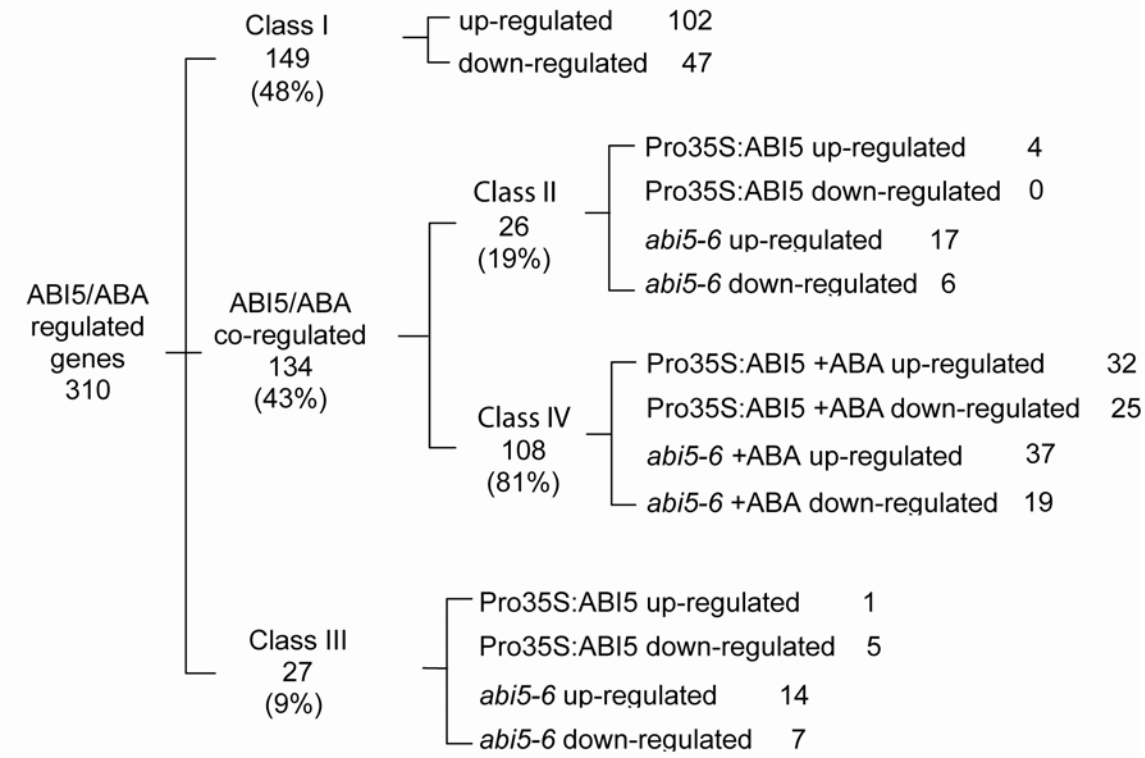


Figure 3-7. Classification of ABA/ABI5 regulated genes. Percentages in parentheses were calculated with total numbers of ABI5/ABA regulated genes identified by cDNA microarray analysis.

within the set of ABI5/ABA regulated genes. Class IV is made up of genes that respond to ABA in the *ABI5* overexpression lines or in the *abi5-6* knockout line, but not in WT lines. One hundred and sixty one of these genes (Class II, III, and IV) were regulated by ABI5. Of these, the 134 genes (Class II and IV) were controlled by ABI5/ABA in a co-regulated fashion. This indicates that there is a tight relationship between ABI5 and ABA on gene regulation. A large number of ABI5/ABA regulated genes encode proteins of unknown function. The characterization of these functionally unknown genes may be particular interest for future studies.

Class II genes

Twenty six (19%) of the one hundred and thirty-four ABI5/ABA co-regulated genes were found to be regulated by *ABI5* expression level and also regulated in response to ABA in WT seedlings. These were designated Class II genes (Figs. 3-6, 3-7 and Table 3-4).

As previously described, the majority of ABA-regulated genes identified by our cDNA microarray experiments were not regulated either in Pro35S:ABI5 seedling or *abi5-6* mutant seedling. Therefore, ABI5 may be a part of the ABA response regulatory network, but there could be other regulons in the network.

Class III genes

We identified an additional 27 genes that were regulated by ABI5 in Pro35S:ABI5 or *abi5-6* seedlings (Fig. 3-7), but did not show any changes in response to ABA treatment in WT seedlings (Fig. 3-6). These were designated

Table 3-4. Functional classification of Class II genes

Functional Category	No.	Description
Trnascriptional Regulation	1	ABI5 bZIP transcription factor
Stressresponse	11	cold-acclimation protein COR413 pm-1, dehydrin (ERD10), cold-responsive protein (COR6.6), pathogen-responsive alpha-dioxygenaseperoxidase 2, annexin 1
Hormone Response	1	1-aminocyclopropane-1-carboxylate oxidase
Transport	4	lipid transfer proteins
Carbohydrate metabolism	1	actoylglutathione lyase
Unknown function	8	vacuolar calcium-binding protein, fasciclin-like arabinogalactan (FLA9), curculin-like lectin

Class III genes. These ABI5-regulated genes could be a part of regulons other than ABA response regulatory network in *Arabidopsis* vegetative tissues. This group is composed of 6 regulated genes, including 1 up-regulated and 5 down-regulated in Pro35S:ABI5 seedling and 21 regulated genes, including 14 up-regulated and 7 down-regulated in *abi5* seedling. The largest group of genes (30%) in this class encoded proteins involved in stress responses including protein phosphatase 2C (At3g11410), heat shock protein 70 (At1G79930) and ACC oxidase (At1g62380). Another fifteen percent encoded proteins involved in defense response including osmotin-like protein OSM34 (At4G11650) and Bet v I allergen family protein (At1G70850) (Table 3-5).

Class IV genes

The class IV genes are ABA responsive in Pro35S:ABI5 or *abi5-6*, but not in WT. This group is composed of 57 regulated genes, including 32 up-regulated and 25 down-regulated in Pro35S:ABI5 and 56 regulated genes, including 37 up-regulated and 19 down-regulated in *abi5-6* in response to ABA (Fig. 3-7). In the class IV regulated genes, the majority of genes (27%) are predicted to function in metabolic pathways, followed by genes involved in stress response regulatory networks (18%) (Table 3-4). Those stress responsive genes encoded early-response to dehydration stress protein ERD4 (At1g30360), low temperature and salt responsive protein LTI6A (At3g05880), protein phosphatase 2C ABI2 (At5g57050) and ABI1 (At4g26080). These protein phosphatases ABI1 and ABI2 have been previously reported to be up-regulated in response to ABA (Leung et al., 1997; Merlot et al., 2001), but it was proven by our microarray experiment and northern blot analysis (data not shown) that these protein phosphatase are regulated by ABI5 in the presence of ABA. We also identified many genes in this class encoding proteins of unknown function (Table 3-6).

Table 3-5. Functional classification of Class III genes

Functional Category	No.	Description
Trnascriptional Regulation	1	Speckle-type POZ protein
Stress response	8	protein phosphatase 2C, alcohol dehydrogenase, annexin 1
Defense response	4	osmotin-like protein (OSM34), Bet v I allergen
Embryonic development	2	late embryogenesis abundant protein
Hormone response	1	1-aminocyclopropane-1-carboxylate oxidase
ell organization and biogenesi	1	xyloglucan:xyloglucosyl transferase
Photosynthesis	2	chlorophyll A-B binding protein / LHCII type I (LHB1B1)
Transport	1	lipid transfer protein 4
Carbohydrate metabolism	1	lactoylglutathione lyase
Other metabolism	1	allantoinase
Unknown function	5	glycine-rich protein, expressed protein

Table 3-6. Functional classification of Class IV genes

Functional Category	No.	Description
Transcriptional regulation	4	homeobox-leucine zipper 12, myb transcription factor, NAM protein, putative bZIP69
Stressresponse	20	protein phosphatase 2C ABI1, ABI2, dehydrin ERD4, glutathione S-transferase 6
Defense response	4	trypsin and protease inhibitor, mannitol dehydrogenase, hydroxyproline-rich glycoprotein
Embryonic development	2	Late embryogenesis abundant protein Lea14-A, LEA3 protein
Development	2	senescence-associated protein
Hormone Response	2	auxin-responsive GH3 protein, gibberellin-regulated protein 4 (GASA4)
Signal transduction	4	leucine-rich repeat transmembrane protein kinase, Ras-related GTP-binding protein
Cell organization and biogenesis	1	xyloglucan:xyloglucosyl transferase
Transport	6	mitochondrial substrate carrier family protein, ipid transfer protein (LTP) family protein
Regulation of biological process	2	Bax inhibitor-1 (BI-1)
Protein metabolism	6	chaperonin, cullin, subtilase protein, cathepsin B-like cysteine protease
Carbohydrate metabolism	4	beta-galactosidase, glycosyl hydrolase 3, alkaline alpha galactosidase
Secondary metabolism	1	cinnamoyl-CoA reductase
Other metabolism	17	Cys/Met metabolism pyridoxal-phosphate-dependent enzyme, RuBisCO small subunit 1B, RuBisCO small subunit 2B, isochorismatase hydrolase, D-3-phosphoglycerate dehydrogenase
Unknown	33	PUR alpha-1, phosphate-responsive protein, acid phosphatase, vacuolar calcium-binding protein OUT cysteine protease, actin-depolymerizing factor 6, alcohol acyltransferase

Promoter analysis of ABI5/ABA regulated genes

Co-regulated genes are likely to share common regulatory elements or motifs in the promoter. As previously described, sequences 1.5 kb upstream of known or predicted translation start sites of ABI5/ABA regulated genes were obtained to apply computational approaches for these regulated gene promoter sequences. To identify common regulatory sequences statistically over-represented in the promoters of ABI5/ABA regulated genes, the motif-finding program Weeder (Pavesi et al., 2004) and Clover (Frith et al., 2004) programs were employed.

First, we analyzed the promoters of the Class II genes to identify conserved motifs among these regulated genes. Our analysis showed that DPBF core motif containing ACACCTGG and a novel motif containing ATGCCGTCTA are significantly over-represented with statistical significance in the promoters of the combined Class II genes. In the promoter sequences of Pro35S:ABI5 up-regulated genes, two novel motifs and SORLIP motifs were significantly over-represented. The DPBF core-like element containing ACACCTGG, an ABRE motif containing ACGTGG and a novel motif were also statistically over-represented in the up-regulated genes of *abi5-6* (Table 3-7). Furthermore, in the promoters of *abi5-6* down-regulated genes, a novel motif and an ABRE motif were significantly over-represented. These indicated that ABI5 recognizes more divergent ABRE-like motif sequences including classical G-box containing ABRE and DPBF core motifs.

In the promoters of the combined Class III genes, the Site II element responsible for the expression of cytochrome genes in *Arabidopsis* (Welchen and Gonzalez, 2005) and the Myb core motif, binding site for MYB transcription factors involved in the regulation of genes that are responsive to water stress in *Arabidopsis* (Urao et al., 1993) were statistically over-represented (Table 3-8). In the

Table 3-7. Statistically enriched motifs in the promoters of Class II genes

Coregulated Class	Sequence	Motifs (P<0.05)	Genes with the motif
ABI5 or ABA Regulated	ACACCTGG	DPBFCOREDCDC3	96%
	ATGCCGTCTA	N/A	88%
	CGTGGCGGACAC	SORLIP1AT	30%
35S:ABI5 Induced	GCGGAC	N/A	100%
	TAGCGTGG	N/A	100%
	GCCACGCTAT	SORLIP1AT	100%
	ATGCCACGCTAT	Light-Induced Promoters SORLIP1AT	100%
abi5 Induced	ACGTGG	ABRELATERD1	100%
	ACACCTGG	DPBFCOREDCDC3	100%
	CGGTGAACGA	MYCCONSENSUSAT GTGANTG10 pollen	56%
	CCGAATAACACC	N/A	100%
abi5 Repressed	TGCTACCT	N/A	100%
	CGTTGACGTG	ABRELATERD1	100%
	CCAATATAAGCT	WBOXATNPR1 CAATBOX1	100%

Table 3-8. Statistically enriched motifs in the promoters of Class III genes

Coregulated Class	Sequence	Motifs (P<0.05)	Genes with the motif
ABI5 Regulated	TGGGCTGC	SITEIIATCYTC	70%
	GGCAACAGCT	MYBCORE	80%
	GGCAACAGCTAT	MYBCORE	60%
35S-ABI5 Repressed	GGACGT	ACGTATERD1	100%
	GCCTTGAT	N/A	100%
	GTCAATGCCT	CAATBOX1	100%
	AGGCTCTTTAGT	DOFCOREZM	100%
abi5 Induced	CGTTACCG	N/A	100%
	AGGTGTGTCG	N/A	80%
	ATGGGCTGCACC	SITEIIATCYTC	50%
abi5 Repressed	GGTTTGCA	N/A	100%
	TCTCAGCTCA	N/A	100%
	TAGGAGGATGCG	N/A	100%

promoters of down-regulated genes in Pro35S:ABI5 seedlings, an ABRE-like motif, a novel motif containing GCCTTGAT sequence and DOF core motif required for the binding of Dof zinc finger proteins in maize (Yanagisawa and Schmidt, 1999; Yanagisawa, 2000) were found to be significantly over-represented. We also identified additional novel motifs in the promoters of up-regulated and down-regulated genes in *abi5-6* seedlings. These suggest that the genes in this class might be regulated by other signaling pathways.

We tested promoters of the Class IV genes in the class of ABI5/ABA co-regulated genes. Conserved ABRE motifs with ACGT core motifs were found to be significantly over-represented with statistical significance in the genes of Pro35S:ABI5 seedlings up-regulated in response to ABA and other conserved ABRE motifs with ACGT were also found to be statistically over-represented in the genes of *abi5-6* seedlings up-regulated in response to ABA. This suggests that the ABRE sequence element is closely correlated with regulation by both ABI5 and ABA in the set of ABI5/ABA co-regulated genes (Table 3-9).

We also identified CGCG box sequences in the promoters of genes of Pro35S:ABI5 seedlings down-regulated in response to ABA (Table 3-9). The CGCG box containing (A/C/G)CGCG(G/T/C) sequence has been reported to be recognized by AtSR1, Ca^{2+}/calmodulin binding protein and was found in promoters of genes such as those involved in ethylene signaling, abscisic acid signaling, and light signal perception (Yang and Poovaiah, 2002). Our analysis also showed that a novel motif containing GTGCGC sequence and GATA box sequences were significantly over-represented in the promoters of genes of *abi5-6* seedlings down-regulated in response to ABA. The GATA elements have been shown to be conserved in the promoter regions of all LHCII Type I chlorophyll a/b binding protein (Cab) genes in Petunia and other dicotyledonous plants (Gidoni et al., 1989; Lam and Chua, 1989). Small *p*- values of these

Table 3-9. Statistically enriched motifs in the promoters of Class IV genes

Coregulated class	Sequence	Motifs ($P<10^{-8}$)	Genes with the motif
ABI5 and ABA Regulated	CCTGTGGC	D1GMAUX28	67%
		SORLIP1AT	
	ACCGCGCACT	CACTFTPPCA1	38%
		CGCGBOXAT	
	TGGAGACCAGTA	CACTFTPPCA1	30%
		SURECOREATSULTR11	
35S:ABI5 +ABA Induced	CTTACCACGT	ABRELATERD1	76%
	TACGTGGGATGC	ABRELATERD1	56%
35S:ABI5 +ABA Repressed	ACCGCGCA	CGCGBOXAT	100%
	TTACCGCGCA	CGCGBOXAT	100%
	TTACCGCGCACT	CACTFTPPCA1	81%
abi5 +ABA Induced	ACACGT	ABRELATERD1	100%
	TACGTGGG	ABRELATERD1	94%
	TATCAACGGC	MYB2CONSENSUSAT	74%
	AACAGCTCGACT	N/A	61%
abi5 +ABA Repressed	GTGCGC	N/A	100%
	CGCGCACT	CACTFTPPCA1	100%
	TTCATGATAA	GATABOX	84%
	CGTTATCATGAA	GATABOX	79%

identified motifs suggest that the observed motifs are statistically over-represented in the promoters of regulated genes.

In the promoter sequences of the combined Class IV genes, Sequences Over-Represented in Light-Induced Promoters (SORLIPs) motif was statistically over-represented in the majority of the Class IV genes (Table 3-9). Additionally, the tetranucleotide containing CACT sequence that is responsible for the mesophyll-specific expression of phosphoenolpyruvate carboxylase (*ppcA1*) was significantly over-represented (Gowik et al., 2004).

DISCUSSION

Transcription profiling of genes responsive to ABA

The plant hormone ABA plays an important role in the adaptation of vegetative tissues to abiotic stresses including drought, water stress, salinity and low temperature by regulating stomatal aperture (Ingram and Bartels, 1996; Himmelbach et al., 1998). The physiological response to ABA is achieved through the transcriptional regulation of stress-responsive genes (Bohnert et al., 1995). However, while few of the regulatory components have been identified so far, considerable insights have been gained on ABA-mediated transcriptional regulation. We employed cDNA microarray analysis to investigate gene expression in response to ABA at the genome level and identified 175 ABA-responsive genes corresponding to 2.5% of the total genes on the cDNA microarray. These include many genes previously reported to be ABA responsive indicating that cDNA microarray analysis was accurate in identifying

ABA responsive genes. In addition, we discovered a large number of ABA responsive genes not previously reported.

The set of ABA induced genes included a large number of genes that are classified as stress response genes and that are known to protect plants against stress conditions such as drought-induced Di21, cold-regulated protein COR6.6, dehydration protein ERD7, ERD14 and ERD10. Likewise, many lipid transfer protein (LTP) genes were identified in this set. These genes, not classified specifically as stress response genes have been shown to be involved in protecting membranes from stress by incorporating lipid molecules into the membrane (Hughes et al., 1992). Interestingly, the promoters of barley *LTP* genes contain putative ABRE and DRE/CRT elements (White et al., 1994). Furthermore, we identified genes for several enzymes involved in the detoxification of secondary metabolites produced against stress and pathogen attack. In addition, there are a number of transcription factors induced by ABA treatment suggesting that these transcription factors may be responsible for stress induced gene regulation. Also, several enzymes involved in protein degradation were induced suggesting that ABA is involved in the degradation of cellular regulatory proteins. Finally, ABA induction of a large number of metabolism related genes indicates that metabolic reprogramming might be initiated by ABA.

In the set of ABA repressed genes, there were genes for many GA regulated proteins. It is known that ABA and GA often play antagonistic roles in regulating plant growth, development and stress responses (Swain et al., 1997; Debeaujon and Koornneef, 2000). We also identified many genes for RubisCO (ribulose bisphosphate carboxylase) enzyme involved in catalyzing the first major step of carbon fixation in the Calvin cycle. ABA is known to reduce the photosynthetic

capacity of a leaf through an apparent inhibition of RubisCO activity, in addition to promoting stomatal closure (Seemann and Sharkey, 1987).

Although microarray analysis is a powerful and sensitive technique, it is still necessary to evaluate the validity of microarray data by alternative methods. By performing northern blot analysis on selected genes, we validated the cDNA microarray data by showing consistency between two different expression analysis approaches. However, the expression data from the cDNA microarray did not correspond well with qRT-PCR of the ABI5 subfamily of bZIP transcription factors (ABI5/AtDPBF1, AtDPBF2, AREB3/AtDPBF3, EEL/AtDPBF4 and ABF3/AtDPBF5). qRT-PCR indicated that *ABI5/AtDPBF1, AtDPBF2* and *ABF3/AtDPBF5* were induced at each time point and *EEL/AtDPBF4* was induced at two points during the course of experiment, but only *ABI5/AtDPBF1* and *ABF3/AtDPBF5* were found to be induced as measured by cDNA microarray analysis. The observed inconsistency between the two different methods might be due to high background on the microarray slide, weak expression of the genes, and/or cross-hybridization among these highly homologous genes.

Identification of ABA responsive genes by transcription analysis combined with the known sequence of the *Arabidopsis* genome allowed us to look for regulatory elements in the promoters of these genes. Several *cis*-elements are known to regulate ABA responses such as ABA-responsive elements (ABREs), coupling elements (CEs) and MYB and MYC consensus sequences (Busk and Pagès, 1998). In our study, one or more classical G-box containing ABRE motifs and DPBF core motifs were identified in the promoters of most of the ABA induced genes suggesting that ABA-responsive elements are the main *cis*-regulatory elements regulating ABA-induced gene regulation. Furthermore, we identified multiple classical G-box containing ABA-responsive elements (ABRE)

in all the promoters of the ABA regulated genes including both ABA induced and repressed genes. This clearly suggests that ABRE motifs are the main *cis*-elements involved in regulating ABA-dependent gene expression.

Transcription profiling of Pro35S:ABI5 and *abi5-6* mutant seedlings in response to ABA

Our cDNA microarray analysis revealed an intimate relationship between the ABA signaling network and ABI5-mediated gene regulation. We identified a number of known ABA-dependent signaling components that were regulated by ABI5 and/or ABA. Two negative regulators of ABA signaling, *ABI1* and *ABI2*, were induced in response to ABA in Pro35S:ABI5 and *abi5-6* vegetative tissues, respectively. Thus, this indicates that ABI5 interacts with the ABA signaling pathway to control expression of downstream genes through ABI1/ABI2 regulation. However, other studies have reported that both *ABI1* and *ABI2* were induced by ABA alone (Leung et al., 1997; Merlot et al., 2001) contradicting our result. This may be due to different experimental conditions such as different concentrations of ABA, different duration of ABA treatment or different stages of plant development. It is possible that different modes of gene regulation might result from ABA dosage, treatment time and plant growth state.

By analyzing cDNA microarrays, we could only identify a small number of genes showing changes in Pro35S:ABI5 vegetative tissues in the absence of ABA compared to Pro35S:ABI5 vegetative tissues treated with ABA. The difference in the number of regulated genes suggests that the ABI5 protein requires post-translational modification for further activation. Interestingly, it has been shown that ABI5 protein accumulates about 10 fold higher in the presence of ABA, and ABA induces ABI5 phosphorylation, activating it (Lopez-Molina et al., 2001).

In each class of ABI5/ABA regulated genes, there are genes regulated in Pro35S:ABI5 or *abi5-6*, and only small number of the genes identified from each genetic background were the same. Since bZIP proteins bind to specific DNA elements in the promoter region as homodimers or heterodimers, there might be certain proteins preferentially binding to specific promoter sequences. Vinson's group used mammalian bZIP proteins to predict dimer formation based on amino acids in a certain position of the leucine zipper region and found that dimer formation is more robust for homodimers than for heterodimers (Vinson et al., 2002). Additionally, mammalian bZIP proteins binding to specific DNA elements were predicted to be controlled by reduction/oxidation and phosphorylation of certain amino acid residues in the basic region of bZIP proteins, and these modifications were sufficient to block binding to DNA elements (Amoutzias et al., 2006). Based on the findings from Vinson's group, the genes that showed changes in the *ABI5* overexpressing genetic background might be exclusively regulated by ABI5 homodimers, and the genes that showed changes in *abi5-6* might be substantially regulated by homodimers of other ABI5-related bZIP proteins. Bensmihen's group reported that ABI5 and EEL form heterodimers and compete for the same binding sites within the AtEm1 promoter (Bensmihen et al., 2002). Furthermore, we also proved heterodimer formations among some proteins in the ABI5 bZIP subfamily and binding of these heterodimers to ABRE-like DNA element by electrophoretic mobility shift assay (EMSA), as discussed in chapter V.

From the promoter analysis of ABI5/ABA regulated genes, the DPBF-core like elements along with ABRE-like elements were identified from the promoters of Class II genes. However, only conserved ABRE elements were identified from the promoters of Class IV genes. These results suggest that ABI5 recognizes more divergent forms of ABRE sequences in Class II genes than in Class IV

genes. Furthermore, different *cis*-regulatory elements including Myb core motifs were statistically over-represented in the promoters of Class II genes. This suggests that ABI5 may interact with other signaling pathways involving the Myb transcription factor.

MATERIALS AND METHODS

Plant materials and ABA treatment

Arabidopsis thaliana seeds were surface-sterilized by treatment with 70% ethanol for 1 min followed by 50% household bleach for 7 min with rotation, washed 4 times with sterile water, and plated on MS solid (Murashige and Skoog, 1962) medium or placed in MS liquid medium. The plates and liquid medium containing sterilized seeds were incubate 3 days at 4°C. Seeds were germinated and grown on MS medium containing 1% sugar and 0.25% phytoagar or incubate in liquid MS medium supplemented with 1% sucrose with gentle shaking for 10 days under continuous light at 22°C. For ABA treatment, seedlings grown on MS solid medium were transferred to plates containing 50 μM ABA (+/- mixed (*cis-trans*) Isomers, PhytoTechnology Laboratories, Shawnee Mission, KS), and seedlings grown in liquid medium were supplemented with 50 μM ABA to treat seedlings with ABA at 4 intervals (2, 12, 24 and 48 hr).

RNA isolation and gel blot analysis

Whole *Arabidopsis* seedling tissues were collected and stored at –90°C. RNA

extraction was performed from frozen tissues using the RNeasy Plant Kit (Qiagen, Valenci, GA) or modified hot phenol/SDS extraction and LiCl precipitation protocol (Ausubel et al., 1994). Contaminating DNA was removed from each RNA preparations using DNA-*free*™ kit (Ambion, Austin, TX) followed by the manufacturer's instructions. Three independent biological replicates were used to isolate total RNA. The isolated total RNA (15 μg) was denatured at 65°C for 15 min with a mixture of 2 μl 10X MOPS, 3.5 μl formaldehyde and 10 μl deionized formamide, resolved in 1% agarose/MOPS-formaldehyde gels, subsequently capillary transferred to nylon membrane (Hybond N^+ TM, Amersham Pharmacia Biotech, Arlington, IL) using 20X SSC, and fixed using Stratagene UV crosslinker as described (Maniatis et al., 1982). The membranes were hybridized with ^{32}P-labelled cDNA probes generated as previously described. Hybridization was performed at 42°C in 5X SSC, 5X Denhardt's solution, 1% SDS, 100 μg salmon sperm DNA, 5% Dextran Sulfate, and 50% formamide for 14-16 hr. After hybridization, filters were washed with 2X SSS, 0.1% SDS for 10 min and 0.2X SSC, 0.1% SDS for 5 min at 65°C respectively.

Expressed sequence taq (EST) cDNA clones

The *Arabidopsis* cDNA microarray was generated from 7,060 ESTs of the CD4-27 non-redundant *Arabidopsis* EST library from ABRC (The Arabidopsis Biological Resource Center, The Ohio State University, Columbus, OH). Each EST clone was blasted against TAIR databases, and 5,061 were found unique based on the matches to the Arabidopsis Gene Identification (AGI) numbers.

Amplification of cDNA clones

In the cDNA microarray analyses, 7,060 cDNA clones of the CD4-27 non-redundant *Arabidopsis* EST library were included and 8 transgenes, 10 spiking controls and 39 constitutively expressed genes were included as internal control. pZL-1 and pBluescript vectors were used for cDNA library construction. All cDNA clones were inoculated into a 96-well V-bottom plate containing LB/Ampicillin (50 mg/ml) and were placed in a shaking incubator (200 rpm) overnight at 37°C. PCR reactions (50 µl) consisted of 10 µM dNTP mix, 1 O.D. primer mix (SP6 primer, T3 primer, and T7 primer) and 1 µl of overnight culture. Amplifications were carried out using Home made Taq polymerase in 10X reaction buffer. The PCR amplification was performed with cycling conditions followed: an initial three-minute denaturation at 95°C to disrupt cells and release the plasmid DNA, 45 cycles (94°C for 30 sec, 45°C for 45 sec, 72°C for 1 min), followed by 3 min at 72°C. PCR products were ethanol precipitated and resuspended in 50 µl of TE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA). One-tenth of the PCR product was analyzed on a 0.8% agarose gel containing ethidium bromide (EtBR) to confirm amplification quality.

Microarray fabrication

PCR product in 96-well plates were dissolved in water, and then mixed with 20X SSC (final concentration 3X SSC) for microarray spotting. DNA printing was performed using an OmniGrid Microarrayer (GeneMachines, San Carlos, CA) equipped with Telechem SMP3 Pins (TeleChem International, Inc., Sunnyvale, CA). PCR products were deposited onto poly-L-lysine-coated glass slides (CEL associates, Inc., Pearland, TX). Duplicated spots were made for each PCR product. Printing was carried out at 21°C at 60% humidity. After printing, the

microarray slides were allowed to dry completely at ambient conditions and stored in a desiccator at room temperature.

Microarray hybridization and scanning

Three different total RNA samples were used as template to synthesize Cyanine3 (Cy3)- or Cyanine5 (Cy5)-conjugated dUTP (Amersham Biosciences, Piscataway, NJ) labeled cDNA probe using FluoroScript cDNA Labeling System (Invitrogen life technologies, Carlsbad, CA). 25 μg of total RNA was used for reverse transcription and labeling with Cy-dyes followed by manufacturer's instructions. The successfully labeled cDNA samples were combined to perform microarray hybridization. Microarray hybridization was followed as described by Quackenbush's group (Hegde et al., 2000). For probe hybridization, one slide was hybridized with one pair of labeled probes; the other slide was hybridized with same probe pair but oppositely labeled. Cy-3 or Cy-5 labeled probes (10 μl) were mixed with 2 μl of 10 mg/ml sheared salmon sperm DNA and heated at 95°C for 3 min and then added 22 μl of 2X hybridization buffer (10X SSC, 50% formamide, 0.2% SDS) that has been preheated at 42°C. For array hybridization, each array was supplied with 44 μl of denatured probe pair to the surface of prehybridized array. The slide was then covered with coverslip and incubated in a sealed hybridization chamber at 42°C for 16-20 hr. During the incubation, the hybridization chamber was humidified with pre-added 3X SSC. At the end of incubation, the slide was washed with wash buffer 1 (1X SSC, 0.25% SDS) at 42° for 4 min and wash buffer 2 (0.1X SSC, 0.2%v SDS) at room temperature for 4 min with agitation followed by washing in 0.1X SSC for 4 min with agitation. After washing, the slide was dried by centrifugation at 1000g for 2 min. Hybridized microarray slide was scanned for Cy-3 at 523 nm and Cy-5 at 635 nm with an Affymetrix Array scanner 428 (Affymetrix, Santa Clara, CA). Two

separate TIFF images were generated for each channel and loaded into image analysis program.

Data analysis

Spot intensities were quantified using GenePix 4.0 (Axon instruments, Foster city, CA). This program automatically aligns feature-indicators with feature, associates individual microarray features with gene name and IDs, calculates and subtracts local background effects, and plots scatter plots of the extracted data for all the features on a microarray. The spots that were weak and have a small number of bright pixels were marked absent (A) and good spots were marked present (P). Total net signal intensities of each of two channels from good spots (present) were obtained and the median value of Cy-3 and Cy-5 signals were further analyzed by using GeneSpring (Agilent Technologies, Inc., Santa Clara, CA). GeneSpring is a powerful visualization and analysis solution designed for use with genomic expression data.

For normalization, intensity dependent LOWESS (locally weighted regression scatter plot smoothing) normalization was applied to adjust intensity-dependent variation due to dye properties (Quackenbush, 2002). Fold changes were calculated for each sample harvested after series of ABA treatment compared with each of samples harvested before ABA treatment, generating six measurements for each gene at each time point during ABA treatment. For the comparison of WT and Pro35S:ABI5 or *abi5-6*, fold changes were calculated for Pro35S:ABI5 or *abi5-6* seedling generating six measurements for each comparison. Genes showed significant 2-fold or greater changes were determined being up-regulated and down-regulated at any time point of treatment and were selected for further analysis. Differentially expressed genes

were filtered by only selecting genes that exhibited at least 2-fold changes in four of six microarray hybridizations and then applied another filtering to remove genes that had t-test *p*-value > 0.05. Then genes showed statistically significant changes at each time point of ABA treatment were combined and the average values of each replicate were used to perform clustering analysis. K-means clustering was used to group genes based on their expression pattern in response to ABA. This produced groups of genes with high degree of similarity within each group and a low degree of similarity between groups.

Promoter analysis

Sequences 1.5 kb upstream of known or predicted coding sequences that present on cDNA microarray were obtained from Genbank and used to search for *cis*-regulatory elements of ABA responsive and ABI5/ABA regulated genes using PLACE motif database for plant *cis*-acting regulatory DNA elements. The query sequence was used to search for motifs identical to, or similar to the previously reported *cis*-element motifs in the PLACE (Higo et al., 1999) database using the homology search tools. In addition to searching the presence of known motifs in the promoter, sequence elements conserved in a set of promoter sequences were detected using Weeder (Pavesi et al., 2004) which is based on the exhaustive enumeration technique. It automatically starts different runs of the program, each one with different parameters, and then provides an overall summary of the results as well as some advice based on statistical significance and some simple considerations (Pavesi et al., 2004). Then we further applied a C++ program called Clover (*Cis*-Element Over-representation) to determine which motifs identified from Weeder are statistically over-presented in a set of promoter sequences. First, a raw score is calculated to quantify the degree of the motif's presence in the test sequences. Then, *P*-values are

estimated for the raw score by several different ways to assess the probability of obtaining a raw score of the size or greater by chance (Frith et al., 2004). Furthermore, graphical representation of nucleic acid multiple sequence alignment was performed using WebLogo. Sequence logos were generated and each logo consists of stacks of symbols, one stack for each position in the sequence.

CHAPTER IV
IDENTIFICATION OF ABI5-REGULATED GENES USING MULTIPLE MICROARRAY PLATFORMS

INTRODUCTION

The invention of high-throughput genomic technologies, specifically DNA microarrays or gene chips, has enabled the simultaneous evaluation of mRNA levels for thousands of genes in any biological sample (Lobenhofer et al., 2001). With DNA microarrays, a huge amount of genetic information generated by DNA sequencing projects can be analyzed in a facile and economical manner (Case-Green et al., 1998). Because physiological responses involve changes in the complex regulatory networks that affect gene expression, DNA microarrays help to elucidate regulatory networks that affect a large number of genes. Furthermore, DNA microarrays can help to understand the modes of action of compounds and can improve basic science research as well as biomedical research including disease diagnosis and new drug development (Lobenhofer et al., 2001).

The most common microarray platforms are based on a collection of cDNA clones or synthesized oligonucleotides. Spotted microarray platforms include: cDNA microarrays (Schena et al., 1998) that contain completely sequenced or partially sequenced cDNA clones with known or unknown functions; and oligonucleotide arrays that are fabricated by academic laboratories and industrial manufacturers such as Agilent Technologies, GE Healthcare Codelink™, Operon and Illumina (Kane et al., 2000). Another common microarray platform, the Affymetrix GeneChip^{TM} system (Affymetrix, Santa Clara, CA), employs 20-25

mer oligonucleotide sequences that are synthesized using a combination of photolithography and oligonucleotide chemistry directly on the surface of the array as well as mismatched (MM) sequences to ensure hybridization specificity (Lockhart et al., 1996).

These different types of microarrays have their advantages and disadvantages. Generally, oligonucleotide microarrays offer high specificity for distinguishing highly conserved sequences like members of gene families (Kane et al., 2000; Hughes et al., 2001) and the capacity to distinguish single-nucleotide polymorphisms (Guo et al., 1994). However, extensive knowledge of the genome sequence is required for the design of oligonucleotides, and accurate and specific oligonucleotide sequences are required to avoid cross-hybridization (Rouillard et al., 2003). Conversely, sequence information is not required for cDNA microarray fabrication. However, intensive labor is required for preparing bacterial stocks of cDNA clones, amplifying cDNA clones, purifying the reactions, and preparing the DNA solution for printing. Also, there is the potential of misplacing clones when handling thousands of clones necessary for the construction of a cDNA microarray (Kehoe et al., 1999).

Due to the number and variety of microarray platforms and data analysis methods, it is important to determine how and whether results can be compared across platforms (Pylatuik and Fobert, 2005). To address the question, a number of studies have been performed to compare data from different microarray formats and different laboratories. In the comparison of cDNA and oligonucleotide microarrays, substantial variation and a low level of correlation were found (Kothapalli et al., 2002; Kuo et al., 2002; Woo et al., 2004; Zhu et al., 2005). However, the evaluation of commercially available platforms revealed statistical significance of common genes (Barczak et al., 2003; Woo et al., 2004). Unexpectedly, one group found that there is a high degree of agreement

between a mouse cDNA microarray and the Affymetrix Gene Chip (Dabrowski et al., 2006). Based on the information from published literature, the comparisons of cross-platform and cross-laboratory could not resolve the issue of reproducibility between different microarray platforms.

The *Arabidopsis thaliana* genome is the first plant genome to be fully sequenced (The Arabidopsis Genome Initiative, 2000). This sequence information, together with a large collection of expressed sequence tags (ESTs), has facilitated high-throughput genomic technologies to study gene expression on a global scale in *Arabidopsis* (Schaffer et al., 2000).

In this study, we present a cross-platform comparison of three *Arabidopsis* DNA microarrays: a cDNA microarray, the Agilent *Arabidopsis* 2 Oligo microarray and the Affymetrix ATH1 genome arrays. The three platforms were evaluated using identical RNA samples isolated from WT and *abi5-6* dry seeds with the goal of identifying ABI5 bZIP transcription factor-regulated genes. ABI5 is a key transcription factor regulating gene expression during embryogenesis. We focused on comparing the expression of 4,518 unique genes represented in all three microarray platforms. The Pearson correlation coefficient ranged from 0.53 to 0.83 depending on the microarray pair used for analysis. To validate the microarray data, quantitative real-time PCR (qRT-PCR) was performed with 53 selected genes. We found that the best correlation was between the Affymetrix ATH1 genome array and qRT-PCR.

RESULTS

Common genes across three microarray platforms

ABI5-regulated genes in seeds were identified using three different microarray platforms. These include a cDNA microarray, the Agilent *Arabidopsis* 2 Oligo microarray and the Affymetrix ATH1 genome array. RNA was obtained from three independent biological samples taken from WT and *abi5-6* dry seeds. For dual-color microarrays including cDNA and Agilent, two separate hybridizations were performed with independent microarray slides using identical RNA samples, but reversing the labeled Cy3 and Cy5 tags. For the Affymetrix array, two separate single-color hybridizations were performed for each WT and *abi5-6* dry seeds. The data from three experiments allowed a cross-platform comparison to be made.

Probes/cDNA clones present in the microarrays were compared across platforms based on the *Arabidopsis* Genome Initiative (AGI) locus identifiers (locus ID). Affymetrix ATH1 genome array and Agilent *Arabidopsis* 2 oligonulceotide array probe sequences were annotated with AGI locus ID numbers by the manufacturer, but the in-house fabricated cDNA microarray was annotated with Genbank IDs. Therefore, sequences of the EST clones present in the cDNA microarray were queried with the BLAST algorithm against the *Arabidopsis* (TAIR) DNA sequence database, and the AGI locus ID was assigned to the corresponding EST clones.

In the Affymetrix ATH1 genome array, 22,810 elements representing 22,353 unique genes were included, and each probe synthesized on the array is composed of a 25-mer oligonucleotide. The Agilent *Arabidopsis* 2 oligonulceotide array contains 22,575 features representing 21,500 unique

Table 4-1. Gene sets represented in 3 microarray platforms

	cDNA Microarray	Agilent Oligo Array	Affymetrix ATH1 Array
Total features	7123	22575	22810
Unique genes	5335	21500	22591

	cDNA vs Agilent	cDNA vs Affymetrix	Agilent vs Affymetrix
Genes in common	4789	4756	18739
Genes in common in all 3 formats	4518	4518	4518

genes and each probe is composed of a 60-mer oligonucleotide (Table 4-1). By performing BLAST searches of the EST sequences present on the cDNA microarray against the TAIR database, we found that 6,864 ESTs were assigned an AGI locus ID representing 5,076 unique genes. The other 259 ESTs did not show a significant hit. Therefore, the cDNA microarray used in this study represents 5,335 unique genes (Table 4-1).

The majority of genes represented in the Affymetrix array were included in the Agilent array. Additionally, most of the AGI locus IDs assigned to ESTs were present in both the Affymetrix array and the Agilent array. In conclusion, 4,518 unique genes are represented in all three microarrays platforms. We used these 4,518 genes to compare gene expression across three microarray platforms (Fig 4-1 and Table 4-1).

Intra-platform comparison

To compare technical replicates and different microarray platforms, the Pearson correlation coefficient was calculated to measure the strength and direction of a linear relationship between two variables. The sample correlation coefficient is designated as *r* and ranges from -1 to +1. A positive value implies a positive association, and a negative value implies a negative association between samples. For intra-platform comparison, the correlation was calculated for both ratios and $\log_2$ ratios from each sample. Hybridization ratios of WT to *abi5-6* for the Affymetrix ATH1 genome array used data obtained from chips hybridized to WT and other chips hybridized to *abi5-6.* These ratios were compared to the ratios obtained from other platforms that were hybridized to WT and *abi5-6* simultaneously. It was previously reported that low intra-platform concordance has been caused by the combination of low intra-platform consistency and poor

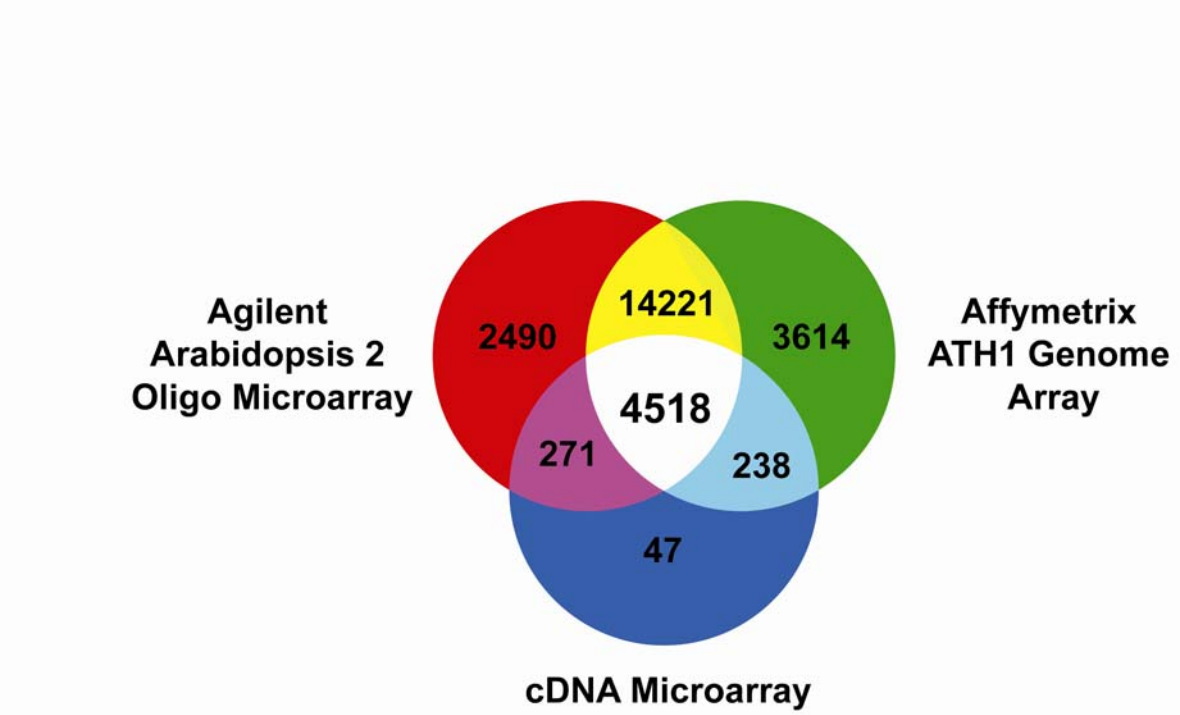


Figure 4-1. Venn diagram with numbers of genes present on each microarray platform. Overlapping gene sets are indicated by distinct colors. Genes common to the three microarray platforms are indicated in the white sector.

data analysis, rather than due to technical differences between the platforms (Shi et al., 2005). Therefore, we first tested intra-platform consistency before comparing gene expression across platforms. For the cDNA microarray, the overall correlation coefficient value between replicates was low for the absolute ratio and $\log_2$ ratios. However, removing genes with poor spot quality (spot quality filtering) and low confidence (<95%) (confidence level filtering) increased the intra-platform correlation level except for the cDNA microarray hybridized with sample 3 (Table 4-2). Therefore, sample 3 was eliminated from the cDNA microarray data set for further comparison among the different microarray platforms. One set of Agilent arrays showed a good correlation between replicates, but the other set had very low correlation value for absolute ratio and $\log_2$ ratio indicating poor hybridization performed with one of the duplicate slides. However, high correlation was observed when two RNA samples were compared with application of spot quality filtering (Table 4-2). For intra-platform comparison of the single-color microarray, Affymetrix arrays were identically paired as other dual-color microarray platforms. In this microarray platform, high correlation was observed between replicates of same samples, and relatively good correlation was observed between independent biological samples after spot quality filtering (Table 4-2).

Intra-platform comparison from these microarray platforms demonstrated that the consistency of gene expression between technical replicates was generally good for absolute ratio and $\log_2$ ratio following application of spot quality and/or confidence level filtering.

Table 4-2. Correlation coefficient of intra-platform comparison

		Present Only		Present and t-test *p* -value <0.05	
		Ratio	Log2	Ratio	Log2
cDNA	sample 1	0.16	0.42	0.79	0.78
	sample 2	0.20	0.30	0.83	0.80
	sample 3	-0.03	-0.27	-0.17	-0.17
Agilent	sample 1	0.77	0.69	-	-
	sample 2	0.27	0.27	-	-
	1 vs 2	0.84	0.76	0.49	0.68
Affymetrix	sample 1	0.83	0.82	-	-
	sample 2	0.88	0.85	-	-
	1 vs 2	0.48	0.53	0.37	0.40

Pearson correlation coefficient was calculated from ratio and $\log_2$ ratio of microarray data for each platform. For Affymetrix ATH1 genome array, ratio and $\log_2$ ratios are generated by identical pairing as cDNA microarray and Agilent oligo array. Results are shown for unfiltered and filtered data for flags and t-test *p*-value.

Inter-platform comparison

4,518 unique genes present in all three microarrays were used in the comparison across platforms, and the correlations between microarray platforms were calculated based on the averaged $\log_2$ ratios for each comparison. To compare different microarray platforms, dual-color microarray platforms were normalized as in the intra-platform comparison by the LOWESS method. In the case of single-color microarrays, normalization was performed differently. First, each measurement was divided by the 50th percentile of all measurements in that sample. Then, eight microarrays including four chips hybridized with WT seed and four chips hybridized with *abi5-6* dry seed were normalized against the median of the WT dry seed. All expression ratios obtained from each microarray analysis were transformed to $\log_2$ scale to compensate for the difference in signal intensities among the different microarray platforms (Kuo et al., 2006).

The correlation coefficient between the Affymetrix and Agilent microarrays was 0.238 without filtering, while it was 0.832 after filtering for spot quality and confidence level (Table 4-3). Also, the comparisons between the cDNA microarray and the oligonucleotide microarrays (either Affymetrix or Agilent) showed a relatively good correlation coefficient (0.524 and 0.528) after filtering for spot quality and confidence level.

Validation of microarray data by qRT-PCR

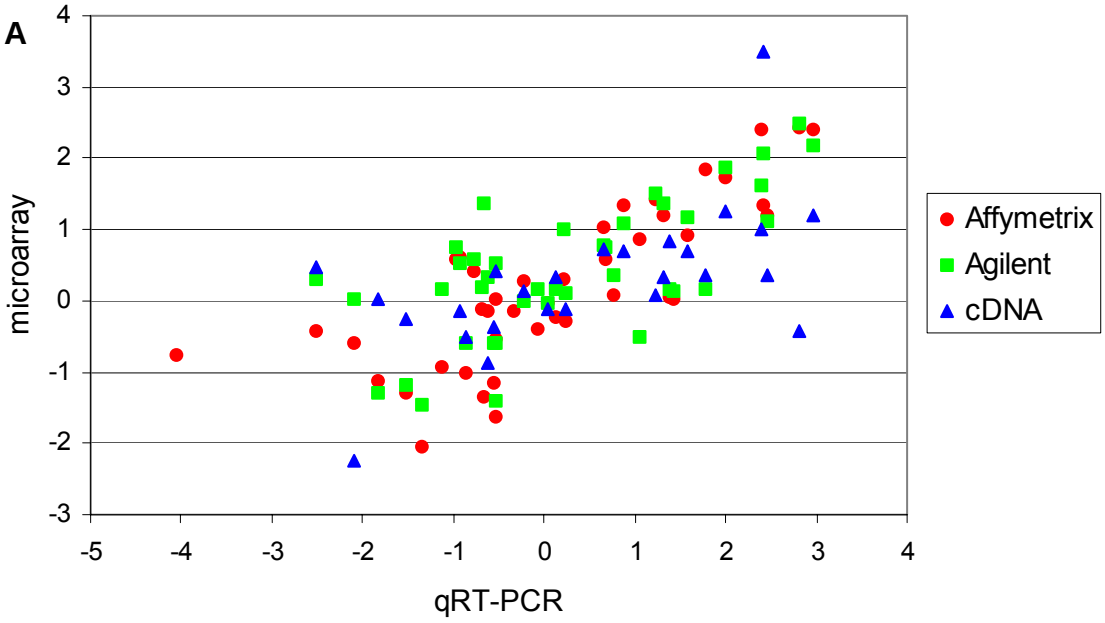
To validate the expression of the ABI5-regulated genes identified by microarray analyses, we performed quantitative RT-PCR (qRT-PCR) with 53 selected genes (Fig. 4-2)(Table A3). Transcript levels obtained by qRT-PCR were normalized to that of the 18S Ribosomal RNA and used as the fold change

Table 4-3. Correlation coefficients of cross platform comparison.

	All values		Present only	
Platform	common genes	t-test *p* -value <0.05	common genes	t-test *p* -value <0.05
Affymetrix vs Agilent	0.204	0.702	0.495	0.832
Affymetrix vs cDNA microarray	0.074	0.276	0.204	0.524
Agilent vs cDNA microarray	0.139	0.560	0.203	0.528

Pearson correlation coefficients were calculated from the common genes present in each pair of microarray platforms. Results are shown for unfiltered and filtered data for flags and t-test *p*-value.

A



B

Microarray format	Affymetrix	Agilent	cDNA
Correlation coefficient	0.79	0.57	0.60

Figure 4-2. Scatter plot from qRT-PCR analysis versus three microarray formats. (A) Log_2 ratios of 53 selected genes from qRT-PCR analysis were plotted (*y* axis) and the corresponding Log_2 ratios of 53 selected genes from microarray analysis were plotted (*x* axis). (B) Correlation coefficient was calculated between each microarray measurement format and qRT-PCR measurement.

relative to that of the control sample. The fold-difference obtained from microarray analyses and qRT-PCR were transformed to a $\log_2$ scale to bring the values to a uniform scale. To evaluate the accuracy of the microarray platforms employed in this study, the correlation coefficients were calculated by comparing qRT-PCR data against the measurements from each microarray platform (Fig. 4-2). Overall, the measurements from the three microarray platforms showed a relatively good correlation (0.57 to 0.79) with the qRT-PCR results. In particular, the Affymetrix ATH1 array showed the highest correlation with qRT-PCR analysis, having a Pearson correlation coefficient of 0.79. This indicates that there is significant concordance between these two different expression analysis approaches. Therefore, we decided to further analyze the Affymetrix ATH1 array data to study ABI5-regulated genes in seeds.

Microarray analysis of ABI5-regulated gene expression in seeds

Because of the high correlation between the preliminary Affymetrix and qRT-PCR results and because the Affymetrix ATH1 Genome Array represents approximately 80% of the *Arabidopsis thaliana* genome, we used the Affymetrix ATH1 Genome array to identify ABI5-regulated genes in seeds. RNA samples were prepared from three independent of WT and *abi5-6* mutant dry seeds, and the fold change was calculated for *abi5-6* mutant seeds compared to WT seeds.

To identify ABI5-regulated genes in *Arabidopsis* seeds, we performed statistical analysis using the Genespring (Agilent Technologies, Santa Clara, CA) visualization and analysis program. The genes were designated as being up-regulated or down-regulated if those transcripts showed more than a 1.7-fold difference with a t-test p-value < 0.05 in *abi5-6* seeds relative to WT seeds. A one way ANOVA test was applied to identify significant changes in *abi5-6* seeds.

Using these criteria, 729 genes were determined to be ABI5-regulated, including 482 up-regulated genes and 247 down-regulated genes in *abi5-6* seed. Therefore, ~3.3% of the annotated *Arabidopsis* genes were identified to be ABI5-regulated genes in seeds.

These ABI5-regulated genes were classified into 18 categories using Gene Ontology at TAIR with some modifications (Fig. 4-3). There were a large number of genes without significant similarity to known genes or functions, but we focused on the genes assigned with a known function. Of the 482 genes that were scored as being up-regulated in *abi5-6*, the largest group of genes was involved in metabolic pathways. The next largest group of ABI5-regulated genes involved in transcriptional regulation including AtDPBF2 (At3g44460), EEL (At2g41070), ATHB-7 (homeobox-leucine zipper transcription factor) (At2g46680), HB-14 (At2g34710), HAT-5 homeobox-leucine zipper protein (At3g01470), no apical meristem proteins (At1g69490, At4g01550 and At5g13180) and ATL4 C3HC4-type RING finger (At3g60220). In addition, many stress responsive genes were also induced in *abi5-6* dry seeds including eEarly-Responsive to Dehydration ERD4 (At1g30360), ERD6 (At1g54730), ERD14 (At1g76180), ERD15 (At4g14270), drought-induced protein Di21 (At4g15910), cold acclimation WCOR413 (At2g15970), and low-temperature-responsive protein 78 (At5g52310). In other words, ABI5 is involved in suppressing the expression of a large number of genes including genes involved in metabolic processes and transcription factors in WT seeds. Additionally, 162 genes (34%) of induced genes in *abi5-6* dry seeds encoded functionally unknown proteins.

Of the 729 genes that were scored as ABI5-regulated in dry seeds, expression of 247 (33%) genes were down-regulated in *abi5-6* dry seeds. The largest group of down-regulated genes was involved in metabolic processes as were up-

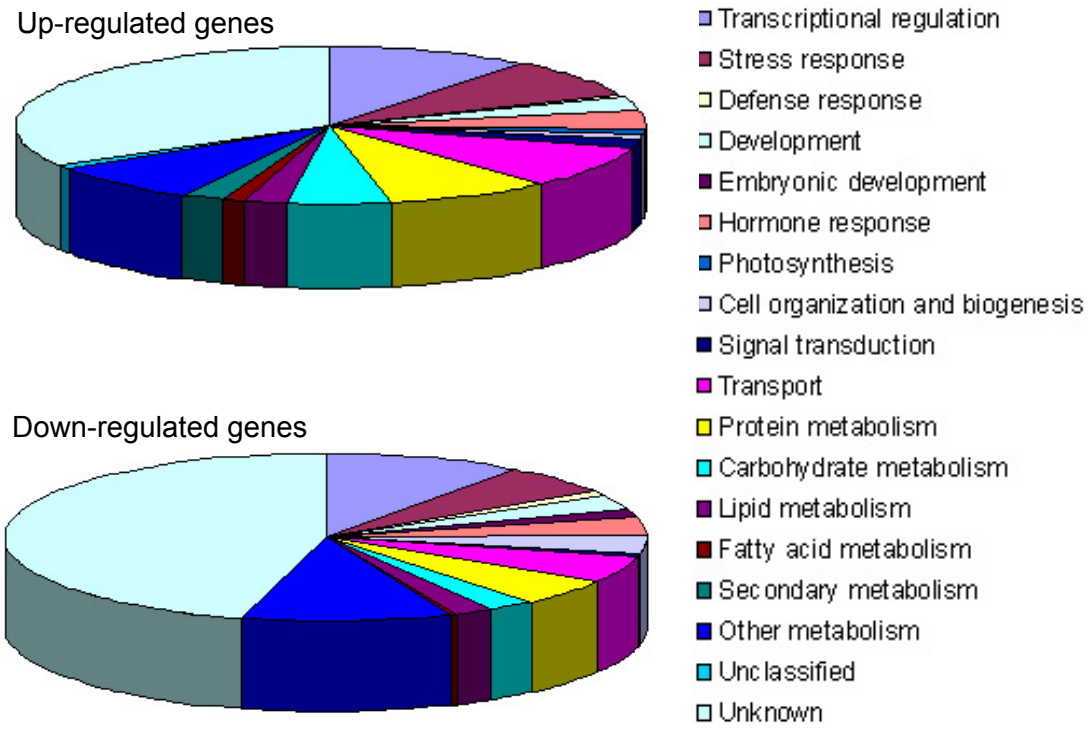


Figure 4-3. Functional classification of differentially regulated genes by ABI5 identified from Affymetrix ATH1 genome array. Functional categories are based on Gene Ontology at TAIR and some modifications were applied if needed. Differential expression was determined by comparison of normalized means from *abi5-6* Dry seed and WT dry seed arrays. The genes were determined as up-regulated or down-regulated by exhibiting at least a 1.7-fold change and a t-test *p*-value < 0.05.

regulated genes. The next largest groups were transcription factors, including bHLH transcription factor, zinc finger C3HC4-type RING finger protein ATL5 (At3g62690), ATL6, no apical meristem (NAM) proteins (At1g77450, At3g49530 and At5g63790), G-box binding factor 2 (At4g01120), G-box binding factor 3 (At2g46270), and Myb family transcription factors (At3g50060 and At5g67300). Many genes involved in stress responses were also repressed including the dehydration-responsive protein RD22 (At5g25610), protein phosphatase 2C ABI2 (At5g57050) and glutathione S-transferase (At5g62480). Approximately half of the down-regulated genes in *abi5-6* dry seeds encoded functionally unknown proteins.

DISCUSSION

DNA microarrays enable the analysis of gene expression on a global scale with any biological sample. This high-throughput technology has evolved rapidly over the last decade and has extended the area of application from basic science research to clinical practice. Currently, there are many high-density microarray platforms available. In this study, we employed three types of microarray platform; an in-house printed *Arabidopsis* cDNA microarray, the Agilent *Arabidopsis* 2 Oligo microarray and the Affymetrix ATH1 genome arrays. The cDNA microarray technique is considered to provide high sensitivity and specificity, but a low specificity has been observed possibly due to cross-hybridization among highly homologous transcripts (Duggan et al. 1999). Since cDNA sequences are long and double-stranded, they might have a higher chance to have non-specific hybridization and cross-hybridization. In addition, short (25-30 mer) and long (60-70 mer) oligonucleotide microarrays containing

highly specific oligonucleotide sequences for each gene are considered to have high specificity as well as high sensitivity.

It has been reported that there is poor correlation between cDNA microarrays and oligonucleotide microarrays (Carter et al., 2003; Kuo et al., 2006). In our study, we also discovered a poor correlation between the cDNA microarray and the oligonucleotide microarray platforms from Agilent and Affymetrix. This discordance might be caused by some variables such as different array manufacturing, target size, labeling method, hybridization process, image processing and normalization. However, several other studies have revealed a good correlation between cDNA microarrays and oligonucleotide microarrays (Hughes et al., 2001; Yuen et al., 2002; Petersen et al., 2005; Dabrowski et al., 2006). Thus, the results of previous comparative studies across platforms are variable among laboratories, and further investigation is essential to resolve the issue of reproducibility.

In the comparison of the two oligonucleotide microarray platforms, Agilent and Affymetrix, we found good correlation between them. For the manufacturing of the Agilent *Arabidopsis* 2 Oligo microarray, a 60-mer sequence for each gene was selected from the 3′ end of the open reading frame (ORF) sequence, with the lowest similarity to other sequences in the genome, as well as highest specificity**.** The Affymetrix ATH1 genome array contains 11 different probe sets for each gene of which each probe has a 25-mer oligonucleotide selected from the region within 300-500 bp of the 3′ end of the transcript. Significant correlation between the two different types of oligonucleotide microarrays might be attributed to similar strategies of probe design. Furthermore, this concordance might be due to the highly specific oligonucleotide sequences present in the array. This might prohibit cross-hybridization and offer high specificity by discerning between genes belonging to the same family.

To validate the microarray data and evaluate which microarray platform is more reliable among the three microarray platforms, we performed qRT-PCR for a selected gene set. qRT-PCR showed a greater dynamic range than any microarray platform included in the analysis. In general, there was good correlation between the microarray and qRT-PCR analysis, and Affymetrix array had a significantly higher correlation with the qRT-PCR compare to the other platforms. Previously, it was also shown that the Affymetrix single-color microarray had a better correlation with qRT-PCR compared to the dual-color microarray platforms (Petersen et al., 2005; Kuo et al., 2006). For qRT-PCR analysis, we evaluated only a small number of genes from thousands of genes present on the microarray. Therefore, it is hard to conclude that the general correlation between qRT-PCR and the Affymetrix array is better than that of the others. Further research is required to decide which microarray platform is most appropriate for the analysis and which microarray platform is most reliable and desirable, depending on specific applications.

We further analyzed data generated from the Affymetrix ATH1 genome array to identify ABI5-regulated gene expression in seeds. ABI5 is a bZIP transcription factor that is a key player in regulating ABA-triggered responses and in regulating gene expression during embryogenesis (Kim et al., 1997; Hobo et al., 1999; Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001; Kang et al., 2002; Kim et al., 2002). Employing Affymetrix microarray platform, we identified 729 genes that were ABI5-regulated in dry seeds including 482 up-regulated and 247 down-regulated genes in *abi5-6* dry seeds (Appendix 1 and 2). These include a large number of genes encoding proteins with diverse known or proposed functions and proteins of unknown function. Characterization of the functionally unknown genes may provide additional insight into the mechanism of ABI5 regulation in seeds.

The genes up-regulated in *abi5-6* seeds include many stress tolerance genes involved in regulating the response to dehydration and many genes encoding transcription factors. In addition, a large number of genes involved in metabolic pathways were identified including several enzymes involved in protein degradation and cell wall component modification. These imply that ABI5 is involved in suppressing the expression of the genes involved in metabolic processes and stress response in WT seeds. Furthermore, many genes encoding auxin-responsive proteins were identified. The roles of auxin in *Arabidopsis* seed germination have not been clearly revealed, but microarray analysis showed the induction of a number of auxin biosynthesis genes and genes encoding auxin carrier proteins (Ogawa et al., 2003). This suggests that ABI5 might be involved in suppressing the expression of auxin-responsive genes to prevent seed germination. A smaller number of genes were determined to be down-regulated than up-regulated in dry seeds of *abi5-6* mutant. Several genes encoding proteins involved in embryo development were down-regulated indicating that ABI5 induces the expression of theses genes in WT dry seeds. Taken together, the Affymetrix microarray data are consistent with dry mature seeds being in a metabolically quiescent state, and ABI5 plays a significant role in repression of these genes.

MATERIALS AND METHODS

RNA isolation

RNA isolation from dry seeds of WT and *abi5-6* was performed using a modified hot phenol/SDS extraction and LiCl precipitation protocol (Ausubel et al., 1994).

Three independent biological samples were used to isolate total RNA. The tissues were ground in 0.75 ml of extraction buffer (2% Cetyl Trimethyl Ammonium Bromide (CTAB), 2% polyvinylpyrrolidone K30 (PVP), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 0.5 g/L spermidine, 2% beta-mercaptoethanol) and spun for 10 min at 14,000 rpm. The upper aqueous phase was extracted with equal volume of chloroform:isoamylalcohol (24:1) and spun for 10 min at 14,000 rpm. One-third volume of 8M LiCl was added to the supernatant, and the mixture was precipitated overnight at 4°C followed by centrifugation at 14,000 rpm at 4°C. After centrifugation at 14,000 rpm for 20 min, the pellet was dissolved in 500 μl SSTE (1.0 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.5% SDS) and extracted with an equal volume of chloroform:isoamylalcohol (24:1). The supernatant was precipitated with two volumes of 100% EtOH at -80°C overnight. The pellet was dissolved in nuclease-free H_2O. Contaminating DNA was removed from each RNA preparations using the DNA-*free*™ kit (Ambion, Austin, TX) according to the manufacturer's instructions. Total RNAs (20-200 ng) from embryos were analyzed using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) to determine the quality of the RNA samples.

2 μg of total RNA from each sample was amplified using the messageAmp™ aRNA kit (Ambion, Autin, TX) following the manufacturer's instructions. First, total RNA was reverse transcribed and then the generated cDNA was used in an *in vitro* transcription reaction. 200 ng of purified amplified RNA was analyzed using the Agilent 2100 Bioanalyzer.

Microarray hybridization and scanning

To perform cDNA microarray hybridization, three independent biological

samples and two technical replicates for each sample were used. Indirect labeling of aRNA using the Genisphere 3DNA Array 350 RP kit was performed according to the manufacturer's protocol (Genisphere, Hatfield, PA). 200 ng aRNA was reverse transcribed by modified oligo(dT) primers in which a fluorophore/dendrimer-specific sequence occurs at the 5' end of oligo(dT) primer. The produced cDNA was purified using the Qiagen QIAquick® PCR purification kit (Qiagen, Valencia, CA). Subsequently, cDNA samples to be co-hybridized were combined and concentrated using Microcon YM30 microconcentrators (Millipore, Billerica, MA) according to the manufacturer's recommendations. For each array, a hybridization mix was added to a pre-warmed glass microarray for incubation in a 62°C water bath overnight. After washing hybridized slides, secondary hybridization was performed using the complementary 3DNA capture reagents containing fluorescent dyes. The hybridization mix was added to a pre-warmed microarray slide for incubation at 62°C for 3 hr, and then the slide was washed and dried for scanning. Hybridized microarray slides were scanned for Cy-3 at 523 nm and Cy-5 at 635 nm with an Affymetrix Array scanner 428 (Affymetrix, Inc., Santa Clara, CA).

To perform Agilent microarray hybridizations, 2 μg of purified aRNAs from two independent biological samples and two technical replicates were reverse-transcribed as previously described. Purified cDNA was *in vitro* transcribed by combining dNTP mix and amino-allyl UTP (aaUTP). Then, 5 μg of purified aaRNA was coupled with NHS ester dye according to manufacturer's protocol. The hybridization reaction mixture, containing successfully incorporated Cy3- and Cy5-labeled aaRNA, was loaded onto Agilent *Arabidopsis* 2 Oligo Microarray (Agilent, Santa Clara, CA) for incubation at 60°C for 17 hrs. Following incubation, the oligonucleotide slide was washed and dried for scanning. As previously mentioned, hybridized microarray slides were scanned with an

Affymetrix Array scanner 428, and separate TIFF images were generated for each channel.

The Affymetrix *Arabidopsis* ATH1 Genome array (Affymetrix, Inc., Santa Clara, CA) was also used for gene expression analysis. cDNA synthesis, cRNA labeling, hybridization and image analysis were performed by Codon Biosciences (Houston, TX). Three independent biological samples and two technical replicates for each sample were used.

Processing of microarray data

For two-color microarray experiments, spot intensities were quantified using GenePix 4.0 (Axon instruments, INC., Foster city, CA). This program marks the spots that were weak and have a small number of bright pixels as absent (A) and good spots as present (P). Total net signal intensities of each of two channels from good spots (present) were obtained and the median value of Cy-3 and Cy-5 signals were further analyzed by using GeneSpring 7.1 (Agilent Technologies, Inc., Santa Clara, CA). GeneSpring is a powerful visualization and analysis solution designed for use with genomic expression data. The raw data generated by GenePix software were LOWESS normalized as follows: First, values below 0.01 were set to 0.01. To account for dye swaps, the signal channel and control channel measurements were reversed. LOWESS normalization was applied to account for intensity-dependent variation due to dye properties. Fold changes for each gene were calculated for *abi5-6* dry seed compared with WT dry seed. Genes showing a 2.0-fold or greater change were defined as being up-regulated or down-regulated in *abi5-6* dry seed, and these genes were used for further analysis. Differentially expressed genes were filtered by eliminating genes that had a t-test *p*-value >0.05.

For the analysis of Affymetrix ATH1 genome array, raw data was introduced to GeneSpring 7.1 (Agilent Technologies, Inc., Santa Clara, CA) for normalization and further analysis. Affymetrix ATH1 genome arrays were paired identically as cDNA microarray and Agilent oligo array. Normalization was performed as follows: First, values below 0.01 were set to 0.01. Each measurement was divided by the 50th percentile of all measurements in that sample. Each measurement for each gene in *abi5-6* samples was divided by the median of corresponding gene measurement in the WT sample. Genes showing more than 2-fold difference with a t-test *p*-value < 0.05 were selected for the comparison among the different microarray platforms.

For the classification of genes based on their biological function by Gene Ontology at TAIR, genes that showed higher than 1.7-fold difference with $p < 0.05$ were selected. Furthermore, one-way ANOVA was also applied to identify statistically significant differences in gene expression between WT and *abi5-6* dry seeds. The multiple testing correction of Benjamini and Hochberg false discovery rate was applied to further analyze the significance level.

Quantitative RT-PCR

2 μg of total RNA was reverse transcribed with RT reaction mix including 1 X TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM dNTP mixture, 2.5 μM random hexamers, 40 U RNase inhibitor and 125 U Multiscribe™ reverse transcriptase using the TaqMan® reverse transcription kit (Applied Biosystems, Foster City, CA) or SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Primers were designed by using the Primer Express version 1.5 software

(Applied Biosystems, Foster City, CA), and the sequences of each primer pair are given in Table 4-4. PCRs were performed in an optical 96-well plate with the GeneAmp 7500 sequence detector (Applied Biosystems, Foster City, CA), using Power SYBR® Green to monitor dsDNA synthesis. Three identical reactions were repeated on the plates, and each reaction contained 10 µl 2 X Power SYBR® Green Master Mix reagent (Applied Biosystems, Foster City, CA), 20 ng cDNA and 5 µm of each forward and reverse gene-specific primers in a final volume of 20 µl. The following standard thermal profile was used: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 50°C for 1 min. Subsequently, a dissociation stage was followed: 95°C for 15 sec, 50°C for 1 min, and 95°C for 15 sec. Collected data were analyzed using the SDS 1.7 software (Applied Biosystems, Foster City, CA). In order to compare data from different PCR runs or cDNA samples, the C_t values for the genes were normalized to the C_t value of 18S RNA. Quantification of the abundance of each transcript was determined using the comparative C_t method. The amount of target, normalized to 18S RNA and relative to a calibrator, is given by: $2^{-\Delta(\Delta Ct)}$ (user bulletin 2, ABI Prism 7500 sequence detection system; Applied Biosystems).

Table 4-4. Gene-specific primers used for qRT-PCR

AGI ID	Gene name	Forward Primer	Reverse Primer
At1g01060	LHY	TTGGCATTTCCACGCAGATA	TGAGATACCATACCTGAGGGATGATT
At1g01620	PIP1C	GGGACGACCACTGGATATTCTG	TGGAATGGCTCTGATGACAAGT
At1g03970	GBF4	TGGAGGTTCTGATTCCGGTC	AATGGCTCCTGCTTAAGGGC
At1g08230	AtGAT1	GCCGTTGCTGTGGTATACCTACA	CGAAGAGGGAACGCACAACT
At1g09960	SUT4	GCTCTTCCACGGACAAGGATT	CGCTTGACACTCATCTTATATAACAAAA
At1g11260	STP1	TCTCGGAGGCGCGGT	TGTCAGGGAGGACGAGGG
At1g21970	LEC1	GGTCGTCGGGTCAAGATGA	CCGGCATTCCGTTAATGG
At1g22710	SUC2	AGCATAGTACCTGTGAAAACACGAA	GGTGGTTCCAAGAAAAATTTGAGT
At1g31320	LBD4	GCGGGTCACCGTCTTCG	GGCTTCATCCACAATGTCCATAT
At1g45249	ABF2	TTAACGAACAATGTGGGCTTTG	CAGCACCAACGCCTAAAGCT
At1g49720	ABF1	GACTGCCTCCAACTATCTTTCCA	AAACCCCTGTTGACCATATTTACAG
At1g59870	PEN3	GTATAACCCGGCCACTTGGA	GTTTCGCTGGTGCAATGCT
At1g69490	NAP	TCGATGAAACTTCCAAGGACG	TGAGAGACGGGTCCCATGTAA
At1g76180	ERD14	GACACAAGAAACCTGAAGACGGT	TCTTCCACAGGAGGAGGAACAA
At1g77450	NAC	TGCCATCGCCGATAACG	CCACATCTGATTCCCGCC
At2g15970	WCOR413	ACACGCCAGAGAATACCTGGA	AATGTGCCAGCAATCAAATTTG
At2g17770	AtbZIP27	CCATCGGCAACTAAACATTGG	AGGCATGTTGAGGAAATCTTGAA
At2g28300	LEC2	TCAGGCTCAGCCTCATCACTT	CCGCCGCCATCTGCT
At2g36270	ABI5	CGGTGTCTTCAGATGGATTAGGA	CCCCTTAGCCCTCCCATATC
At2g40170	AtEm6	CACCGGAGACAAGCCTGGT	GGTCTTGGTCCTGAATTTGGATT
At2g40220	ABI4	CGAAGACGCCGCACGT	GGGTTAAGTTGAGCTGAGCACG
At2g41070	EEL	GGGTTTATGACATATCCGGTTTG	CCAGGCGCTTGTGGTGTAT
At2g43000	NAC	AACCTTGGCGGTGCACTG	CGCGGCAACATCCTCAA
At2g46270	GBF3	ACGCTCATCCCGGTATTCC	CCCGGAGTTGTTAAAGGTGG
At3g01470	HAT5	CATGACAATGACCGGAGCTG	TCGTGCGACGGTGAAGTG
At3g08550	ABI8	TCTCGCCGATCCTGCCT	GAGATCGGAGCCGCCAG
At3g15670	LEA76	AGTGGGATCTTGGGCCAGA	CTTCACCGCATCAGTAGCTCC

Table 4-4. Continued

AGI ID	Gene name	Forward Primer	Reverse Primer
At3g19290	ABF4	CAGCTGAATCAGCCTCATCCA	TGCTACGTTTGCTTGTTTAGGAAA
At3g22840	ELIP1	TCCTCGGTACAACAGCGATCTT	TCAACGCTTATGCCCTTGAAA
At3g24650	ABI3	CAGGGATGGAAACCAGAAAAGA	AGGTTACCCACGTCGCTTTG
At3g26790	FUS3	GATGTCACCACAAACACAGAGTCTT	CCAAAAAATCGAGAGGAGTATCGT
At3g44460	AtDPBF2	GCCACGTCAAGGGTCGTT	CCAAACCTCGTCGACCGT
At3g51810	AtEm1	GAGGACTCAGTACGATGGAAAAATCT	TGGTGAACTTTGACTCATCGATCT
At3g53420	PIP2A	ATCGCTTGGGCCTTTGG	GGGTTAATGTGACCACCAGAGATAC
At3g56850	AREB3	TGCCA AGCCTCAAGCATT	AACCACCCATCAAAGAAGACTGA
At3g61430	PIP1A	GATGACCACTGGGTGTTTTGG	CTGATGACAACCACATGGTAAAGAG
At4g01120	GBF2	TCCTGTTATGCCCACAGCAA	TTCCATGGCTGTGGCACA
At4g15910	DI21	TCTCCGGTGCCGTTAAATCT	ACTCAATCCTGCTGCTGTTACATT
At4g20260	DREPP	AAAATTGAATTCCCCGGATCA	CCACCGGTATTTCTTTAGTCTTCAC
At4g26080	ABI1	GGAGGAAGTATCTCCGGCG	CGGTGAAATCCATCTGGGTT
At4g34000	ABF3	GGAGAGGCCTTTTCCCAAAC	GTGTTGCAGGTTGAGAACGGT
At4g35770	SEN1	AGCTGATCTTCGAAACTCAAACTTC	ACTGCCTCTGCTGCAACATTT
At4g35900	AtbZIP14	AAACCGACACAGCCCTCATC	TTTTGGTTGTGGTGGTTTTGG
At4g38410	ERD10	TGTAACCAAGGAGCCCAAGG	TCACTTGAAGAGCTGTCAGAAAGG
At5g05410	DREB2A	TCGATGAGCTTCTACGTGACCTAA	CGGGTACCGGTCCTGATTTA
At5g13180	NAC	TTTGCCGAGCTCTCCTTCTT	CAACTACTGGTCTCTTCATCGGAA
At5g13330	RAP2.6	CACGTGTATGGCTTGGGACA	CTGCCGCATCATAGGCTCTT
At5g25610	RD22	ACGGAGGCAAAACTGCGTT	CGAAAACTTCTCCGATCCAAAA
At5g42910	AtbZIP15	TGAACGACCGGAGAGAAAGCT	TCATCCTCCAACTCGGGTTT
At5g44080	AtbZIP13	GCTGCGGTGGAAGACGAA	CACCGGAATCTTCACATCCAA
At5g52310	RD29A	TGTGCCGACGGGATTTG	ATGCCTCACCGTATCCAGGTC
At5g57050	ABI2	AAACGGATTTTACTATGGCATAAGAAG	TCTCCGCCGGAAGCAA
At5g60300	LRK	TGGGTCTGCTCTGTTCAAATATAGTG	GCAAAGGTAGGTTTTTGTTTAGGTACA

CHAPTER V
ABI5 REGULATION OF *AtCOR413 PM-1*

INTRODUCTION

The plant hormone abscisic acid (ABA) plays important roles during plant growth and development. It regulates many processes, including gene expression, closure of stomata to reduce water loss due to transpiration, photosynthesis, and adaptation to environmental stresses. In seeds, ABA is required to maintain the embryonic program and to prevent precocious germination until conditions become favorable. ABA is also involved in the acquisition of desiccation tolerance during late seed maturation (Nambara et al., 1992; Ooms et al., 1993). Many ABA responses involve changes in gene expression, and studies on the promoter sequences of ABA-regulated genes have identified *cis*-regulatory elements, designated ABA-responsive elements (ABREs), which control ABA- and/or stress-responsive gene expression (Giraudat et al., 1994; Busk and Pagès, 1998; Yamaguchi-Shinozaki and Shinozaki, 2005).

Several basic leucine zipper (bZIP) factors, including ABI5, have been isolated on the basis of their interaction with ABREs as candidates for ABA-responsive transcription factors. ABI5 is a key player in ABA-triggered processes and is also responsible for monitoring osmotic status in germinated embryos before initiating vegetative growth (Kim et al., 1997; Hobo et al., 1999; Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001; Kang et al., 2002; Kim et al., 2002). Furthermore, ABI5 is considered a key factor regulating gene expression during embryogenesis, and *ABI5* expression increases during seed maturation reaching a maximum in desiccating seeds (Finkelstein and Lynch, 2000; Brocard

et al., 2002). *Arabidopsis abi5* mutants, like many other ABA-insensitive mutants, were selected on the basis of ABA-resistant germination and showed greatly reduced expression of AtEm6 and AtEm1 in seeds (Parcy et al., 1994; Finkelstein and Lynch, 2000)

Affymetrix transcriptional profiling identified ABI5-regulated genes in dry seeds (Chapter IV). Among these ABI5-regulated genes, the conserved motif of a classical G-box containing ABRE was identified in the promoter regions of these genes. Subsequently, individual promoter analysis was performed on the selected genes. Based on the *cis*-regulatory elements present in the promoters of those genes, we decided to further investigate the regulation of *AtCOR413 pm-1* by ABI5 *in vivo* especially in dry seeds.

COR413-pm1 is a cold-regulated gene and was initially identified from cold-acclimated wheat (*Triticum aestivum*). Using EST sequencing and *in silico* reconstitution, 27 more COR413-related proteins were further identified in other plant species including *Arabidopsis*, maize and barley. The *Arabidopsis* genome encodes COR413-PM (plasma-membrane) and COR413-TM (thylakoid-membrane) which are rich in hydrophobic amino acids, suggesting that they are membrane proteins. Gene regulation studies revealed that the expression of cor413 gene family members was regulated by low temperature as well as by dehydration, light, and abscisic acid (Breton et al., 2003).

Low temperature (LT) is one of the most important environmental factors affecting plant growth and productivity. Many plant species acquire freezing tolerance upon exposure to low non-freezing temperatures, a phenomenon called cold acclimation (Thomashow, 1999). This is accompanied by the accumulation of compatible osmolytes and soluble sugars, change in lipid composition to ensure membrane fluidity and altered expression of many genes

to provide protection against cold stress (Viswanathan and Zhu, 2002; Sharma et al., 2005).

The expression of many cold-regulated genes is altered during the process of cold acclimation and potentially contributes to freezing tolerance. Many cold-regulated genes also can be induced by dehydration. The promoters of many cold- and dehydration-responsive genes in *Arabidopsis* contain C-repeat/dehydration-responsive elements (CRT/DRE) containing a 9 base pair conserved sequence, TACCGACAT, and abscisic acid-responsive elements (ABRE) containing the 8-10 base pair conserved sequence, (T/G/C)ACGT(G/T)GC. These genes are regulated by CRT/DRE binding proteins and ABRE binding proteins, respectively (Yamaguchi-Shinozaki and Shinozaki, 1994; Viswanathan and Zhu, 2002).

In this study, we analyzed the expression of *AtCOR413 pm-1* in WT, *abi5-6* and Pro35S:ABI5 plants and performed a complementation test by crossing Pro35S:ABI5 plants with *abi5-6* plants containing ProAtCOR413pm1-1489:GUS transgene to examine the role of ABI5 on the regulation of *AtCOR413 pm-1* expression in dry seeds. The expression of *AtCOR413 pm-1* in vegetative tissues was analyzed by employing promoter deletions fused to GUS to define *cis*-regulatory elements that confer ABA- and cold-response. Our analysis of *AtCOR413 pm-1* expression *in vivo* suggested that ABI5 is a primary factor negatively regulating *AtCOR413 pm-1* expression in developing seeds.

RESULTS

AtCOR413 pm-1 gene expression is induced by ABA and low temperature treatment in seedlings and is regulated by ABI5 in seeds

AtCOR413 pm-1 expression is induced in dry seeds of *abi5-6* mutant plants (Chapter IV). Previously, it was shown that *AtCOR413 pm-1* can be induced by low temperature (4°C) (LT) treatment, water stress and exogenous ABA treatment (Breton et al., 2003). Quantitative RT-PCR was conducted to investigate temporal expression of *AtCOR413 pm-1* in response to ABA and LT treatment in WT, Pro35S:ABI5 and *abi5-6* seedlings. *ABI5* and *AtCOR413 pm-1* transcript levels were analyzed in two independent biological samples of WT, Pro35S:ABI5 and *abi5-6* seedlings harvested just before seedlings were treated with ABA and then at 2, 4, 12, and 24 hr after ABA treatment. Additionally, transcript levels of *ABI5* and *AtCOR413 pm-1* were also analyzed in the three different genetic background seedlings harvested just before being treated with LT and then at 4, 6, 12, and 24 hr after LT treatment.

After ABA treatment, *ABI5* expression was elevated at 2 hr post-treatment and then decreased over time in WT seedling. In contrast, *ABI5* expression initially decreased in seedlings carrying the Pro35S:ABI5 construct following ABA treatment and then greatly increased at 12 hr post-treatment. *AtCOR413 pm-1* expression was highly induced at every time point during the course of the experiment in WT, Pro35S:ABI5 and *abi5-6* (Fig. 5-1). During LT treatment, *ABI5* expression gradually increased to a peak at 24hr post-treatment in WT. *ABI5* expression decreased following LT treatment initially and then increased to maximum at 24 hr post-treatment in Pro35S:ABI5 lines. As in the experiments with ABA treatment, *AtCOR413 pm-1* expression was highly induced by LT treatment at every time point during the course of experiment in all three genetic

backgrounds. These results suggested that there could be regulatory proteins other than ABI5 that play major roles in regulating the expression of *AtCOR413 pm-1* in response to ABA and LT treatment in seedlings (fig. 5-1).

To investigate the regulation of *AtCOR413 pm-1* expression by ABI5 in developing embryos, we also analyzed the expression of *AtCOR413 pm-1* in WT, Pro35S:ABI5 and *abi5-6* mutant embryos using qRT-PCR and RNA blot analysis (Fig. 5-2). It has been previously shown that the *ABI5* expression is observed starting at the heart stage embryo and increases in dry seeds to a maximum (unpublished data from Thomas Lab.).

Consistent with our previous observation, qRT-PCR analysis revealed that *ABI5* transcript level reached a maximum in dry seeds of WT, but *AtCOR413 pm-1* expression was down-regulated as the embryo matured (Fig. 5-2). However, *AtCOR413 pm-1* transcript level significantly increased in *abi5-6* embryos with the highest expression in dry seeds as previously shown by microarray analysis. In Pro35S:ABI5 dry seeds, the *AtCOR413 pm-1* transcript levels were similar to WT dry seeds. These expression patterns corresponded to RNA blot analysis of WT, Pro35S:ABI5 and *abi5-6* mature embryo, dry seeds and seedlings using a ^{32}P-labelled 3' region of the *ABI5* cDNA and full length *AtCOR413 pm-1* cDNA (Fig. 5-2). These results suggest that ABI5 is a negative regulator of *AtCOR413 pm-1* in developing seeds, especially in the desiccating seed.

ABI5 binds to *AtCOR413 pm-1* promoter

ABI5 appears to negatively regulated *AtCOR413 pm-1*. We wanted to identify potential ABI5 regulatory element in the promoter of *AtCOR413 pm-1* gene. One

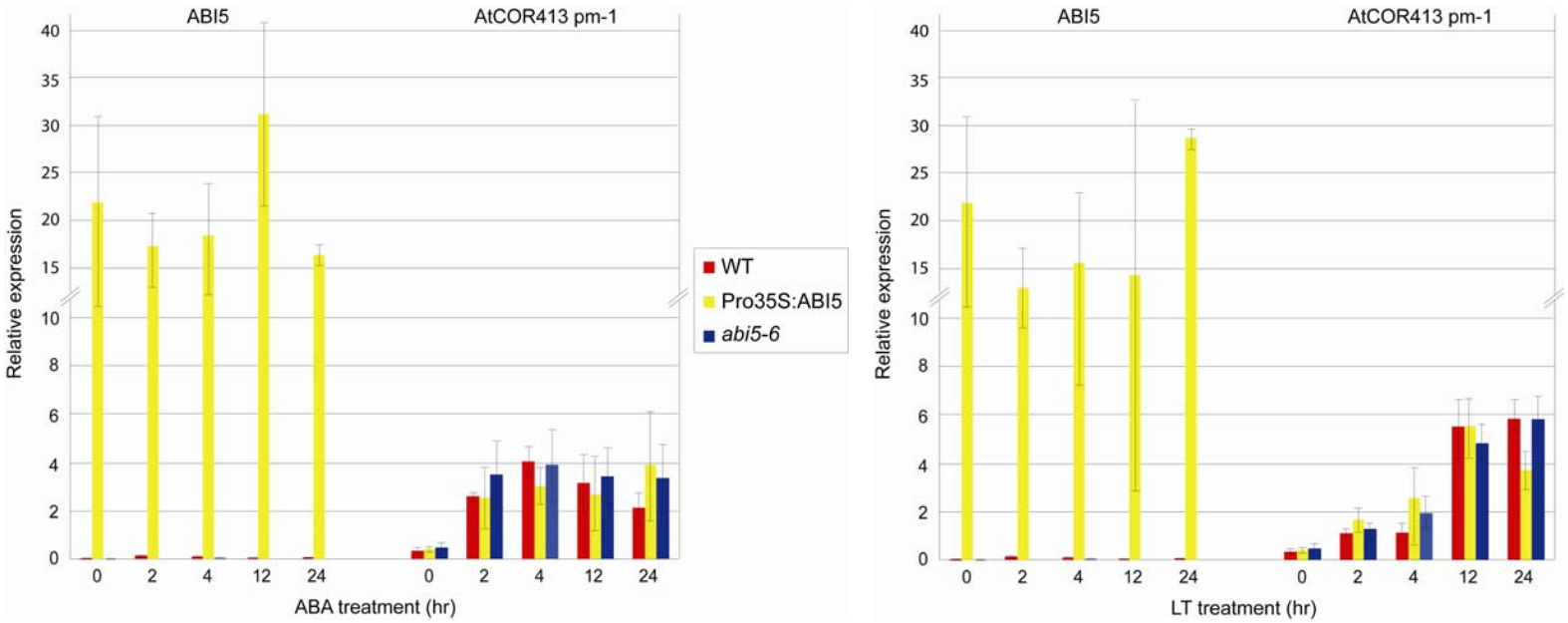


Figure 5-1. Expression of *ABI5* and *AtCOR413 pm-1* in WT, *abi5-6* and Pro35S:ABI5 seedlings.
11-days-old seedlings grown on MS plates were treated with 100 μM ABA and LT (low-temperature) treated at 4°C, and tissues were harvested at the time points indicated. Transcript levels were measured by qRT-PCR, and the relative amount of transcript levels were calculated and normalized to the expression of 18S ribosomal RNA. Data shown represent mean values obtained from two independent RNA samples.

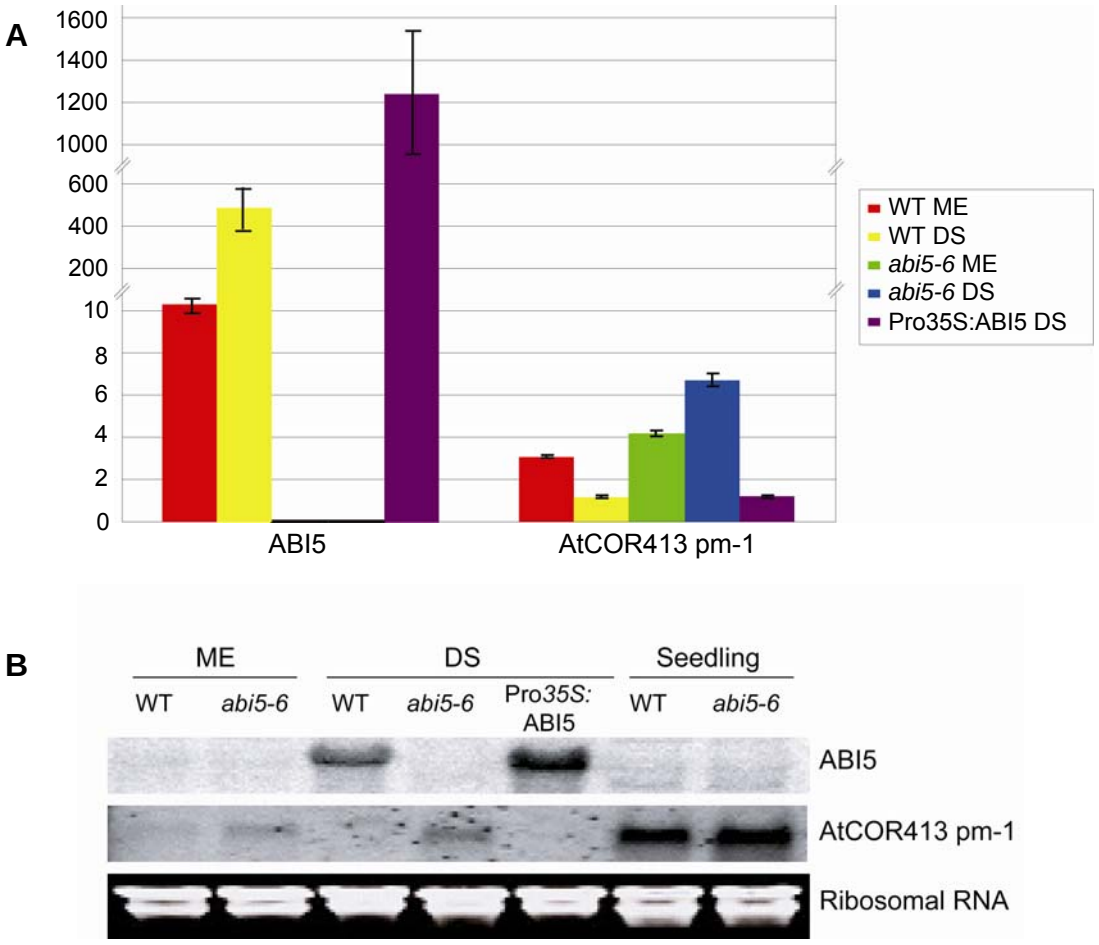


Figure 5-2. Expression of *ABI5* and *AtCOR413 pm-1* in WT, *abi5-6* and Pro35S:ABI5 seeds.
(A) Total RNA was isolated from mature embryo and dry seeds of WT, *abi5-6* and Pro35S:ABI5. qRT-PCR was performed to measure transcript level of *ABI5* and *AtCOR413 pm-1*, and relative amount of transcript levels were calculated and normalized to the expression of 18S ribosomal RNA. Data shown represent mean values obtained from two independent RNA samples.
(B) RNA-gel blots were performed using identical total RNA used for qRT-PCR. 15 µg total RNA was used for blot and the blot was hybridized with ^{32}P-labeled probes of *ABI5* and *AtCOR413-pm1*.

approach we took was to search for conserved motifs among a large set of promoters from genes regulated by ABI5. We used the motif-finding program Weeder (Pavesi et al., 2004), which carries out an algorithm for the automatic discovery of conserved motifs in a set of related regulatory DNA sequences to identify over-represented motifs that occurred with statistical significance in the promoters of the ABI5-regulated genes in seeds. A highly conserved ABRE-like motif with the consensus sequence ACGT was identified from a set of promoter sequences of genes that showed induction of transcript level in *abi5-6* dry seeds. We tested the binding of ABI5 to the ABRE-like motif identified by the motif-finding program by electrophoretic mobility shift assay (EMSA). As shown in figure 5-3, the *in vitro* transcribed/translated ABI5 and EEL/AtDPBF4 products bound to the ABRE-like motif containing the consensus sequence ACGT, but no binding was observed with AtDPBF2 product. We also tested binding of the DPBF-core motif with ABI5 since it has been shown that ABI5 can interact with the DPBF-core motif which does not contain the ACGT core sequence in the context (Kim et al., 1997; Kim et al., 2002). However, the ABI5 *in vitro* transcribed/translated product failed to bind to the oligonuclotides containing the DPBF-core motif identified in the *AtCOR413 pm-1* promoter. Furthermore, *in vitro* transcribed/translated ABI5, AtDPBF2 and EEL/AtDPBF4 products failed to bind to an oligonucleotide containing a mutation in the ABRE motif (Fig. 5-3). Therefore, these data indicate that ABI5 and EEL/AtDPBF4 can bind to the promoter of *AtCOR413 pm-1* and possibly regulate gene expression.

It was shown that the ABI5 subfamily of bZIP proteins bind *cis*-regulatory elements in DNA as dimers (Kim et al., 1997; Bensmihen et al., 2002; Kim et al., 2002). Therefore, we also tested binding of *in vitro* co-transcribed/translated products of ABI5 subfamily of bZIP proteins to the ABRE motif in the promoter of *AtCOR413 pm-1*. When an EMSA was performed with co-transcribed/translated products of ABI5 and EEL proteins, an additional shifted band was observed that

A 5' TTCATAACTGCC**ACGT**GGCGTAAACGTATC 3' ABRE
5' GTTCATGTGTGAGAAGTTGGTGTCGACATGTGTCTA 3' DPBF
5' TTTCATAACTCCGCGGTCAGGTAAACGTAT 3' ABRE mutated

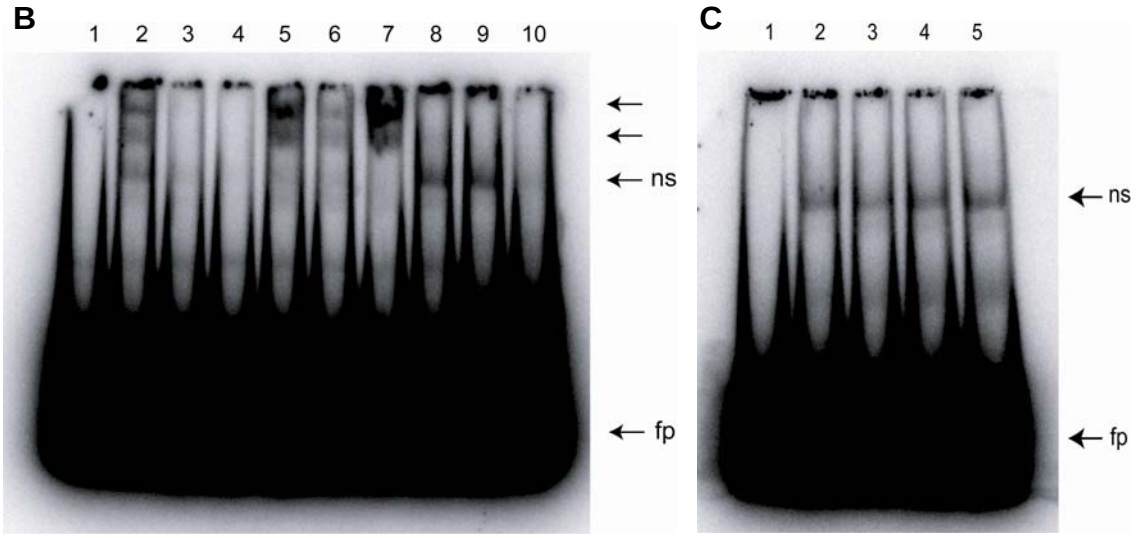


Figure 5-3. ABI5 binds to *AtCOR413 pm-1* promoter.
(A) Sequence of *AtCOR413 pm-1* promoter containing ABRE-motif. ABRE and mutated motif in the oligo-sequences are underlined and were used as probes for EMSA. (B) ABI5 binds to ABRE-motif in the *AtCOR413 pm-1* promoter. ABRE-motif containing oligo was incubated without protein extract (free probe; lane 1) or with *in vitro*–transcribed and translated vector pCiTE4C (negative control; lane 8). ABI5 *in vitro* transcribed and translated protein was incubated with labeled ABRE-motif containing oligo (lane 2) and unlabeled ABRE-motif containing oligo and non-specific oligonucleotides for competitive binding (lane 3 and 4: 4X and 8X unlabeled ABRE containing oligo, lane 5 and 6: 4X and 8X unlabeled D, lane 7: unlabeled DPBF-core motif containing oligo). ABI5 *in vitro* transcribed and translated protein was also incubated with labeled DPBF-core motif containing oligo (lane 9) and unlabeled ABRE-motif containing for competitive binding (lane 10). Bands shifted by ABI5 indicated by arrows. ns: non-specific band shifted by reticulocyte proteins, fp: free probe. (C) Mutation in the ABRE abolishes binding of ABI5. The ABRE mutated oligo was incubated without protein extract (free probe; lane 1) or with *in vitro* transcribed and translated vector pCiTE4C (negative control; lane 5). ABI5 (lane 2), AtDPBF2 (lane 3) and EEL (lane 4) *in vitro* transcribed and translated proteins were incubated with labeled ABRE mutated containing oligo.

had mobility between those of ABI5 and EEL (Fig. 5-4). However, we could not detect binding of the co-transcribed/translated ABI5 and AtDPBF2 products. These results indicate that the ABI5/EEL heterodimer can form and bind to the ABRE motif with the consensus sequence ACGT. However, ABI5 may dimerize with AtDPBF2, but the resulting heterodimer can not bind to the ABRE motif. It is possible that AtDPB2 inhibits the binding of ABI5 to the ABRE *cis*-regulatory element. In addition, the co-transcribed/translated products of AtDPBF2 and EEL showed similar mobility with EEL protein alone indicating that AtDPBF2 forms heterodimer with EEL, but the resulting heterodimer fails to bind to the ABRE motif in the promoter of *AtCOR413 pm-1.* It was previously shown that AtDPBF2 and EEL form a functional heterodimer and bind to the Dc3 proximal promoter region (Kim et al., 2002). These binding assay results indicate that ABI5 can form heterodimers with other ABI5 subfamily members of bZIP proteins, and these resulting heterodimer complexes have different binding specificities.

Analysis of *AtCOR413 pm-1* promoter:GUS in the seedlings of WT, *abi5-6* and Pro35S:ABI5 in response to stress treatment

We analyzed the effect of ABI5 on the regulation of *AtCOR413 pm-1* expression in WT, *abi5-6* and Pro35S:ABI5 seedlings in response to abiotic stress. The construct containing the *AtCOR413 pm-1* promoter region from -1489 to +76 was fused to a GUS reporter gene (ProAtCOR413pm1-1489:GUS) and was introduced into WT, *abi5-6* and Pro35S:ABI5 plants (Fig. 5-5). ProAtCOR413pm1-1489:GUS exhibited a significant level of transgene activation in *abi5-6* and Pro35S:ABI5 lines under untreated control conditions which was not clearly different from WT. Quantification of GUS activity indicated that the level of induction by ABA treatment in *abi5-6* and Pro35S:ABI5 lines was increased compared to that of WT which also showed induction by ABA

A 5' TTCATAACTGCC**ACGT**GGCGTAAACGTATC 3' ABRE

B

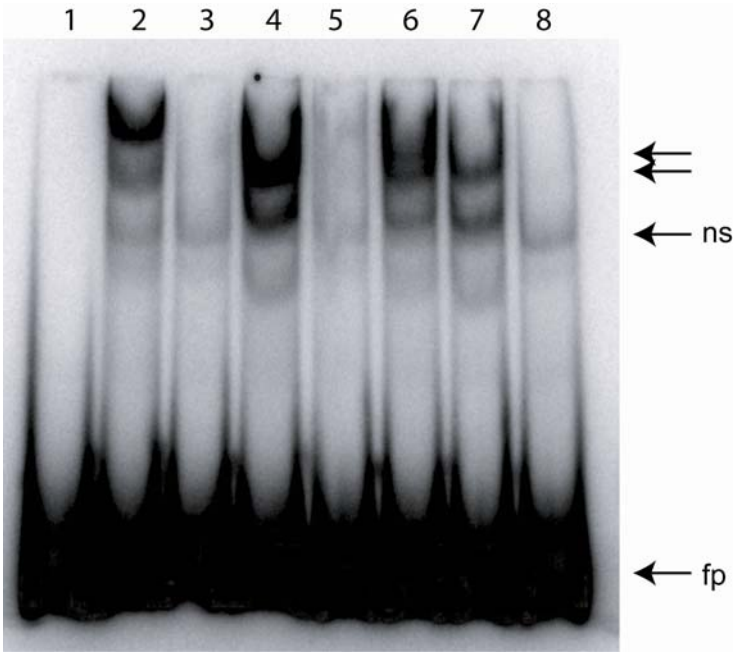


Figure 5-4. ABI5 and EEL bind to the ABRE motif.
(A) Sequence of *AtCOR413 pm-1* promoter containing the ABRE-motif. The ABRE motif in the oligo-sequence is underlined and was used as probes for EMSA.
(B) ABI5 and EEL/AtDPBF4 bind to the ABRE-motif in the *AtCOR413 pm-1* promoter. ABRE-motif containing oligo was incubated without protein extract (free probe; lane 1) or with *in vitro* transcribed and translated vector pCiTE4C (negative control; lane 8). ABI5 (lane 2), AtDPBF2 (lane 3), EEL (lanes 4) proteins were incubated with labeled ABRE motif containing oligo. Additionally, *In vitro* co-transcribed and translated ABI5 and AtDPBF2 (lane 5), ABI5 and EEL (lane 6) and AtDPBF2 and EEL (lane 7) were incubated with labeled ABRE motif containing oligo. The shifted bands are indicated by arrows. ns: non-specific band shifted by reticulocyte proteins, fp: free probe.

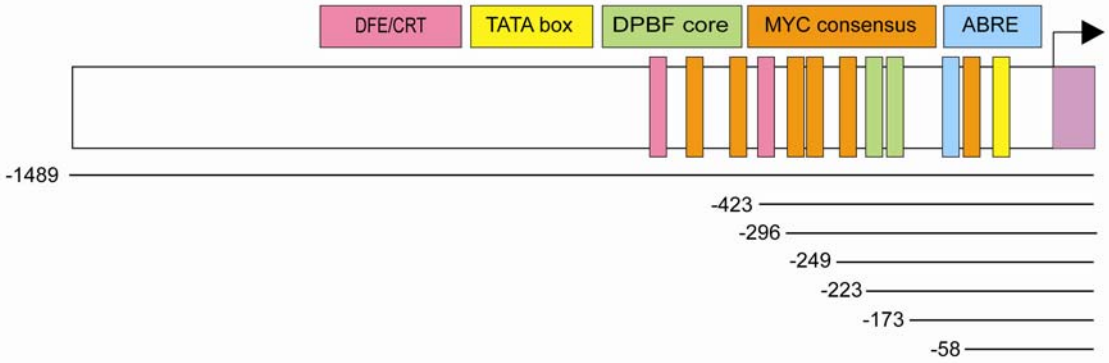


Figure 5-5. Structure of *AtCOR 413 pm-1* promoter. Schematic representation of constructs carrying different length of *AtCOR413 pm-1* promoters and GUS coding region. Numbers indicate upstream end of the promoter fragment in each construct with respect to the transcription start site (+1). Different 5´-deletions of *AtCOR413 pm-1* promoters were cloned into pBI101 upstream of the GUS coding region. Plants transformed with pBI101 (without construct) were used as a control.

treatment (Fig. 5-6). These results suggest that ABI5 is not the major protein regulating ABA response on *AtCOR413 pm-1* in seedling. However, transgene expression was decreased by LT treatment in all three genetic backgrounds (Fig. 5-6). In contrast to the GUS quantification assay, qRT-PCR revealed that *AtCOR413 pm-1* expression was highly induced by LT treatment at every time point during the course of experiment in all three different backgrounds. This discordance might be caused by the suppression of the protein synthesis rate and metabolic activities at low temperature.

Analysis of *AtCOR413 pm-1* promoter: Identification of *cis*-regulatory elements responsible for abiotic stress response

To further investigate the regulation of *AtCOR413 pm-1* gene *in vivo* and define the promoter region responsible for the ABA and LT response, we performed transactivation assays using *AtCOR413 pm-1* promoter:GUS fusion constructs in *Arabidopsis* dry seed and seedlings. A series of 5´-deletions of *AtCOR413 pm-1* promoter was created, and each deletion fragment was fused to the GUS reporter gene (Fig. 5-5). Seven constructs containing 5´ sequence-deleted to -1489, -423, -296, -249, -223, -173, and -58 from the transcription start point and 5' UTR region fused to GUS transgene were introduced into *Arabidopsis*. GUS activity was analyzed in 11-day old seedlings treated with ABA and LT stress. The response to ABA and LT stress were analyzed in 8 to 10 independent transgenic T2 lines for each construct. GUS expression was assayed by histochemical and fluorometric analysis.

Using a series of 5´-deletions of *AtCOR413 pm-1* promoter constructs, we identified several *cis*-regulatory elements involved in the regulation of ABA response. In particular, the transgenic line carrying ProAtCOR413pm1-249:GUS

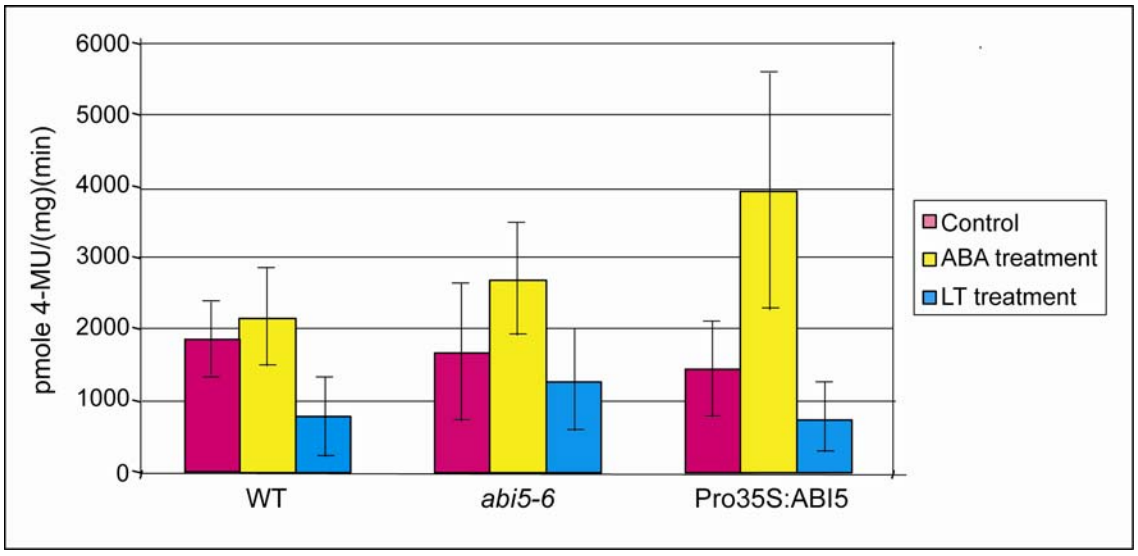
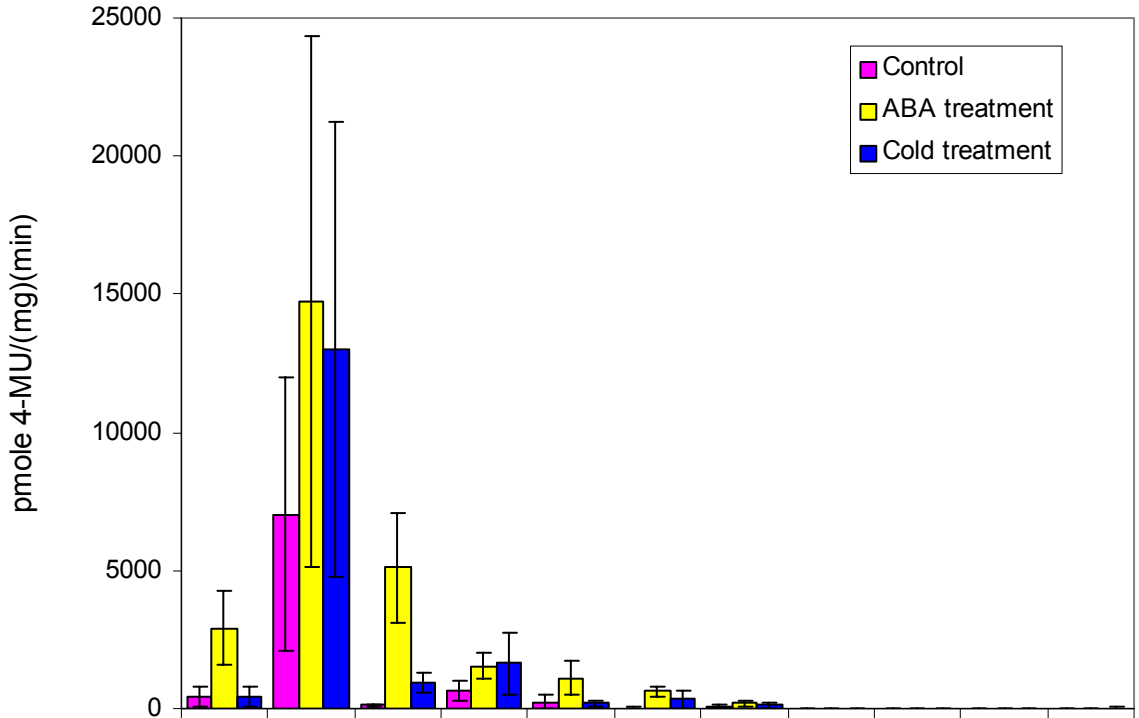


Figure 5-6. GUS expression driven by *AtCOR413 pm-1* promoter in WT, *abi5-6* and Pro35S:ABI5 seedling. GUS expression levels in seedlings ProAtCOR413pm1-1489:GUS treated with ABA and LT (low-temperature). The results depict the mean (± SD) of 10 independent lines of T2 seedlings for each genetic background.

and ProAtCOR413pm1-296 lines showed a 14-fold and 44-fold induction of GUS activity, respectively after ABA treatment compared to ProAtCOR413pm1-223:GUS line (Fig. 5-7). These results indicated that the promoter sequence located between -249 and -223, containing a MYC consensus motif, is responsible for ABA response in *AtCOR413 pm-1*. In addition, base substitution analysis in the ABRE motif of ProAtCOR413pm1-1489:GUS showed higher transgene expression than the non-mutated ABRE construct, and the transgene expression was increased after ABA treatment (Fig. 5-7). These results suggested that other *cis*-regulatory elements in the promoter of *AtCOR413 pm-1* are responsible for ABA response in vegetative tissues. Furthermore, base substitution constructs that have mutations in the ABRE and DRE motif in the ProAtCOR413pm1-423:GUS construct were tested to test whether these *cis*-regulatory elements are involved in the regulation of *AtCOR413 pm-1* in response to ABA. The mutation in ABRE and DRE motifs resulted in decreased transgene activity compared to control construct after ABA treatment. (Fig. 5-7) However, these mutated lines still exhibited significant levels of GUS expression after ABA treatment indicating the involvement of ABRE, DRE and MYC *cis*-regulatory elements in the regulation of *AtCOR413 pm-1* in response to ABA.

To identify *cis*-regulatory elements for LT response, we also used 5' deletion constructs containing base substitution in the ABRE motif of ProAtCOR413pm1-423:GUS. Significant level of GUS expression was detected in ProAtCOR413pm1-423:GUS/ABRE mutated line after LT treatment, but transgene activity was decreased in ProAtCOR413pm1-423:GUS/DRE mutated line compared to ProAtCOR413pm1-423:GUS line (Fig. 5-7). These results indicated that DRE motif located at -423 is responsible for LT response of *AtCOR413 pm-1*.



	-1489	1489mutABRE	-423	423mutABRE	423mutDRE	-296	-249	-223	-173	-58	pBI101
Control	426	7031	128	644	229	32	62	15	20	17	6
ABA treatment	2925	14746	5102	1529	1111	622	195	14	6	3	2
Cold treatment	434	13013	941	1635	184	345	130	2	3	2	2

Figure 5-7. GUS expression driven by truncated forms of *AtCOR413 pm-1* promoter. GUS expression levels were tested in seedlings carrying different deletion constructs and mutations in ABRE and DRE motif treated with ABA and cold. The results indicate the mean (± SD) of approximately 10 independent lines of T2 seedlings for each construct.

Taking the GUS assay data together, it could be concluded that the DRE motif is responsible for LT stress response and the ABRE motif is involved in ABA stress response of *AtCOR413 pm-1*. We further showed that other *cis*-regulatory elements present in the promoter of *AtCOR413 pm-1* such as the DRE and the MYC consensus motifs are also involved in the regulation of *AtCOR413 pm-1* in response to ABA.

Promoter analysis of *AtCOR413 pm-1*: Transcriptional regulation of ABI5 in seeds

We have shown that ABI5 protein binds to the consensus motif of the ABRE *cis*-regulatory element containing (T/G/C)ACGT(G/T)GC which was identified from ABI5-regulated genes in seed using EMSA. To investigate the regulation of *AtCOR413 pm-1* expression by ABI5 protein in seeds, we performed a transactivation assay using *AtCOR413 pm-1* promoter:GUS fusion constructs, ProAtCOR413pm1-1489:GUS and ProAtCOR413pm1-173:GUS that includes the ABRE *cis*-regulatory motif (Fig. 5-5). The expression in ProAtCOR413pm1-1489:GUS and ProAtCOR413pm1-173:GUS line was analyzed in 10 independent T2 homozygous seeds.

The ProAtCOR413pm1-1489:GUS line did not show any detectable staining in WT and Pro35S:ABI5 mature embryos and seeds, but showed strong GUS transgene activity in *abi5-6* mature embryos and seeds (Fig. 5-8). Quantification of GUS activity revealed that the level of transgene induction in *abi5-6* seeds was 5 times higher than WT seeds (Fig. 5-9), indicating that ABI5 possibly acts as negative regulatory component on *AtCOR413 pm-1* expression in seeds. Furthermore, we analyzed transgene activity in ProAtCOR413pm1-173:GUS line to test whether the identified ABRE *cis*-regulatory sequence is responsible for

Figure 5-8. GUS expression of the *AtCOR413 pm-1* in WT, Pro35S:ABI5 and *abi5-6* seeds. GUS expression in WT, Pro35S:ABI5 and *abi5-6* mature embryo and dry seeds carrying ProAtCOR413pm1-1489:GUS construct. Histochemical staining was performed with dry seeds which were imbibed for 12 hr to separate embryos from seed coat.

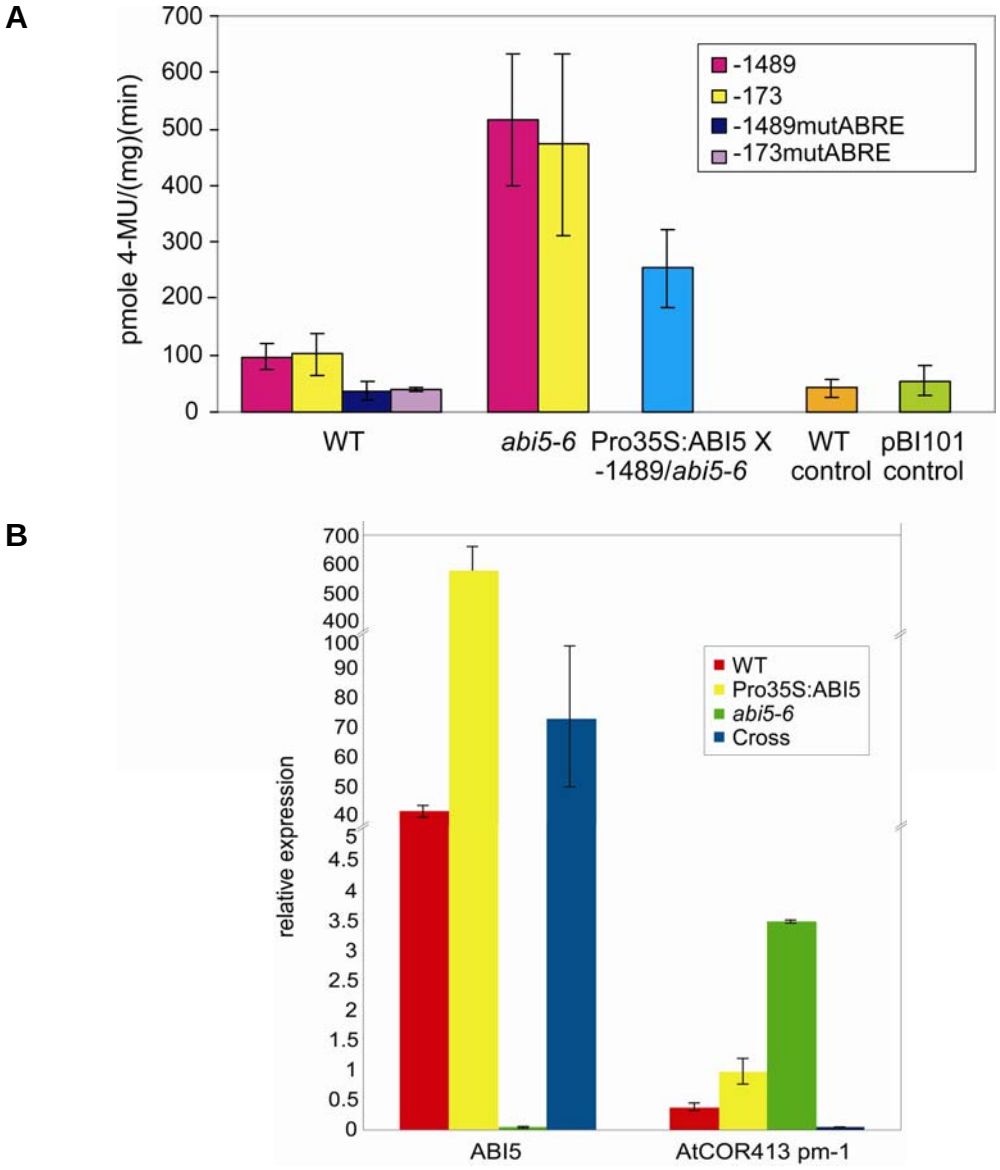


Figure 5-9. GUS expression of the *AtCOR413 pm-1* promoter in WT, Pro35S:ABI5, *abi5-6*, and Pro35S:ABI5 X ProAtCOR413pm1-1489:GUS/*abi5-6* seeds. (A) GUS expression levels in WT and *abi5-6* seeds carrying different deletion construct and in cross between Pro35S:ABI5 and ProAtCOR413pm-1-1489:GUS/*abi5-6*. The results indicate the mean (± SD) of 10 independent lines of T2 seeds and the mean (± SD) of F3 seeds of Pro35S:ABI5 X ProAtCOR413pm1-1489:GUS /*abi5-6* seeds. (B) Total RNA was isolated from dry seeds of WT, *abi5-6*, Pro35S:ABI5 and Pro35S:ABI5 X ProAtCOR413pm1-1489:GUS/*abi5-6*. qRT-PCR was performed to measure transcript level of *ABI5* and *AtCOR413 pm-1*, and relative amount of transcript levels were calculated and normalized to the expression of 18S ribosomal RNA. Data shown represent mean values obtained from two independent RNA samples.

the regulation of *AtCOR413 pm-1* by ABI5. Strong transgene activity was detected in ProAtCOR413pm1-173:GUS/*abi5-6* seeds, but no detectable transgene activity was observed in WT seeds. Further, a quantitative assay of ProAtCOR413pm1-173:GUS line indicated that the level of induction was 5 times higher in *abi5-6* seeds than WT seeds (Fig. 5-9). These results indicated that the ABRE motif located at -173 is essential for the regulation of *AtCOR413 pm-1* in seeds, and ABI5 might be the major protein regulating the expression of *AtCOR413 pm-1* in seeds. Furthermore, base-substitution analysis in the ABRE motif was performed to confirm whether the ABRE motif located in the *AtCOR413 pm-1* promoter is responsible for the regulation in seeds. The seeds carrying ProAtCOR413pm1-1489:GUS and ProAtCOR413pm1-173:GUS constructs with mutated ABRE motifs showed a lower level of transgene activity than the seeds carrying non- mutated ABRE motifs (Fig. 5-7). These analyses suggested that the mutation in the ABRE motif may hinder binding of other bZIP transcription factors such as EEL/AtDPBF4 as it was shown in Figure 5-3, and the ABRE motif is the regulatory element responsible for the regulation of *AtCOR413 pm-1* in seeds.

Complementation assay

To test whether ABI5 is a key factor for the regulation of *AtCOR413 pm-1* in seeds, reciprocal crosses were performed between ProAtCOR413pm1-1489:GUS/*abi5-6* and Pro35S:ABI5. Quantification of GUS activity showed that transgene activity was reduced to half of ProAtCOR413pm1-1489:GUS/*abi5-6* levels indicating that over-expression of *ABI5* could complement to certain level, but could not completely complement the *abi5-6* mutation (Fig. 5-9). Furthermore, we checked the transcript level of ABI5 in ProAtCOR413pm1-1489:GUS/*abi5-6* X Pro35S:ABI5 by qRT-PCR to check whether *ABI5*

expression is as highly induced as in Pro35S:ABI5 seeds. It revealed that *ABI5* expression in ProAtCOR413pm1-1489:GUS/*abi5-6* X Pro35S:ABI5 seeds was induced about 2-fold more than in WT seeds containing ProAtCOR413pm1-1489:GUS construct, but it was greatly reduced by 10-fold of Pro35S:ABI5 seeds containing ProAtCOR413pm1-1489:GUS construct (Fig. 5-9). These phenomena could be caused by homology-dependent gene silencing in plants. Taking these together, it could be concluded that ABI5 is the main player negatively regulating the expression of *AtCOR413 pm-1* in seeds.

DISCUSSION

The *Arabidopsis* basic leucine zipper transcription factor ABI5 is considered a key factor regulating gene expression during embryogenesis and is involved in the activation of several Late Embryogenesis–Abundant (LEA) genes, including *AtEm1* and *AtEm6* (Parcy et al., 1994; Finkelstein and Lynch, 2000). LEA proteins are highly accumulated in the seed during the final desiccation stage of seed development and are also expressed in vegetative tissues during periods of water, osmotic, and low-temperature stress (Bray, 1993; Wise and Tunnacliffe, 2004). They have been proposed to protect cellular structures in the mature embryo during seed desiccation and in water-deficient vegetative tissues by sequestering ions and protecting other proteins in the membrane (Baker et al., 1988).

Previously, we showed that *AtCOR413 pm-1* is induced in *abi5-6* seeds by microarray analysis and validated these results by qRT-PCR and RNA gel blot analysis. Also, we showed that ABI5 binds to the promoter of *AtCOR413 pm-1 in vitro,* and the ABRE motif in the promoter region is a *cis*-regulatory element

responsible for the regulation of *AtCOR413 pm-1* in seeds. These results suggested that ABI5 acts as a negative regulator for *AtCOR413 pm-1* in seeds.

The cold-regulated (COR) 413 pm-1 is known to encode a protein with five transmembrane domains potentially targeted to the plasma membrane and is correlated with the development of freezing tolerance in cereals and *Arabidopsis* (Breton et al., 2003). In plants, the membrane is the primary site of freezing injury, and the freeze-induced membrane damage includes expansion induced lysis, hexagonal phase II transition, and fracture jump lesions, resulting from the severe dehydration associated with freezing (Steponkus, 1984; Uemura and Steponkus, 1997). However, multiple mechanisms appear to be involved in the membrane stabilization including lipid composition changes, the accumulation of sucrose or simple sugars, and protein composition changes (Strauss and Hauser, 1986; Gilmour et al., 1988; Uemura and Steponkus, 1997). Therefore, AtCOR413 pm-1 membrane protein might be involved in stabilizing the membrane against freezing injury.

Furthermore, we investigated the expression of *ABI5* and *AtCOR413 pm-1* during late embryogenesis and early germination by examining their expression profiles at AtGenExpress. *ABI5* expression increased during seed maturation showing its peak in desiccating dry seed and then decreasing by imbibition of seed with its lowest expression at 3 hr after imbibition. In contrast, *AtCOR413 pm-1* expression was repressed as the *Arabidopsis* embryo matured and then was highly induced within 1 hr of seed imbibition. During the germination of seeds, rapid initial uptake of water into the cells of dry seeds results in temporary structural perturbations, particularly to the membranes. Subsequently, it leads to an immediate and rapid leakage of solutes into imbibing solution (Bewley, 1997). However, the membrane becomes more stable within a short time after rehydration. Therefore, AtCOR413 pm-1 protein might be involved in

maintaining or enhancing membrane integrity by stabilizing membrane structure against turgor pressure built in the cell by rapid water uptake upon the imbibition of the seed.

By the promoter deletion analysis of *AtCOR413 pm-1*, we have shown that the promoter region between -249 and -223 is responsible for the induction of *AtCOR413 pm-1* in response to ABA. The fragment was further analyzed using an *in silico* promoter analysis approach and revealing a 27-bp sequence containing the MYC consensus motif. Previously, it was shown that a Myc-binding site participates in ABA induction by binding a drought-inducible Myc protein (Abe et al., 1997; Abe et al., 2003). We also revealed that the DRE motif located at -423 in the promoter of *AtCOR413 pm-1* has been shown to be responsible for LT response. It was also shown that the CBF (CRT binding factor) or DREB1 (DRE binding) family proteins bind to the CRT/DRE element and activate transcription (Gilmour et al., 1998; Liu et al., 1998; Chinnusamy et al., 2003). Since it was proved in this study that ABI5 is not the main protein regulating ABA response of *AtCOR413 pm-1* in vegetative tissue, other stress inducible transcription factors such as MYB/MYC and DREB proteins may be responsible for the expression of *AtCOR413 pm-1* in response to ABA and LT stress.

MATERIALS AND METHODS

Plant materials

Arabidopsis seeds were surface-sterilized with 70% ethanol for 5 min, then with 50% bleach containing 0.2% (v/v) triton for 7 min with rotation, washed 4 times

with sterile water, and then plated on MS (Murashige and Skoog, 1962) medium. The plates containing sterilized seeds were incubated for 3 days at 4°C to ensure uniform germination. Seeds were germinated and grown on MS medium containing 1% sucrose and 0.7% Agar (Sigma-Aldrich Co., St. Louis, MO) for 11 days under continuous light at 22°C. For ABA treatment, WT, 35:ABI5, and *abi5* seedlings grown on MS solid medium were transferred into liquid MS medium supplemented with 100 μM ABA and incubated with gentle shaking under continuous light at 22°C. ABA treated seedlings were harvested after 2, 4, 12 and 24 hr. For cold treatment, WT, Pro35:ABI5, and *abi5-6* seedlings grown on MS solid medium were placed at 4°C under continuous light. Cold-treated seedlings were harvested after 4, 6, 12 and 24 hr. Two independent biological samples for each ABA-treated and cold-treated time points were harvested.

AtCOR413 PM-1 **promoter constructs**

The *AtCOR413 pm-1* promoter fragments used in this study were comprised of regions from the ATG start codon up to positions -58, -173, -223, -249, -296, -423 and -1489, relative to the transcription initiation site. These fragments were obtained by PCR amplification of the *Arabidopsis* wild-type (ecotype Columbia) genomic DNA using forward primers and reverse primers (Table 5-1) which contain *Xba*I and *Bam*HI restriction sites at their 5' ends respectively. After sub-cloning into pTOPO (Invitrogen Life Technologies, Carlsbad, CA), the sequence was confirmed. The promoter-containing fragments were isolated by a double restriction digest with *Xba*I and *Bam*HI and cloned into the pBI101 (Clontech, Mountain View, CA) binary vector containing the ß-glucuronidase reporter gene (*GUS*).

Table 5-1. Primer sequences for *AtCOR413 pm-1* promoter analysis

Primer	Sequence
-1489	GGGGGTCTAGAAAGTTAGTGATTTTATCTGAAGTCA
-423	GGGGGTCTAGATGACTTTAATGTATACCGACATACC
-296	GGGGGTCTAGAAAAAAAAATACAACTGGACAGCTGG
-249	GGGGGTCTAGACCACCAATTACATTTGTTAAAGCAA
-223	GGGGGTCTAGATCTATGTCAGAAATCGGATTAGCTT3
-173	GGGGGTCTAGATCTATGTCAGAAATCGGATTAGCTT3
-58	GGGGGTCTAGAAACGTATCCATCGAGTCACTTGTAA
Reverse	GGGGGGGATCCAAGAGAGAACTATGAATAATTGATG
ABRE mutated	GGGGGTCTAGATACCTGACCGCGGAGTTATGAAA
DRE mutated	GGGGGTCTAGATGACTTTAATGTATCAGCATACACCC

To generate mutated *cis*-elements at the ABRE and DRE motif in the *AtCOR413 pm-1* promoter, fragments of the *AtCOR413 pm-1* promoter region were synthesized by PCR using forward primers (-423 and -1489) and a reverse primer which contained an ABRE shuffled sequence with *Xba*I sites at the 5' end. The shuffled fragments were cloned into the pBI101 binary vector in which the minimal promoter of *AtCOR413 pm-1* had previously been cloned. Additionally, a base-shuffled DRE *cis*-element fragment was synthesized by PCR using a mutated DRE-F primer and reverse primer which contained *Xba*I and BamHI sites at their respective 5' ends. This base-substituted fragment was cloned into the pBI101 binary vector containing the ß-glucuronidase reporter gene (*GUS*) and subsequently confirmed by sequencing.

Arabidopsis thaliana **transformation and selection**

Plant transformation was performed by the infiltration method (Clough and Bent, 1998) with some modifications using the *Agrobacterium tumefaciens* strain GV3101 carrying the constructs described above. 250 mL liquid culture was grown at 28°C in LB (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter water) with kanamycin (30 $\mu g \cdot mL^{-1}$) and gentamycin (50 $\mu g \cdot mL^{-1}$) started from 5 mL of cultures containing the constructs described above. Cells were harvested by centrifugation for 20 min at room temperature at 5500 *g* and then resuspended in 250 mL of MS media containing 5.0% sucrose (w/v), 0.02% (v/v) of Silwet L-77 detergent (Lehle Seeds, Round Rock, TX) and 2.5 ul of Benzoa (10 $mg \cdot mL^{-1}$ in DMSO).

For floral dip, the inoculum was added to a beaker, plants were inverted into the suspension to submerge floral tissues, and plants were then removed after 2 min. Dipped plants were placed in a plastic tray and covered with plastic wrap to

maintain humidity. Plants were left in a low light environment overnight and plastic wrap was gradually removed over days. Plants were grown for a further 3–5 weeks until siliques were brown and dry. Seeds were harvested, dried under desiccation for additional days and then stored at 4°C.

To select transformed plants, seeds were sterilized as described above. The sterilized seeds were plated on MS pates containing kanamycin (30 $\mu g \cdot mL^{-1}$) and carbenicillin (250 $\mu g \cdot mL^{-1}$), incubated for 3 days at 4°C, and then grown for 7–10 days under continuous light at 22°C. Transformants were identified as kanamycin-resistant if seedlings produced green leaves and possessed well-established roots. The results presented in this study are derived from the T_2 or T_3 generation.

Genetic crosses

Reciprocal crosses of Pro35S:ABI5 plants with ProAtCOR413pm-1-1489:GUS/*abi5-6* were performed. Two random individuals were chosen to produce two independent hybrid plants and the resulting F_1 plants were self-pollinated to produce F_2 progeny. To select successful crossed plants, seeds were plated on MS Kanamycin (30 $\mu g \cdot mL^{-1}$) and Hygromycin (25 $\mu g \cdot mL^{-1}$) selection plates, incubated for 5 days at room temperature in the dark, and then grown for 7–10 days in a controlled environment at under continuous light at 22°C. The resistant seedlings were then transplanted to soil (Sun Gro Horticulture, Vancouver, Canada) and sprayed with BASTA (240 $\mu g \cdot mL^{-1}$) for selection.

Fluorometric GUS assays

GUS enzyme activity in transgenic *Arabidopsis* seedlings was determined fluorometrically according to Jefferson (1987) with some modifications. For GUS fluorometric analysis, approximately 10 independent transgenic lines for each construct were ground in 200 µL of GUS extraction buffer containing 50 mM $NaP0_4$, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine (w/v), and 0.1% Triton X-100 (w/v). This suspension was then centrifuged for 20 min at 4°C. 10 µL of the crude extract was combined with 10uL of substrate buffer (extraction buffer with 4 mM of 4-methylumbelliferyl β-D-glucuronide, triethylamine salt (4-MUG) (Rose Scientific Ltd, Edmonton, Canada). The reaction mixture was incubated for 60 min at 37°C and then was terminated by the addition of 180 µL of stop buffer (0.2 M Na_2CO_3). GUS activity was determined using a Synergy HT multi-detection microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Also, protein concentration was measured using the Bio-Rad protein assay kit and determined utilizing BSA as a standard protein as described by Bradford (Bradford, 1976). GUS activity was reported as picomoles of 4-methylumbelliferone (4-MU) formed per minute per milligram of protein from the initial velocity of the reaction.

Histochemical assay of GUS reporter gene

For histochemical analyses of GUS expression, transgenic lines for each construct were vacuum-infiltrated with the GUS assay solution, which included 1 mM X-GLUC (5-bromo-4-chloro-3-indolyl-β-glucronide) (Rose Scientific Ltd, Edmonton, Canada**)**, 50 mM NaPO4, pH 7.0, 0.6 mM $K_3Fe(CN)_6$, 0.6 mM $K_4Fe(CN)_6$, 0.1% (v/v) Triton X-100, 6 mM Na_2EDTA and 2% dimethylformamide, and incubated for overnight at 37°C. Then the samples were soaked in 70%

(w/v) ethanol to stop the reaction and remove chlorophyll. Photographs were taken with a Zeiss Axiophot microscope (Carl Zeiss Inc., Oberkochen, Germany).

RNA isolation and gel blot analysis

Whole *Arabidopsis* seedling tissues were collected and stored at –90°C. RNA extraction was performed from frozen tissues using a modified hot phenol/SDS extraction and LiCl precipitation protocol (Ausubel et al., 1994). Plant tissues were homogenized with TLE buffer [180 mM Tris-HCl (pH 8.2), 90 mM LiCl and 4.5 mM EDTA] containing 1 % SDS, 0.1 % β-mercaptoethanol, 0.18 M NaOAc (pH 4.0). The same volume of TLE buffered Phenol and Chloroform was added and vortexed vigorously. The homogenized samples were centrifuged for 6 min at 13,000 rpm and the supernatant was extracted with an equal volume of phenol:chloroform: iosamylalcohol (25:24:1) and further with an equal volume of chloroform. Total RNA was precipitated with 1/3 volume of 8M LiCl and the pellet was dissolved in 100 µL of nuclease-free H_2O. Contaminating DNA was removed from each RNA preparation by using the DNA-*free*™ kit (Ambion, Austin, TX) according to the manufacturer's instructions. For quantitative real-time PCR, the isolated total RNA (50-100 ng) was qualitatively analyzed using The total ENA nano Assay on a Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

The isolated total RNA (15 µg) was also analyzed by electrophoresis on 1% agarose/MOPS-formaldehyde gels by denaturing at 65°C for 15 min with a mixture of 2 µl 10 X MOPS, 3.5 µl formaldehyde and 10 µl deionized formamide. The gels were subsequently transferred to nylon membrane by capillary action (Nytran ® N SuperCharge, Schleicher & Schuell BioScience, Inc. USA, Keene, NH) using 20 X SSC. The membranes were hybridized with ^{32}P-labelled cDNA

probes generated by random primer extension using Klenow Fragment (3´→5´ exo$^-$) (New England BioLab, Ipswich, MA)**.** Hybridization was performed at 65°C in the hybridization oven for 14-16 hr. After hybridization, filters were washed with 2X SSC, 0.5% SDS for 5 min at room temperature and washed with 2X SSC, 0.5% SDS 15 min and 0.2X SSC, 0.1% SDS for 15 min at 60°C. Additionally, filters were washed with 2X SSC for 5 min at room temperature. Signal detection was carried out using a Fuji BAS-1500 Phosphorimager (Fuji Film Co., Tokyo, Japan). Blots were stripped between hybridizations in 0.1% SDS at 90 to 95°C for 5-10 min.

Quantitative real-time RT-PCR

2 μg of total RNA was reverse transcribed with RT reaction mix including 1 X TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM dNTP mixture, 2.5 μM random hexamers, 40 U RNase inhibitor and 125 U Multiscribe^{TM} reverse transcriptase using the TaqMan^{R} reverse transcription kit (Applied Biosystems, Foster City, CA) or SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Primers were designed using the Primer Express version 1.5 software (Applied Biosystems, Foster City, CA), and the sequences of each primer pair are given in Table 5-2.

PCRs were performed in an optical 96-well plate with GeneAmp 7500 sequence detector (Applied Biosystems, Foster City, CA), using Power SYBR® Green to monitor dsDNA synthesis. Three identical reactions were repeated on the plates and the reactions contained 10 μl 2 X Power SYBR® Green Master Mix reagent (Applied Biosystems, Foster City, CA), 20 ng cDNA and 5 μM of each forward and reverse gene-specific primers in a final volume of 20 μl. The following

Table 5-2. Primers Used in Gene-Specific qRT-PCR

	Forward Primer	Reverse Primer
ABI5	CGGTGTCTTCAGATGGATTAGGA	CCCCTTAGCCCTCCCATATC
AtCOR413 pm-1	ACACGCCAGAGAATACCTGGA	AATGTGCCAGCAATCAAATTTG
18S rRNA	GTCATCAGCTCGCGTTGACTAC	GAGCGACGGGCGGTG

standard thermal profile was used: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 50°C for 1 min. Subsequently, a dissociation curve was developed using the following program: 95°C for 15 sec, 50°C for 1 min, and 95°C for 15 sec. Data were analyzed using the SDS 1.7 software (Applied Biosystems, Foster City, CA). In order to compare data from different PCR runs or cDNA samples, C_t values for the genes were normalized to the C_t value of 18S RNA. Quantification of the abundance of each transcript was determined using the comparative C_t method. The amount of target, normalized to 18S RNA and relative to a calibrator, is given by: $2^{-\Delta(\Delta Ct)}$ (user bulletin 2, ABI Prism 7500 sequence detection system; Applied Biosystems).

Gel-shift assay

For *in vitro* transcription/translation, ABI5 and AtDPBFs coding regions were cloned into pCITE vectors (Novagen, Madison, WI) (Kim et al., 2002). Extracts containing pCiTE4C, ABI5, AtDPBF2, EEL/AtDPBF4, ABI5 and AtDPBF2, ABI5 and EEL/AtDPBF4 and AtDPBF2 and EEL/AtDPBF4 were prepared by coupled *in vitro* transcription/translation employing 1 μg of each construct and the TNT® Quick Coupled Transcription/Translation Kit (Promega, Madison, WI) according to the manufacturer's instruction. EMSAs were performed as previously described (Kim et al., 1997). Probes were obtained by annealing single-stranded oligonucleotides (*AtCOR413 pm-1* ABRE, DPBF core and mutated ABRE motif) in TEN buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl). The mixture was incubated at 95°C for 10 min and cooled down slowly to room temperature. Oligonucleotides were end-labeled by T4 Polynucleotide Kinase with γ-^{32}P-ATP, and purified with Performa®DTR Gel Purification cartridges (Edge Biosystems, Gaitherburg, MA, USA). Binding reactions (20 μl) were performed using 100 ng of radio-labeled probe, 2.5 μL of *in vitro*

transcribed/translated product, 5X binding buffer (50 mM Tris, 250 mM NaCl, 2.5 mM DTT, 2.5 mM EDTA, 5 mM $MgCl_2$, 20% glycerol), and 100 ng poly-(d[I-C]) (Roche Diagnostics Corporation, Mannheim, Germany) for 20 min at RT followed by 15 min incubation on ice. Reactions were electrophoretically separated on a 6% non-denaturing polyacrylamide gel using 1X Tris-borate buffer (44.58 mM Tris, 43.67 mM boric acid, and 1 mM EDTA, pH 8.0) at 4°C (80V/1 h). Gels were dried at 80°C for 30 min and exposed to BAS-IP MS 2025 Phophoimaging plate (Fuji Film Co., Tokyo, Japan) and signal detection was carried out using a Fuji BAS-1500 Phosphorimager (Fuji Film Co., Tokyo, Japan)

CHAPTER VI
CONCLUSION AND FUTURE PERSPECTIVES

ABI5 encodes a transcription factor that belongs to a seed-specific subfamily of bZIP transcription factors and is a key factor in regulating gene expression during embryogenesis and in response to ABA. The *Arabidopsis abi5* mutants, including *abi5-6,* were selected on the basis of ABA-resistant germination and vegetative growth. However, these mutants have a mild phenotype and do not exhibit any morphological defects. Therefore, high-throughput genomic technology was employed to study the molecular mechanism of ABI5-regulated gene expression in developing embryos and vegetative tissues. Molecular genetic tools were also applied to elucidate ABI5-regulated gene expression in *Arabidopsis*.

ABI5 AND ABA INHIBIT GROWTH FOLLOWING GERMINATION

To understand the molecular mechanism of ABI5 on a global scale, an *ABI5* over-expressing line and the *Arabidopsis abi5-6* mutant were first characterized with respect to ABA sensitivity during germination and seedling development. The over-expression of ABI5 was sufficient to confer hypersensitivity to high concentrations of ABA and a resulting increase in the inhibition of root growth. In contrast, the *abi5-6* mutant did not display a significant difference in ABA sensitivity or root growth compared to WT. Moreover, seeds of the *ABI5* over-expressing line germinated into seedlings with green cotyledons and long roots on medium lacking ABA. About 20% of the Pro35S:ABI5 seeds could germinate in the presence of high concentrations of ABA, but further development into

seedlings was arrested. Thus, these results suggested that the ABI5 protein needs to be further activated by modifications for appropriate function. In addition, ABA and ABI5 might play a major role in growth inhibition following germination, rather than in controlling seed germination. Our finding corresponds to a previous report from Lopez-Molina's group showing that ABI5 blocks the growth of germinated embryos protecting the plants from unfavorable conditions (Lopez-Molina et al., 2001). However, further study is needed to reveal the molecular mechanism underlying the growth arrest by ABI5 in combination with ABA.

THE ABA SIGNALING NETWORK AND ABI5-MEDIATED GENE REGULATION ARE CLOSELY RELATED

We employed cDNA microarrays to analyze the global effects of ABI5 on ABA regulated gene expression and also studied ABA-mediated gene expression in vegetative tissues on a genomic scale. This high-throughput genomic technology allowed us to monitor the response of thousands of genes simultaneously and to identify a large number of ABA responsive genes and ABA/ABI5 regulated genes. Of 310 genes identified as ABI5/ABA regulated genes, 175 genes, corresponding to 2.5% of all the genes on the cDNA microarray, were found to represent ABA-responsive genes. A large number of these have not previously been reported to be ABA-responsive. The ABA induced genes identified from the cDNA microarray analysis included a large number of genes that are classified as stress response genes and several enzymes involved in the detoxification of secondary metabolites produced against stress and pathogen attack. Many metabolism related genes were also induced by ABA treatment suggesting the initiation of metabolic reprogramming by ABA. In the set of ABA repressed genes, many GA responsive genes were

down-regulated by ABA treatment, showing the negative regulation of ABA on the GA regulatory network. Many genes encoding the Rubisco enzyme were also down-regulated suggesting that the photosynthetic capacity of leaves may be reduced in response to ABA through an apparent inhibition of Rubisco gene expression. In the promoters of the ABA regulated genes, multiple classical G-box containing ABA-responsive elements (ABREs) were identified indicating that ABRE motifs are the main *cis*-elements involved in regulating ABA-dependent gene expression.

Among 310 genes identified as ABI5/ABA regulated, 161 were regulated by ABI5. One hundred thirty-four of the 161 ABI5-regulated genes were co-regulated by ABA showing a tight relationship between ABI5 and ABA in regulating gene expression in vegetative tissue. A small number of ABA-responsive genes were regulated by the ABI5 regulatory network, but the majority was not, indicating the existence of other networks in *Arabidopsis* vegetative tissues. Through cDNA microarray analysis, we identified only a small number of differentially expressed genes in Pro35S:ABI5 vegetative tissues in the absence of ABA compared to these treated with ABA. This suggests that ABI5 protein might require post-translational modification(s) for further activation. This finding is consistent with a previous report that ABI5 protein is highly accumulated in the presence of ABA, and that ABA induces ABI5 phosphorylation to activate it (Lopez-Molina et al., 2001). Moreover, in each class of ABI5/ABA regulated genes, only a small number of the genes identified from Pro35S:ABI5 or *abi5-6* were regulated in both genetic backgrounds. This might be due to preferential binding of the bZIP protein homodimers or heterodimers to specific promoter sequences. We identified a large number of ABI5/ABA regulated genes encoding proteins of unknown function in this study. Thus, the characterization of these functionally unknown genes may be of particular interest for future studies.

THE AFFYMETRIX ATH1 GENOME ARRAY SHOWED A SIGNIFICANT LEVEL OF CORRELATION WITH QRT-PCR

Three *Arabidopsis* DNA microarray platforms including a cDNA microarray, the Agilent *Arabidopsis* 2 Oligo microarray and the Affymetrix ATH1 genome array were used to determine which platform is more reliable and desirable for the identification of ABI5 bZIP transcription factor regulated genes in seeds. Identical RNA samples isolated from WT and *abi5-6* seeds were used, and expression comparison across platforms was performed using 4,518 genes present on all three microarray platforms. There was a good correlation between the Agilent and Affymetrix platforms. However, the results obtained with the cDNA microarray did not correspond well with those from the other platforms. Several other studies have revealed a good correlation between cDNA microarrays and oligonucleotide microarrays. Therefore, further investigation is essential to resolve the issue of reproducibility. In addition, qRT-PCR was performed for a selected gene set to validate the microarray data and evaluate the microarray platforms included in this study. In general, there was good correlation between the microarray and qRT-PCR analysis, and the Affymetrix array showed a significantly higher correlation with the qRT-PCR data than did the data from other platforms. However, it is hard to conclude that the general correlation between qRT-PCR and the Affymetrix array is better than that of the others because of the limited sample size of the qRT-PCR analysis. Further study is required to conclude which microarray platform is most reliable and desirable for the analysis.

ABI5 NEGATIVELY REGULATES THE EXPRESSION OF *ATCOR413 PM-1* IN SEEDS

A small number of genes were found to display consistent regulation patterns across the three different microarray platforms hybridized with WT and *abi5-6* seed RNA. The cold-regulated *(COR) 413 pm-1* gene is one of the genes which showed consistent expression patterns across platforms. AtCOR41 pm-1 is known to encode a protein with five transmembrane domains and is potentially targeted to the plasma membrane. This protein may be involved with the development of freezing tolerance in plants by stabilizing the membrane against freezing injury. The consensus sequence of the classical G-box containing ABRE *cis*-regulatory element was identified in the promoter regions of these consistently regulated genes across platforms, and a strong ABRE *cis*-regulatory element was identified in the promoter of *AtCOR413 pm-1* gene. We further showed that *AtCOR413 pm-1* is up-regulated in *abi5-6* seeds by qRT-PCR, RNA gel blot analysis, and in a *AtCOR413 pm-1* promoter:GUS transgene activation assay. Furthermore, the binding of ABI5 to the promoter of *AtCOR413 pm-1* was shown *in vitro* by EMSA. The ABRE motif in the promoter region was identified as a *cis*-regulatory element responsible for the regulation of *AtCOR413 pm-1* in seeds. Thus, it was concluded that ABI5 acts as a negative regulator for *AtCOR413 pm-1* in seeds.

AtCOR413 pm-1 expression was highly induced in vegetative tissues by ABA and LT treatment regardless of whether *ABI5* was over-expressed or knocked-down. This suggested that there might be regulatory proteins other than ABI5 involved in the regulation of *AtCOR413 pm-1* in response to ABA and LT treatment in seedlings. Furthermore, the expression of *ABI5* and *AtCOR413 pm-1* during late embryogenesis and early germination was investigated by examining their expression profiles with AtGenExpress Visualization Tool. It was

shown that *ABI5* expression increased during seed maturation peaking in desiccating seed and then decreasing during the imbibition of seed, with lowest expression at 3 hr after imbibition. In contrast, *AtCOR413 pm-1* expression was low as the *Arabidopsis* embryo matured and then increased within 1 hr of seed imbibition. The expression pattern of *AtCOR413 pm-1* suggested that this protein might be involved in maintaining or enhancing membrane integrity by stabilizing membrane structure against turgor pressure built up in the cell by rapid water uptake upon the imbibition of the seed. To better understand the function of AtCOR413 pm-1, the sub-cellular localization of AtCOR413 pm-1 protein needs to be determined under abiotic stress conditions, seed development, and seed germination. Furthermore, the characterization of *AtCOR413 pm-1* mutants also may facilitate elucidation of the role of this protein in the stress response and also in plant development.

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APPENDIX

Table A-1. Up-regulated genes in *abi5-6* seeds

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT1G64970	248118_at	16.31	G TMT	expressed protein	Other Metabolism
AT1G80110	266274_at	13.01	ATPP2 B11	expressed protein	Unknown
AT4G32870	266977_at	7.60		expressed protein	Unknown
AT4G35550	264244_at	6.92		homeobox-leucine zipper protein (HB-2)	Transcriptional regulation
AT2G23950	259295_at	6.90		leucine-rich repeat family protein	Protein metabolism
AT1G80440	251646_at	6.60		kelch repeat-containing F-box family protein	Unknown
AT4G37550	249580_at	6.45		formamide amidohydrolase	Other Metabolism
AT2G23030	265735_at	6.31		protein kinase 3	Protein metabolism
AT2G31980	262940_at	6.13		cysteine proteinase inhibitor	Unknown
AT3G28740	262727_at	5.95		cytochrome P450	Other Metabolism
AT5G24470	259382_s_at	5.72	APRR5	pseudo-response regulator 5 (APRR5)	Stress response
AT1G69490	263404_s_at	5.50	NAP	no apical meristem (NAM) family protein	Transcriptional regulation
AT5G06690	255070_at	5.45		thioredoxin family protein	Unknown
AT1G33700	258402_at	5.24		hypothetical protein	Unknown
AT1G71250	261718_at	5.15		GDSL-motif lipase/hydrolase	Fatty acid metabolism
AT2G15970	250483_at	5.08	COR413 PM1	cold acclimation WCOR413-like protein	Stress response
AT2G22680	252210_at	5.06		Zinc finger, C3HC4 type (RING finger)	Transcriptional regulation
AT3G01470	255829_at	5.01	ATHB 1	homeobox-leucine zipper protein 5 (HAT5)	Transcriptional regulation
AT5G62800	248238_at	5.01		seven in absentia (SINA) family protein	Protein metabolism
AT3G19800	264338_at	4.93		expressed protein	Unknown
AT1G78500	264586_at	4.80		pentacyclic triterpene synthase	Lipid metabolism
AT1G51115	261937_at	4.76			Unknown
AT3G48360	248759_at	4.66		speckle-type POZ protein	Transcriptional regulation
AT1G07610	264790_at	4.56	MT1C	metallothionein-like protein 1C (MT-1C)	Stress response
AT4G19230	250430_at	4.52	CYP707A1	cytochrome P450	Hormone Response
AT3G28220	255585_at	4.47		meprin and TRAF protein	Unknown
AT5G47240	251987_at	4.34		MutT/nudix family protein	Unknown
AT5G59590	264365_s_at	4.30		UDP-glucoronosyl/UDP-glucosyl transferase	Other Metabolism
AT3G26220	259308_at	4.26	CYP71B3	cytochrome P450 monooxygenase (CYP71B3)	Other Metabolism
AT5G47610	265345_at	4.23		zinc finger (C3HC4-type RING finger) family protein	Transcriptional regulation
AT5G47020	255561_at	4.23		glycine-rich protein	Unknown
AT1G26920	262061_at	4.20		expressed protein	Unknown
AT4G39780	252411_at	4.10		ERF/AP2 transcription factor family	Transcriptional regulation
AT1G71000	262028_at	3.99		DNAJ heat shock protein	Protein metabolism

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT2G46710	250775_at	3.96		rac GTPase activating protein	Signal transduction
AT2G39420	247788_at	3.95		esterase/lipase/thioesterase	Other Metabolism
AT4G12110	249864_at	3.93	SMO1 1	sterol desaturase	Lipid metabolism
AT5G10120	266324_at	3.90		ethylene insensitive	Hormone Response
AT2G46680	248091_at	3.90	ATHB 7	homeobox-leucine zipper protein 12 (HB-12)	Transcriptional regulation
AT5G26731	247628_at	3.83		expressed protein	Unknown
AT5G07200	265648_at	3.81	YAP169	gibberellin 20-oxidase	Hormone Response
AT5G14640	260570_at	3.74		protein kinase	Protein metabolism
AT4G03280	260339_at	3.74	PETC	cytochrome B6-F complex iron-sulfur subunit	Stress response
AT5G35630	247467_at	3.73	GS2	glutamine synthetase	Development
AT5G55620	250293_s_at	3.73		expressed protein	Unknown
AT5G13180	257966_at	3.66	ANAC083	no apical meristem (NAM) family protein	Transcriptional regulation
AT2G16660	264575_at	3.63		nodulin family protein	Unknown
AT3G25690	252912_at	3.56	CHUP1	hydroxyproline-rich glycoprotein	Unknown
AT5G52450	249741_at	3.55		MATE efflux protein-related	Stress response
AT3G59060	257628_at	3.55	PIL6	phytochrome-interacting factor 4 (PIF4)	Transcriptional regulation
AT1G15670	264986_at	3.54		kelch repeat-containing F-box family protein	Unknown
AT1G01490	262080_at	3.54		heavy-metal-associated domain-containing protein	Transport
AT5G14450	252315_at	3.54		GDSL-motif lipase/hydrolase	Lipid metabolism
AT2G24180	245330_at	3.49	CYP71B6	cytochrome P450	Other Metabolism
AT5G50180	256417_s_at	3.49		protein kinase ATN1	Protein metabolism
AT1G06040	260302_at	3.46	STO	transcriptional control putative zinc finger protein	Transcriptional regulation
AT1G21820	253145_at	3.46		unknown protein	Unknown
AT3G61430	258366_at	3.44	PIP1A	aquaporin PIP1.1 (PIP1.1) (AQ1)	Stress response
AT1G48660	256765_at	3.43		auxin-responsive GH3 family protein	Hormone Response
AT3G24240	253758_at	3.41		leucine-rich repeat transmembrane protein kinase	Signal transduction
AT2G20630	251031_at	3.38		protein phosphatase 2C	Unknown
AT1G03080	262952_at	3.36		kinase interacting family protein	Unknown
AT4G26590	255674_at	3.33	ATOPT5	oligopeptide transporter OPT	Transport
AT4G35770	248028_at	3.32	SEN1	senescence-associated protein (SEN1)	Development
AT3G51910	259882_at	3.31	AT HSFA7A	heat shock transcription factor	Transcriptional regulation
AT5G23790	266118_at	3.30		galactinol synthase	Stress response
AT1G80530	251202_at	3.30		nodulin family protein	Unknown
AT5G06570	261785_at	3.28		expressed protein	Unknown

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT3G48690	264661_at	3.27		expressed protein	Unknown
AT1G73680	262238_at	3.21		pathogen-responsive alpha-dioxygenase	Stress response
AT4G38460	252678_s_at	3.20		geranylgeranyl pyrophosphate synthase	Lipid metabolism
AT1G22710	261084_at	3.20	SUC2	sucrose-proton symporter (SUC2)	Transport
AT5G47560	256221_at	3.19	ATSDAT	tonoplast malate/fumarate transporter	Transport
AT5G17540	255550_at	3.19		transferase	Unknown
AT3G51420	248879_at	3.18		strictosidine synthase	Secondary metabolism
AT4G36360	247722_at	3.17	BGAL3	beta-galactosidase	Carbohydrate metabolism
AT3G53160	257232_at	3.14		UDP-glucoronosyl/UDP-glucosyl transferase	Stress response
AT3G60140	261410_at	3.13	DIN2	glycosyl hydrolase family 1 protein	Stress response
AT3G01820	255435_at	3.13		adenylate kinase	Other Metabolism
AT1G68500	261109_at	3.13		expressed protein	Unknown
AT1G23310	253131_at	3.11	GGT1	glutamate:glyoxylate aminotransferase 2 (GGT2)	Photosynthesis
AT2G42600	250318_at	3.11	ATPPC2	phosphoenolpyruvate carboxylase	Stress response
AT1G75580	256231_at	3.09		auxin-responsive protein	Hormone Response
AT1G17830	250611_at	3.08		expressed protein	Unknown
AT3G04630	257236_at	3.08	WDL1	BRI1-KD interacting protein 116	Development
AT3G30350	251118_at	3.08		expressed protein	Unknown
AT1G03687	245748_at	3.06		DTW domain-containing protein	Unknown
AT1G23800	251144_at	3.06	ALDH2B7	aldehyde dehydrogenase	Other Metabolism
AT1G56300	259758_s_at	3.04		DNAJ heat shock protein	Protein metabolism
AT5G57630	266363_at	3.02	CIPK21	CBL-interacting protein kinase 21	Other Metabolism
AT3G47800	266495_at	2.99		aldose 1-epimerase	Carbohydrate metabolism
AT4G27260	248338_at	2.98	GH3.5	IAA-amido synthase	Hormone Response
AT3G26290	247340_at	2.96	CYP71B26	cytochrome P450 71B26	Unknown
AT5G14500	246219_at	2.96		aldose 1-epimerase	Carbohydrate metabolism
AT1G49600	258744_at	2.96	ATRBP47A	RNA-binding protein 47 (RBP47)	Unknown
AT3G53420	248793_at	2.94	PIP2A	aquaporin PIP2.2 (PIP2.2)	Stress response
AT1G48000	253501_at	2.94	MYB112	myb-related transcription factor (cpm10)	Transcriptional regulation
AT1G74670	264783_at	2.93		gibberellin-responsive protein	Hormone Response
AT4G01970	253161_at	2.91		raffinose synthase	Unknown
AT2G07050	256070_at	2.88	CAS1	cycloartenol synthase (CAS1)	Secondary metabolism
AT1G60190	264662_at	2.85		armadillo/beta-catenin repeat family protein	Protein metabolism
AT2G43710	251428_at	2.83	SSI2	acyl-(acyl-carrier-protein) desaturase	Defense response

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT5G15780	256514_at	2.83		pollen Ole e 1 allergen and extensin	Unknown
AT1G75240	245745_at	2.81		zinc finger homeobox family protein	Transcriptional regulation
AT5G08380	266925_at	2.81		alpha-galactosidase	Carbohydrate metabolism
AT4G20260	250180_at	2.80		DREPP plasma membrane polypeptide	Stress response
AT2G15570	249093_at	2.79	ATHM3	thioredoxin M-type 3, chloroplast (TRX-M3)	Unknown
AT3G27190	249848_at	2.78		uracil phosphoribosyltransferase	Other Metabolism
AT2G40230	258823_at	2.77		transferase family protein	Unknown
AT5G05460	253873_at	2.76		glycosyl hydrolase	Unknown
AT5G26340	260956_at	2.76	MSS1	hexose transporter	Transport
AT3G13450	247313_at	2.75	DIN4	2-oxoisovalerate dehydrogenase /	Stress response
AT3G57780	247867_at	2.73		expressed protein	Unknown
AT5G09980	256710_at	2.73		expressed protein	Unknown
AT1G21400	249134_at	2.72		2-oxoisovalerate dehydrogenase	Carbohydrate metabolism
AT5G13360	253090_at	2.71		auxin-responsive GH3	Hormone Response
AT5G41070	257174_at	2.71		double-stranded RNA-binding protein	Unknown
AT1G29240	256744_at	2.71		expressed protein	Unknown
AT4G33010	252092_at	2.70		glycine dehydrogenase (decarboxylating)	Other Metabolism
AT1G08980	252859_at	2.69	ATAMI1	amidase	Hormone Response
AT5G52310	262988_at	2.69	COR78	low-temperature-responsive protein 78 (LTI78)	Stress response
AT1G04410	260194_at	2.69		malate dehydrogenase	Carbohydrate metabolism
AT3G01640	259162_at	2.68		GHMP kinase	Other Metabolism
AT1G53580	258920_at	2.66		glyoxalase II	Stress response
AT5G66170	267075_at	2.65		senescence-associated family protein	Development
AT5G26010	251412_at	2.65		protein phosphatase 2C	Protein metabolism
AT3G53280	261053_at	2.62	CYP71B5	cytochrome P450 71B5 (CYP71B5)	Other Metabolism
AT4G36380	245987_at	2.62	ROT3	cytochrome P450 90C1 (CYP90C1)	Cell organization and biogenesis
AT2G01940	249710_at	2.60		zinc finger protein ID1	Transcriptional regulation
AT1G49660	265584_at	2.60		expressed protein	Unknown
AT1G75170	264469_at	2.60		SEC14 cytosolic factor-related	Transport
AT1G73910	266860_at	2.59	ATARP4A	actin-related proteins	Unknown
AT1G49500	256053_at	2.58		expressed protein	Unknown
AT3G47430	250029_at	2.58		peroxisomal biogenesis factor 11	Cell organization and biogenesis
AT5G12200	247136_at	2.57		dihydropyrimidinase / hydantoinase (PYD2)	Unknown
AT2G41070	258210_at	2.56	EEL	basic leucine zipper transcription factor (BZIP12)	Transcriptional regulation

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT2G45740	259803_at	2.55		peroxisomal biogenesis factor 11	Cell organization and biogenesis
AT1G09570	250155_at	2.54	PHYA	phytochrome A (PHYA	Photosynthesis
AT5G16150	258359_s_at	2.53		hexose transporter	Transport
AT3G26240	251291_at	2.53		DC1 domain-containing protein	Unknown
AT2G02130	252831_at	2.53	LCR68	plant defensin-fusion protein (PDF2.3)	Defense response
AT1G76450	257824_at	2.53		oxygen-evolving complex-related	Unknown
AT1G78610	263491_at	2.52		mechanosensitive ion channel protein	Unknown
AT1G78080	246055_at	2.52	RAP2.4	AP2 transcription factor family (RAP2.4)	Transcriptional regulation
AT2G47730	252264_at	2.51	ATGSTF8	glutathione S-transferase 6 (GST6)	Stress response
AT1G01620	253585_at	2.51	PIP1C	plasma membrane intrinsic protein 1C (PIP1C)	Stress response
AT3G09910	262762_at	2.51		Ras-related GTP-binding protein	Signal transduction
AT5G43150	257702_at	2.50		expressed protein	Unknown
AT1G32450	267254_at	2.49		proton-dependent oligopeptide transport protein	Transport
AT1G08230	250937_at	2.49		amino acid transporter family protein	Transport
AT1G76180	267471_at	2.49	ERD14	dehydrin (ERD14)	Stress response
AT2G19800	259163_at	2.49	MIOX2	expressed protein	Unknown
AT3G06850	252033_at	2.48	DIN3	branched chain alpha-keto acid dehydrogenase	Stress response
AT5G10300	246205_at	2.47		hydrolase, alpha/beta fold family protein	Unknown
AT5G19440	253188_at	2.46		Eucalyptus gunnii alcohol dehydrogenase	Unknown
AT5G20950	247480_at	2.45		glycosyl hydrolase	Carbohydrate metabolism
AT3G14415	266882_at	2.45		(S)-2-hydroxy-acid oxidase	Other Metabolism
AT1G75190	256017_at	2.44		expressed protein	Unknown
AT5G55400	253042_at	2.44		fimbrin-like protein	Unknown
AT5G53580	248237_at	2.44		aldo/keto reductase family protein	Unknown
AT4G23750	260602_at	2.43		ERF/AP2 transcription factor	Transcriptional regulation
AT3G63350	258522_at	2.43	HSFA7B	heat shock transcription factor	Transcriptional regulation
AT5G60300	253984_at	2.42		lectin protein kinase	Protein metabolism
AT1G29980	245523_at	2.42		expressed protein	Unknown
AT2G46660	260378_at	2.42	CYP78A6	cytochrome p450 (CYP78A9)	Other Metabolism
AT3G60910	253908_at	2.41		expressed protein	Unknown
AT3G54050	260264_at	2.41	fbp	fructose-1,6-bisphosphatase	Carbohydrate metabolism
AT1G55850	265249_at	2.40	ATCSLE1	cellulose synthase family protein	Carbohydrate metabolism
AT5G53900	263382_at	2.39		expressed protein	Unknown
AT2G34710	246103_at	2.38	PHB	homeobox-leucine zipper transcription factor (HB-14)	Transcriptional regulation

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT3G54820	261603_at	2.34	PIP2D	aquaporin	Stress response
AT4G27180	251497_at	2.34	ATK2	kinesin-like protein B (KATB)	Cell organization and biogenesis
AT1G70410	264741_at	2.33		carbonic anhydrase	Unknown
AT2G34340	257499_at	2.32		expressed protein	Unknown
AT1G07440	259165_at	2.31		short-chain dehydrogenase/reductase (SDR)	Carbohydrate metabolism
AT3G15353	254089_at	2.31	MT3	metallothionein protein	Other Metabolism
AT4G15910	249442_at	2.31	ATDI21	drought-induced protein (Di21)	Stress response
AT5G14040	250527_at	2.31		mitochondrial phosphate transporter	Transport
AT5G55050	247401_at	2.30		GDSL-motif lipase/hydrolase	Lipid metabolism
AT4G23060	246990_at	2.30		calmodulin-binding family protein	Unknown
AT5G04380	267003_at	2.30		S-adenosyl-L-methionine:carboxyl methyltransferase	Unknown
AT1G33790	257940_at	2.30		jacalin lectin family protein	Unknown
AT3G08030	248994_at	2.30		expressed protein	Unknown
AT4G27250	260900_s_at	2.29		dihydroflavonol 4-reductase	Unknown
AT4G01460	258913_at	2.29		basic helix-loop-helix (bHLH) family protein	Transcriptional regulation
AT1G55920	260106_at	2.29	AtSerat2;1	serine O-acetyltransferase	Stress response
AT3G22200	258094_at	2.29	POP2	4-aminobutyrate aminotransferase	Development
AT5G05880	257205_at	2.29		UDP-glucoronosyl/UDP-glucosyl transferase	Unknown
AT2G02100	251890_at	2.28	LCR69	plant defensin-fusion protein (PDF2.2)	Defense response
AT1G10700	264204_at	2.28		ribose-phosphate pyrophosphokinase 3	Other Metabolism
AT5G04370	246428_at	2.28		S-adenosyl-L-methionine:carboxyl methyltransferase	Unknown
AT1G80960	259186_at	2.27		F-box protein-related	Unknown
AT2G36970	256456_at	2.27		UDP-glucoronosyl/UDP-glucosyl transferase	Other Metabolism
AT4G04040	264092_at	2.27		pyrophosphate-dependent 6-phosphofructose kinase	Carbohydrate metabolism
AT5G35790	249606_at	2.27	G6PD1	plastidic glucose-6-phosphate dehydrogenase	Carbohydrate metabolism
AT3G44460	256030_at	2.26	DPBF2	basic leucine zipper transcription factor (BZIP67)	Transcriptional regulation
AT3G16430	248027_at	2.26		jacalin lectin family protein	Unknown
AT5G62350	245939_at	2.25		invertase/pectin methylesterase inhibitor	Unknown
AT2G37760	258641_at	2.25		aldo/keto reductase	Unknown
AT4G22780	255602_at	2.25	ACR7	ACT domain-containing protein (ACR7)	Other Metabolism
AT5G24950	259434_at	2.25	CYP71A15	cytochrome P-450-like protein	Other Metabolism
AT1G60740	261228_at	2.24		peroxiredoxin type 2	Unknown
AT3G16520	248200_at	2.23		UDP-glucoronosyl/UDP-glucosyl transferase	Other Metabolism
AT4G32010	267181_at	2.23		transcriptional factor B3	Transcriptional regulation

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT5G07500	247421_at	2.23	PEI1	zinc finger (CCCH-type) family protein	Transcriptional regulation
AT5G55370	262307_at	2.23		long-chain-alcohol O-fatty-acyltransferase	Unknown
AT1G09950	258204_at	2.22		Transcription factor HBP-1b	Transcriptional regulation
AT5G39080	254073_at	2.22		transferase	Unknown
AT3G13960	256792_at	2.22	AtGRF5	transcription activator GRL5	Transcriptional regulation
AT5G04470	266560_at	2.22		expressed protein	Unknown
AT4G14780	255365_at	2.21		protein kinase ATMRK1	Protein metabolism
AT5G28640	261607_at	2.21	AN3	SSXT protein-related / glycine-rich protein	Transcriptional regulation
AT5G62150	252323_at	2.20		peptidoglycan-binding LysM protein	Unknown
AT1G31820	259981_at	2.20		amino acid permease family protein	Transport
AT1G75770	261195_at	2.20		expressed protein	Unknown
AT4G34030	251036_at	2.20	MCCB	putative protein	Other Metabolism
AT3G48530	245703_at	2.19		CBS domain-containing protein	Unknown
AT1G75180	259502_at	2.19		expressed protein	Unknown
AT1G62810	258026_at	2.19		copper amine oxidase	Unknown
AT4G24150	246042_at	2.19	AtGRF8	expressed protein	Transcriptional regulation
AT1G14340	264923_s_at	2.19		RNA recognition motif (RRM)-containing protein	Unknown
AT2G45190	260287_at	2.19	AFO	axial regulator YABBY1	Development
AT4G12970	249035_at	2.18		expressed protein	Unknown
AT1G58180	258599_at	2.18		carbonic anhydrase	Transport
AT4G17560	267316_at	2.18		ribosomal protein L19	Protein metabolism
AT2G34850	263350_at	2.18		NAD-dependent epimeras	Unknown
AT3G49680	246260_at	2.18		branched-chain amino acid aminotransferase 3	Other Metabolism
AT4G01026	263127_at	2.17		expressed protein	Unknown
AT5G01210	252471_at	2.17		transferase	Unknown
AT1G03220	247131_at	2.17		extracellular dermal glycoprotein EDGP precursor	Unknown
AT4G00430	254794_at	2.16	TMP C	plasma membrane intrinsic protein	Stress response
AT2G41250	264223_s_at	2.16		haloacid dehalogenase-like hydrolase	Other Metabolism
AT4G27540	262135_at	2.16		prenylated rab acceptor (PRA1)	Unknown
AT5G17410	249804_at	2.16		tubulin	Cell organization and biogenesis
AT1G72150	265476_at	2.16	PATL1	SEC14 cytosolic factor	Transport
AT1G73480	262159_at	2.16		hydrolase, alpha/beta fold family protein	Unknown
AT1G75100	266321_at	2.15		expressed protei	Cell organization and biogenesis
AT4G27840	245138_at	2.15		expressed protein	Unknown

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT3G02170	259210_at	2.15		expressed protein	Unknown
AT3G05180	249742_at	2.15		GDSL-motif lipase/hydrolase	Lipid metabolism
AT3G14690	246868_at	2.15	CYP72A15	cytochrome P450	Other Metabolism
AT4G19960	260592_at	2.14		potassium transporter	Transport
AT1G06240	247487_at	2.14		Protein of unknown function	Unknown
AT3G16500	266229_at	2.14	PAP1	auxin-responsive AUX/IAA family protein	Hormone Response
AT1G01320	257054_at	2.14		tetratricopeptide repeat (TPR)-containing protein	Unknown
AT4G02050	256936_at	2.13		glucose transporter	Transport
AT4G30720	265414_at	2.13		expressed protein	Other Metabolism
AT3G16570	262875_at	2.13	RALFL23	rapid alkalinization factor (RALF) family protein	Signal transduction
AT4G01610	254520_at	2.12		cathepsin B-like cysteine protease	Protein metabolism
AT1G70520	265344_at	2.12		protein kinase	Protein metabolism
AT5G63700	246507_at	2.12		zinc finger (C3HC4 type RING finger) family protein	Transcriptional regulation
AT1G79040	262945_at	2.12		photosystem II 10 kDa polypeptide	Photosynthesis
AT4G09020	252117_at	2.12	ISA3	isoamylase	Carbohydrate metabolism
AT1G01060	254860_at	2.11	LHY	LEH MYB transcription factor	Transcriptional regulation
AT5G16120	252387_at	2.11		hydrolase, alpha/beta fold family protein	Other Metabolism
AT3G11170	264191_at	2.11	FAD7	omega-3 fatty acid desaturase	Stress response
AT1G12820	245713_at	2.11	IPS1	transport inhibitor response protein	Unknown
AT3G15450	253732_at	2.11	SEN5	unknown protein	Unknown
AT2G20180	248435_at	2.11	PIL5	basic helix-loop-helix (bHLH) family protein	Transcriptional regulation
AT1G75800	257460_at	2.11		pathogenesis-related thaumatin	Stress response
AT3G63410	254203_at	2.10	APG1	chloroplast inner envelope membrane protein (APG1)	Unknown
AT4G36220	260060_at	2.10	FAH1	cytochrome P450 84A1 (CYP84A1)	Secondary metabolism
AT2G44670	264339_at	2.10		senescence-associated protein	Development
AT4G25500	246216_at	2.10	ATRSP35	arginine/serine-rich splicing factor RSP40 (RSP40)	Other Metabolism
AT5G53890	257734_at	2.10		leucine-rich repeat transmembrane protein kinase	Signal transduction
AT4G28140	250161_at	2.10		ERF/AP2 transcription factor family	Transcriptional regulation
AT5G62790	248817_at	2.09	DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase	Lipid metabolism
AT3G14880	256598_at	2.09		DNA-binding protein-related, TGA-2.1	Unknown
AT1G20050	263869_at	2.09	HYD1	C-8,7 sterol isomerase	Lipid metabolism
AT5G54160	258796_at	2.09	ATOMT1	5-hydroxyferulic acid O-methyltransferase (OMT1)	Secondary metabolism
AT5G23220	260884_at	2.09		isochorismatase hydrolase	Unknown
AT2G30970	260221_at	2.09	ASP1	aspartate aminotransferase	Other Metabolism

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT4G27830	247488_at	2.09		glycosyl hydrolase	Carbohydrate metabolism
AT1G52720	246184_at	2.08		expressed protein	Unknown
AT5G52440	248798_at	2.08	HCF106	HCF106 protein	Transport
AT1G30400	249336_at	2.07	ATMRP1	ATP-binding cassette transport protein	Transport
AT5G08590	254562_at	2.07	ASK2	serine/threonine protein kinase (ASK2)	Stress response
AT5G18310	256225_at	2.07		expressed protein	Unknown
AT5G61820	266119_at	2.07		expressed protein	Unknown
AT5G59150	246508_at	2.07		Ras-related GTP-binding protein	Signal transduction
AT3G60220	253841_at	2.05	ATL4	zinc finger (C3HC4-type RING finger) protein (ATL4)	Transcriptional regulation
AT5G37260	251758_at	2.05		myb family transcription factor	Transcriptional regulation
AT1G76670	261991_at	2.05		glucose-6-phosphate/phosphate-translocator	Transport
AT1G07280	265795_at	2.05		unknown protein	Unknown
AT4G37580	267461_at	2.05	HLS1	N-acetyltransferase	Hormone Response
AT1G05190	256577_at	2.05	EMB2394	ribosomal protein L6 family protein	Protein metabolism
AT5G45250	265672_at	2.04	RPS4	disease resistance protein (TIR-NBS-LRR class)	Defense response
AT4G14930	261421_at	2.04		acid phosphatase survival protein SurE	Unknown
AT1G69040	259935_at	2.04	ACR4	ACT domain containing protein (ACR4)	Other Metabolism
AT2G22000	259431_at	2.04		expressed protein	Unknown
AT5G58730	262667_at	2.04		pfkB-type carbohydrate kinase	Unknown
AT5G02120	260028_at	2.03	OHP	thylakoid membrane one helix protein (OHP)	Stress response
AT4G01550	256305_at	2.03	ANAC069	no apical meristem (NAM) family protein	Transcriptional regulation
AT3G61900	260833_at	2.03		auxin-responsive family protein	Hormone Response
AT1G71140	247450_at	2.03		ripening regulated protein DDTFR18	Transport
AT4G29060	246437_at	2.03	EMB2726	translation elongation factor Ts (EF-Ts)	Protein metabolism
AT1G09960	259173_at	2.03	SUT4	sucrose-proton symporter (SUT4)	Transport
AT1G52310	250680_at	2.03		protein kinase	Protein metabolism
AT3G01590	253610_at	2.03		aldose 1-epimerase	Carbohydrate metabolism
AT1G59650	250649_at	2.03	CW14	expressed protein	Protein metabolism
AT3G10520	254140_at	2.02	AHB2	non-symbiotic hemoglobin 2 (HB2) (GLB2)	Stress response
AT1G34110	257516_at	2.02		leucine-rich repeat transmembrane protein kinase	Signal transduction
AT1G31230	253799_at	2.02		bifunctional aspartate kinase	Other Metabolism
AT4G24840	251858_at	2.02		expressed protein	Unknown
AT5G03940	260303_at	2.01	FFC	54 chloroplast protein / SRP54 (FFC)	Transport
AT3G04520	253088_at	2.01		threonine aldolase	Unknown

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT3G62860	263001_at	2.00		esterase/lipase/thioesterase	Other Metabolism
AT5G60400	262137_at	2.00		expressed protein	Unknown
AT3G54990	256589_at	2.00	SMZ	APETALA2 - like protein	Transcriptional regulation
AT1G62290	266604_at	2.00		aspartyl protease	Protein metabolism
AT2G01540	252003_at	1.99		zinc finger and C2 domain protein	Transcriptional regulation
AT5G02160	259734_at	1.99		expressed protein	Unknown
AT1G12780	253609_at	1.99		Encodes a UDP-glucose epimerase	Carbohydrate metabolism
AT3G29350	264728_at	1.99	AHP2	two-component phosphorelay mediator 1 (HP1)	Signal transduction
AT1G78390	252367_at	1.98	nced9	9-cis-epoxycarotenoid dioxygenase	Hormone Response
AT2G40540	258998_at	1.98	KT2	potassium transporter (KT2)	Transport
AT5G15160	261211_at	1.98		bHLH family protein	Transcriptional regulation
AT3G19290	245602_at	1.98	ABF4	ABA-responsive element-binding protein 2 (AREB2)	Transcriptional regulation
AT3G47650	263112_at	1.98		bundle-sheath defective protein 2	Unknown
AT3G63040	264656_at	1.98		expressed protein	Unknown
AT1G51140	263663_at	1.97		basic helix-loop-helix (bHLH) family protein	Transcriptional regulation
AT4G24800	249493_at	1.97		MA3 domain-containing protein	Unknown
AT3G18370	259670_at	1.97		C2 domain-containing protein	Unknown
AT1G67100	257476_at	1.97		lateral organ boundaries domain protein 40	Unknown
AT5G22830	250746_at	1.96	GMN10	magnesium transporter CorA-like family protein	Transport
AT2G13360	254235_at	1.96	AGT	serine-glyoxylate aminotransferase	Photosynthesis
AT1G27130	251166_at	1.96	ATGSTU13	glutathione S-transferase	Stress response
AT5G24490	247819_at	1.95		30S ribosomal protein	Protein metabolism
AT2G29380	259888_at	1.95		protein phosphatase 2C	Unknown
AT1G07260	258113_at	1.94		UDP-glucoronosyl/UDP-glucosyl transferase	Carbohydrate metabolism
AT4G27490	263711_at	1.94		3' exoribonuclease	Other Metabolism
AT4G35300	251324_at	1.94		hexose transporter	Transport
AT2G46030	251391_at	1.94	UBC6	ubiquitin-conjugating enzyme 6 (UBC6)	Protein metabolism
AT3G52770	257244_at	1.94		expressed protein	Unknown
AT1G69220	252081_at	1.94	SIK1	serine/threonine protein kinase	Protein metabolism
AT4G03110	250141_at	1.93		RNA-binding protein	Unknown
AT3G44300	260986_at	1.93	NIT2	nitrilase 2 (NIT2)	Hormone Response
AT3G23570	263847_at	1.93		dienelactone hydrolase	Other Metabolism
AT1G60440	261055_at	1.93		eukaryotic pantothenate kinase	Other Metabolism
AT5G15240	255590_at	1.92		amino acid transporter	Transport

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT5G51820	251885_at	1.92	PGM	glucose phosphomutase	Carbohydrate metabolism
AT1G77920	253926_at	1.92		bZIP family transcription factor	Transcriptional regulation
AT1G22850	249988_at	1.92		expressed protein	Unknown
AT5G58350	257624_at	1.91	WNK4	protein kinase	Protein metabolism
AT1G68010	264313_at	1.91	HPR	glycerate dehydrogenase	Photosynthesis
AT5G46180	250583_at	1.90	delta OAT	ornithine aminotransferase	Stress response
AT1G35560	245734_at	1.90		TCP family transcription factor	Transcriptional regulation
AT2G29400	260797_at	1.90	TOPP1	serine/threonine protein phosphatase PP1	Protein metabolism
AT5G55120	261075_at	1.89		expressed protein	Stress response
AT2G33830	253867_at	1.89		dormancy/auxin associated family protein	Hormone Response
AT5G56260	252996_s_at	1.89		dimethylmenaquinone methyltransferase	Unknown
AT1G04920	254293_at	1.89		sucrose-phosphate synthase	Other Metabolism
AT3G09150	245199_at	1.89	HY2	phytochromobilin:ferredoxin oxidoreductase	Signal transduction
AT4G29140	263688_at	1.89		MATE efflux protein	Transport
AT2G17820	261169_at	1.89	ATHK1	histidine kinase 1	Stress response
AT3G55770	257830_at	1.89	atl2	LIM domain-containing protein	Unknown
AT1G79510	253836_at	1.88		expressed protein	Unknown
AT2G35780	246519_at	1.88	SCPL26	serine carboxypeptidase	Protein metabolism
AT4G30200	246831_at	1.88		expressed protein	Unknown
AT3G07200	256452_at	1.87		Zinc finger, C3HC4 type (RING finger)	Transcriptional regulation
AT4G26650	252645_at	1.87		RNA recognition motif (RRM)-containing protein	Unknown
AT3G50410	256754_at	1.87	OBP1	Dof-type zinc finger domain-containing protein	Transcriptional regulation
AT1G56220	250884_at	1.87		dormancy/auxin associated family protein	Hormone Response
AT5G67360	258652_at	1.87	ARA12	cucumisin-like serine protease (ARA12)	Protein metabolism
AT3G14220	264653_at	1.87		GDSL-motif lipase/hydrolase	Lipid metabolism
AT5G10460	248335_at	1.87		haloacid dehalogenase-like hydrolase	Other Metabolism
AT3G51950	259618_at	1.87		RNA recognition motif (RRM)-containing protein	Unknown
AT1G33055	245724_at	1.87		expressed protein	Unknown
AT5G02020	251962_at	1.87		expressed protein	Unknown
AT5G60450	255579_at	1.86	ARF4	auxin-responsive factor (ARF4)	Hormone Response
AT5G66190	262001_at	1.86		ferredoxin-NADP(+) reductase	Other Metabolism
AT1G70530	258432_at	1.86		protein kinase	Protein metabolism
AT3G48000	266915_at	1.86	ALDH2B4	aldehyde dehydrogenase (ALDH2)	Other Metabolism
AT2G39730	255411_at	1.86	RCA	ribulose bisphosphate carboxylase	Photosynthesis

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT3G14067	248560_at	1.86		subtilase	Protein metabolism
AT2G30390	252409_at	1.86		ferrochelatase II	Secondary metabolism
AT3G49590	245555_at	1.86		expressed protein	Unknown
AT3G06450	254409_at	1.86		anion exchange family protein,	Transport
AT1G74920	266693_at	1.86	ALDH10A8	betaine-aldehyde dehydrogenase	Stress response
AT2G44120	260362_at	1.86		60S ribosomal protein L7 (RPL7C)	Protein metabolism
AT5G63980	256139_at	1.86	SAL1	inositol polyphosphate 1-phosphatase	Stress response
AT1G70300	260693_at	1.86	KUP6	potassium transporter HAK2p	Transport
AT4G36970	246842_at	1.85		remorin family protein	Unknown
AT4G34190	260367_at	1.85		stress enhanced protein 1 (SEP1)	Stress response
AT3G22480	256263_at	1.85		prefoldin-related KE2 family protein	Unknown
AT4G36760	259927_at	1.84	ATAPP1	aminopeptidase P	Transport
AT2G01140	248756_at	1.84		fructose-bisphosphate aldolase	Stress response
AT3G25290	261864_s_at	1.84		auxin-responsive protein	Hormone Response
AT1G08650	257072_at	1.84	PPCK1	phosphoenolpyruvate carboxylase kinase	Protein metabolism
AT5G55630	257177_at	1.84	KCO1	outward rectifying potassium channel (KCO1)	Transport
AT2G46690	247626_at	1.84		auxin-responsive family protein	Hormone Response
AT1G19180	267213_at	1.84		expressed protein	Unknown
AT3G15095	257626_s_at	1.83		expressed protein	Unknown
AT3G14650	263696_at	1.83	CYP72A11	cytochrome P450	Other Metabolism
AT3G52920	259586_at	1.83		expressed protein	Unknown
AT5G12260	264217_at	1.83		expressed protein	Unknown
AT3G06660	252372_at	1.82		zinc finger (HIT type) family protein	Transcriptional regulation
AT5G47190	250186_at	1.82		ribosomal protein L19	Protein metabolism
AT5G61060	256965_at	1.82		histone deacetylase	Protein metabolism
AT5G51210	263123_at	1.82		glycine-rich protein / oleosin	Lipid metabolism
AT1G01300	267553_s_at	1.82		aspartyl protease family protein	Protein metabolism
AT3G54220	267351_at	1.82	SCR	scarecrow transcription factor	Transcriptional regulation
AT3G27820	251971_at	1.81		monodehydroascorbate reductase	Other Metabolism
AT4G24610	261567_at	1.81		expressed protein	Unknown
AT1G77500	267151_at	1.81		expressed protein	Protein metabolism
AT1G73390	267511_at	1.81		expressed protein	Protein metabolism
AT5G18200	255451_at	1.81		expressed protein	Carbohydrate metabolism
AT1G54360	265188_at	1.81		TATA box-binding protein (TAF)	Transcriptional regulation

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT2G40040	257227_at	1.80		unknown protein	Unknown
AT3G12630	251811_at	1.80		zinc finger (AN1-like) family protein	Transcriptional regulation
AT4G21400	255900_at	1.80		protein kinase	Protein metabolism
AT1G66130	266327_at	1.80		oxidoreductase	Carbohydrate metabolism
AT2G32650	266238_at	1.80		expressed protein	Unknown
AT5G62390	266322_at	1.80		calmodulin-binding family protein	Unknown
AT5G51160	266000_at	1.79		ankyrin repeat family protein	Unknown
AT1G09010	261177_at	1.79		glycoside hydrolase	Carbohydrate metabolism
AT4G02860	253272_at	1.79		phenazine biosynthesis PhzC/PhzF family protein	Other Metabolism
AT1G35420	252023_at	1.79		dienelactone hydrolase family protein	Unknown
AT2G22660	254087_at	1.79		glycine-rich protein	Unknown
AT5G49970	248242_at	1.79		pyridoxamine 5'-phosphate oxidase	Other Metabolism
AT1G21880	261118_at	1.79		peptidoglycan-binding protein	Other Metabolism
AT3G01490	253054_at	1.78		protein kinase	Protein metabolism
AT3G03640	257234_at	1.78	GLUC	glycosyl hydrolase	Carbohydrate metabolism
AT2G26870	246972_s_at	1.78		phosphoesterase	Lipid metabolism
AT4G39200	254300_at	1.78		40S ribosomal protein S25 (RPS25E)	Protein metabolism
AT1G54730	262173_at	1.78		ERD6 protein	Stress response
AT1G18390	260790_at	1.78		protein kinase family protein	Protein metabolism
AT3G30180	251235_at	1.78	CYP85A2	cytochrome p450 enzyme	Other Metabolism
AT4G15390	264508_at	1.77		transferase	Unknown
AT3G05340	256300_at	1.77		pentatricopeptide (PPR) repeat-containing protein	Unknown
AT4G35560	250844_at	1.77		expressed protein	Unknown
AT5G37740	248541_at	1.77		C2 domain-containing protein	Unknown
AT1G07380	246396_at	1.76		ceramidase family protein	Unknown
AT5G19760	253387_at	1.76		dicarboxylate/tricarboxylate carrier (DTC)	Transport
AT1G70290	248337_at	1.76	ATTPS8	trehalose-6-phosphate synthase	Carbohydrate metabolism
AT1G79520	248082_at	1.76		cation efflux family protein,	Transport
AT1G18840	247643_at	1.76		calmodulin-binding family protein	Unknown
AT5G44190	258527_at	1.76	GLK2	myb family transcription factor (GLK2)	Transcriptional regulation
AT1G48300	245577_at	1.76		expressed protein	Unknown
AT2G04100	261487_at	1.76		ripening regulated protein DDTFR18	Transport
AT1G63850	248380_at	1.75		PRLI-interacting factor G	Unknown
AT5G03230	250468_at	1.75		expressed protein	Unknown

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT3G05830	266342_at	1.75		alpha-helical IF (intermediate filament)-like protein	Unknown
AT2G45670	258108_at	1.75		calcineurin B subunit	Unknown
AT5G62130	260889_at	1.75		Per1-like protein	Unknown
AT1G50480	254492_at	1.75	THFS	formate-tetrahydrofolate ligase	Other Metabolism
AT1G30360	267429_at	1.74		early-responsive to dehydration stress (ERD4)	Stress response
AT1G22570	256997_at	1.74		proton-dependent oligopeptide transport protein	Transport
AT3G13920	247558_at	1.74	EIF4A1	eukaryotic translation initiation factor 4A	Transcriptional regulation
AT1G69760	266461_at	1.74		expressed protein	Unknown
AT5G43880	247731_at	1.74		expressed protein	Unknown
AT1G29130	261071_at	1.74		hypothetical protein	Unknown
AT3G51430	253401_at	1.74	YLS2	strictosidine synthase	Secondary metabolism
AT1G75460	261569_at	1.74		ATP-dependent protease La (LON)	Protein metabolism
AT3G46820	264928_at	1.73	TOPP5	serine/threonine protein phosphatase PP1	Unknown
AT4G39980	253907_at	1.73	DHS1	2-dehydro-3-deoxyphosphoheptonate aldolase 1	Stress response
AT1G80310	256451_s_at	1.73		expressed protein	Unknown
AT2G27500	251039_at	1.73		glycosyl hydrolase	Carbohydrate metabolism
AT1G75450	264831_at	1.73	CKX6	FAD-binding domain-containing protein	Hormone Response
AT1G13730	250312_at	1.73		nuclear transport factor 2 (NTF2) family protein	Transport
AT5G39590	265480_at	1.73		expressed protein	Protein metabolism
AT2G45870	260857_at	1.73		expressed protein	Unknown
AT1G76350	249694_at	1.73		RWP-RK domain-containing protein	Transcriptional regulation
AT3G21790	245357_at	1.73		UDP-glucoronosyl/UDP-glucosyl transferase	Other Metabolism
AT3G22150	253279_at	1.73		pentatricopeptide (PPR) repeat-containing protein	Unknown
AT2G28840	245061_at	1.72		ankyrin repeat family protein	Unknown
AT1G28100	248464_at	1.72		expressed protein	Unknown
AT1G05160	261749_at	1.72	CYP88A3	ent-kaurenoic acid hydroxylase (KAO1)	Hormone Response
AT1G67730	260014_at	1.72	glossy8	b-keto acyl reductase (GLOSSY8)	Development
AT1G67530	259743_at	1.72		armadillo/beta-catenin repeat family protein	Protein metabolism
AT3G26690	250206_at	1.72		Diadenosine 5',5'''-P1,P6-hexaphosphate hydrolase	Unknown
AT1G06800	256455_at	1.72		lipase class 3 family protein	Lipid metabolism
AT3G12670	262488_at	1.72	EMB2742	CTP synthase	Embryonic Development
AT3G12290	259104_at	1.71		tetrahydrofolate dehydrogenase/cyclohydrolase	Other Metabolism
AT3G14230	250455_at	1.71	RAP2.2	ERF/AP2 transcription factor family (RAP2.2)	Transcriptional regulation
AT1G04770	262399_at	1.71		male sterility MS5 family protein	Unknown

Table A-1. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT4G14270	253903_at	1.71		dehydration-induced protein (ERD15)	Stress response
AT3G23490	252274_at	1.71	CYN	cyanate lyase	Unknown
AT1G67520	248114_at	1.71		lectin protein kinase	Protein metabolism
AT1G60710	260288_at	1.71	ATB2	aldo/keto reductase	Unknown
AT1G77530	248007_at	1.71		O-methyltransferase	Secondary metabolism
AT4G30190	256310_at	1.70	AHA2	ATPase 2	Transport

Table A-2. Down-regulated genes in *abi5-6* seeds

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT4G36620	246274_at	0.04		zinc finger (GATA type) family protein	Transcriptional regulation
AT1G27030	264987_at	0.04		expressed protein	Unknown
AT4G22753	254333_at	0.04	SMO1 3	sterol desaturase	Other metabolism
AT4G09610	255049_at	0.05	GASA2	gibberellin-regulated protein 2 (GASA2)	Hormone response
AT4G35010	253226_at	0.05	BGAL11	glycosyl hydrolase	Carbohydrate metabolism
AT2G28780	266222_at	0.07		expressed protein	Unknown
AT1G48750	256145_at	0.09		protease inhibitor/seed storage/lipid transfer protein	Transport
AT1G74710	262177_at	0.09	ICS1	isochorismate synthase 1 (ICS1)	Defense response
AT1G02790	262122_at	0.09	PGA4	galacturan 1,4-alpha-galacturonidase (PGA3)	Carbohydrate metabolism
AT5G53990	248209_at	0.10		glycosyltransferase family protein	Other metabolism
AT3G10570	258962_at	0.10	CYP77A6	cytochrome P450	Other metabolism
AT5G67080	247026_at	0.11	MAPKKK19	protein kinase family protein	Protein metabolism
AT3G01240	259266_at	0.11		expressed protein	Unknown
AT5G38780	249532_at	0.12		S-adenosyl-L-methionine:carboxyl methyltransferase	Unknown
AT4G19170	254564_at	0.12	NCED4	9-cis-epoxycarotenoid dioxygenase	Unknown
AT2G47040	266750_s_at	0.13	VGD1	pectinesterase	Development
AT4G29020	253754_at	0.13		glycine-rich protein	Unknown
AT2G34740	267313_at	0.13		protein phosphatase 2C	Unknown
AT1G62840	257466_at	0.13		expressed protein	Unknown
AT5G66985	247024_at	0.14		expressed protein	Unknown
AT3G07820	258639_at	0.15	PGA5	polygalacturonase 3 (PGA3)	Carbohydrate metabolism
AT1G61566	265007_s_at	0.16	RALFL9	rapid alkalinization factor (RALF)	Other metabolism
AT5G07410	250606_s_at	0.17		pectinesterase	Cell organization and biogenesis
AT5G53660	248213_at	0.17	AtGRF7	growth regulating factor	Transcriptional regulation
AT3G26330	256875_at	0.18	CYP71B37	cytochrome P450	Other metabolism
AT4G22100	254339_at	0.18		glycosyl hydrolase	Carbohydrate metabolism
AT1G76690	259875_s_at	0.18	OPR2	12-oxophytodienoate reductase (OPR2)	Hormone response
AT2G38900	266169_at	0.19		serine protease inhibitor	Stress response
AT1G73010	262369_at	0.19		phosphatase, orphan 1	Other metabolism
AT4G20820	254430_at	0.19		FAD-binding domain-containing protein	Other metabolism
AT1G55570	265080_at	0.20	SKS12	multi-copper oxidase	Unknown
AT2G38540	266421_at	0.20	LP1	nonspecific lipid transfer protein 1 (LTP1)	Transport
AT4G02250	255515_at	0.21		invertase/pectin methylesterase inhibitor	Unknown
AT3G29670	257288_at	0.22		transferase	Unknown

Table A-2. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT1G02400	259445_at	0.22		gibberellin 2-oxidase	Hormone response
AT2G41730	260522_x_at	0.22		expressed protein	Unknown
AT3G14330	258355_at	0.23		pentatricopeptide (PPR) repeat-containing protein	Unknown
AT5G02580	251012_at	0.23		expressed protein	Unknown
AT3G45970	252563_at	0.23	ATEXLA1	expansin family protein (EXPL1)	Cell organization and biogenesis
AT2G46270	266555_at	0.24	GBF3	G-box binding factor 3 (GBF3)	Transcriptional regulation
AT1G62620	265108_s_at	0.26		flavin-containing monooxygenase	Other metabolism
AT1G10770	262760_at	0.26		invertase/pectin methylesterase inhibitor	Unknown
AT2G47670	266488_at	0.26		invertase/pectin methylesterase inhibitor	Unknown
AT1G55330	259664_at	0.26	AGP21	arabinogalactan-protein (AGP21)	Unknown
AT1G20823	256093_at	0.27		zinc finger (C3HC4-type RING finger) family protein	Transcriptional regulation
AT3G49300	252253_at	0.28		proline-rich family protein	Unknown
AT3G62100	251246_at	0.28	IAA30	auxin-responsive protein	Hormone response
AT1G54130	263159_at	0.29	RSH3	RelA/SpoT protein (RSH3)	Other metabolism
AT4G22620	254323_at	0.29		auxin-induced protein 10A	Hormone response
AT1G05150	264568_at	0.29		calcium-binding EF hand family protein	Unknown
AT4G11280	254926_at	0.29	ACS6	1-aminocyclopropane-1-carboxylate synthase 6	Hormone response
AT3G63170	251151_at	0.29		expressed protein	Unknown
AT5G45630	248959_at	0.30		expressed protein	Unknown
AT4G39130	252914_at	0.30		dehydrin family protein	Stress response
AT3G28750	256584_at	0.31		expressed protein	Unknown
AT2G01520	266353_at	0.31		major latex protein	Unknown
AT1G11440	261871_at	0.31		expressed protein	Unknown
AT1G31320	257467_at	0.32		lateral organ boundaries domain protein 4 (LBD4)	Unknown
AT3G01270	259269_at	0.32		pectate lyase family protein	Unknown
AT2G25625	265913_at	0.33		expressed protein	Unknown
AT1G20030	261248_at	0.33		pathogenesis-related thaumatin family protein	Defense response
AT2G32190	265674_at	0.33		expressed protein	Unknown
AT2G42000	267579_at	0.33		plant EC metallothionein	Unknown
AT1G51680	256186_at	0.33	4CL1	4-coumarate--CoA ligase 1	Stress response
AT3G21380	256815_at	0.33		jacalin lectin	Unknown
AT3G49160	252300_at	0.34		pyruvate kinase family protein	Carbohydrate metabolism
AT2G45040	266126_at	0.34		matrix metalloproteinase	Protein metabolism
AT5G50360	248505_at	0.34		expressed protein	Unknown

Table A-2. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT4G28460	253796_at	0.34		expressed protein	Unknown
AT1G78780	264301_at	0.34		pathogenesis	Defense response
AT3G60290	251402_at	0.34		oxidoreductase, 2OG-Fe(II) oxygenase	Other metabolism
AT5G58650	247793_at	0.35		expressed protein	Unknown
AT3G15250	257049_at	0.35		protein kinase	Unknown
AT1G21790	262496_at	0.35		expressed protein	Unknown
AT4G17500	245252_at	0.35	ATERF 1	ERF/AP2 transcription factor family (ATERF-1)	Transcriptional regulation
AT3G61040	251350_at	0.36	CYP76C7	cytochrome P450 family protein	Other metabolism
AT5G58160	247861_at	0.36		FH2 domain-containing protein	Cell organization and biogenesis
AT1G19020	259479_at	0.36		expressed protein	Unknown
AT1G30135	256159_at	0.36		expressed protein	Unknown
AT3G03320	259053_at	0.36		expressed protein	Unknown
AT4G18920	254607_at	0.36		expressed protein	Unknown
AT1G72200	259854_at	0.36		zinc finger (C3HC4-type RING finger) family protein	Transcriptional regulation
AT4G13510	254723_at	0.36	AMT1	ammonium transporter 1	Transport
AT4G27940	253824_at	0.36		mitochondrial substrate carrier family protein	Transport
AT5G63790	247351_at	0.37	ANAC102	no apical meristem (NAM)	Transcriptional regulation
AT2G32210	265670_s_at	0.37		expressed protein	Unknown
AT5G10740	250400_at	0.37		protein phosphatase 2C	Protein metabolism
AT1G01480	259439_at	0.37	ACS2	1-aminocyclopropane-1-carboxylate synthase 2	Hormone response
AT3G02040	258856_at	0.37	SRG3	glycerophosphoryl diester phosphodiesterase	Other metabolism
AT1G10970	260462_at	0.38	ZIP4	metal transporter (ZIP4)	Transport
AT3G62690	251190_at	0.38	ATL5	zinc finger (C3HC4-type RING finger) family protein	Transcriptional regulation
AT4G28240	253812_at	0.38		wound-responsive protein	Unknown
AT4G40070	252834_at	0.38		C3HC4-type RING finger protein (ATL6)	Transcriptional regulation
AT5G05250	250828_at	0.38		expressed protein	Unknown
AT4G26740	253930_at	0.38	ATS1	embryo-specific protein 1 (ATS1)	Embryonic development
AT1G23050	264889_at	0.39		hydroxyproline-rich glycoprotein	Unknown
AT1G19440	260667_at	0.39		very-long-chain fatty acid condensing enzyme	Fatty acid metabolism
AT3G62820	251181_at	0.39		invertase/pectin methylesterase inhibitor	Unknown
AT5G07020	250668_at	0.39		proline-rich family protein	Unknown
AT1G62180	264745_at	0.39	PRH	5'-adenylylsulfate reductase 2	Other metabolism
AT4G39100	252925_at	0.39	SHL1	PHD finger family protein	Transcriptional regulation
AT4G02940	255462_at	0.39		oxidoreductase, 2OG-Fe(II) oxygenase	Unknown

Table A-2. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT5G59220	247723_at	0.40		protein phosphatase 2C	Stress response
AT5G60760	247582_at	0.40		2-phosphoglycerate kinase	Unknown
AT1G28640	262734_at	0.40		GDSL-motif lipase	Lipid metabolism
AT2G45600	267503_at	0.40		expressed protein	Unknown
AT4G31590	253533_at	0.40	ATCSLC05	glycosyl transferase	Unknown
AT5G25610	246908_at	0.40	RD22	dehydration-responsive protein (RD22)	Stress response
AT1G05710	263179_at	0.41		bHLH transcription factor	Transcriptional regulation
AT5G44410	249047_at	0.41		FAD-binding domain-containing protein	Other metabolism
AT3G27060	257809_at	0.41		ribonucleoside-diphosphate reductase	Cell organization and biogenesis
AT3G27360	257714_at	0.41		histone H3	Cell organization and biogenesis
AT3G57690	251590_at	0.41		arabinogalactan-protein (AGP23)	Unknown
AT3G27540	257999_at	0.41		glycosyl transferase	Protein metabolism
AT2G27310	265620_at	0.41		F-box family protein	Unknown
AT5G57480	247912_at	0.41		AAA-type ATPase	Unknown
AT2G29550	266295_at	0.41	TUB7	tubulin beta-7 chain (TUB7)	Cell organization and biogenesis
AT5G11970	250301_at	0.42		expressed protein	Unknown
AT1G11700	262811_at	0.42		expressed protein	Unknown
AT1G53790	262255_at	0.42		F-box family protein	Unknown
AT1G03990	265099_at	0.42		alcohol oxidase-related	Unknown
AT1G62610	265107_s_at	0.42		short-chain dehydrogenase/reductase (SDR)	Other metabolism
AT3G25600	256755_at	0.42		calmodulin	Unknown
AT1G22400	261934_at	0.42	UGT85A1	UDP-glucoronosyl/UDP-glucosyl transferase	Other metabolism
AT2G28940	266781_at	0.42		protein kinase	Protein metabolism
AT1G72430	260427_at	0.42		auxin-responsive protein	Hormone response
AT5G20790	246001_at	0.42		expressed protein	Unknown
AT3G25830	256994_s_at	0.43	ATTPS CIN	monoterpene 1,8-cineole synthase	Lipid metabolism
AT1G62420	260635_at	0.43		expressed protein	Unknown
AT5G07070	250673_at	0.43	CIPK2	CBL-interacting protein kinase 2 (CIPK2)	Signal transduction
AT5G58720	247790_at	0.43		PRLI-interacting factor	Cell organization and biogenesis
AT1G67070	255881_at	0.43	DIN9	phosphomannose isomerase, putative (DIN9)	Stress response
AT3G03310	259057_at	0.43		lecithin:cholesterol acyltransferase	Lipid metabolism
AT4G11310	254915_s_at	0.43	RD21	cysteine proteinase	Protein metabolism
AT1G04000	265039_at	0.43		expressed protein	Unknown
AT1G12130	260994_at	0.44		flavin-containing monooxygenase	Other metabolism

Table A-2. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT5G62480	247435_at	0.44	ATGSTU9	glutathione S-transferase	Stress response
AT5G18450	250010_at	0.44		ERF/AP2 transcription factor	Transcriptional regulation
AT1G08170	261755_at	0.44		histone H2B family protein	Cell organization and biogenesis
AT5G50315	248510_at	0.44			Unknown
AT1G77450	259705_at	0.44	ANAC032	no apical meristem (NAM) family protein	Transcriptional regulation
AT2G27940	257429_at	0.44		zinc finger (C3HC4-type RING finger) family protein	Transcriptional regulation
AT5G42960	249182_at	0.44		expressed protein	Unknown
AT1G76070	261748_at	0.44		expressed protein	Unknown
AT1G23430	263015_at	0.45			Unknown
AT3G46620	252474_at	0.45		zinc finger (C3HC4-type RING finger) family protein	Transcriptional regulation
AT1G23070	264888_at	0.45		expressed protein	Unknown
AT1G73020	262379_at	0.45		unknown function	Unknown
AT1G18010	255908_s_at	0.46		expressed protein	Unknown
AT4G09600	255048_at	0.46	GASA3	gibberellin-regulated protein 3 (GASA3)	Hormone response
AT4G11370	254922_at	0.46	RHA1A	zinc finger (C3HC4-type RING finger) family protein	Transcriptional regulation
AT1G14960	262838_at	0.46		major latex protein-related	Unknown
AT3G21400	256816_at	0.46		expressed protein	Protein metabolism
AT5G07360	250627_at	0.46		amidase family protein	Other metabolism
AT5G44670	249011_at	0.46		expressed protein	Unknown
AT5G62220	247470_at	0.47		exostosin family protein	Unknown
AT1G15740	259500_at	0.47		leucine-rich repeat family protein	Unknown
AT1G20870	262854_at	0.47		expressed protein	Unknown
AT2G29300	266278_at	0.47		tropinone reductase	Other metabolism
AT2G28790	266223_at	0.47		osmotin-like protein	Defense response
AT5G48230	248690_at	0.47	EMB1276	acetyl-CoA C-acyltransferase	Embryonic development
AT1G22370	255943_at	0.47		UDP-glucoronosyl/UDP-glucosyl transferase	Other metabolism
AT3G48510	252321_at	0.47		expressed protein	Unknown
AT3G28920	256622_at	0.48		ZF-HD homeobox family protein	Transcriptional regulation
AT2G41230	266364_at	0.48		expressed protein	Unknown
AT1G11710	262810_at	0.48		pentatricopeptide (PPR) repeat-containing protein	Unknown
AT1G02660	260915_at	0.48		lipase class 3 family protein	Lipid metabolism
AT5G23460	249838_at	0.48		expressed protein	Unknown
AT5G10695	246018_at	0.48		expressed protein	Unknown
AT1G16730	255763_at	0.48		expressed protein	Unknown

Table A-2. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT5G65280	247175_at	0.48		lanthionine synthetase C	Unknown
AT5G13460	250233_at	0.48		calmodulin-binding family protein	Unknown
AT5G25180	246925_at	0.48	CYP71B14	cytochrome P450 71B14	Other metabolism
AT3G50060	252193_at	0.48	AtMYB77	myb family transcription factor	Transcriptional regulation
AT5G14380	250174_at	0.48	agp6	hydroxyproline-rich glycoprotein	Unknown
AT4G21620	254372_at	0.49		glycine-rich protein	Unknown
AT1G01360	261056_at	0.49		expressed protein	Unknown
AT4G01120	255625_at	0.49	GBF2	G-box binding factor 2 (GBF2)	Transcriptional regulation
AT4G29950	253647_at	0.49		microtubule-associated protein	Unknown
AT1G30110	256192_at	0.49		diadenosine 5',5'''-P1,P4-tetraphosphate hydrolase	Unknown
AT1G78580	263136_at	0.49	ATTPS1	alpha, alpha-trehalose-phosphate synthase	Cell organization and biogenesis
AT2G25340	263587_at	0.49	ATVAMP712	synaptobrevin family protein	Transport
AT3G24090	256911_at	0.49		glucosamine-fructose-6-phosphate aminotransferase	Other metabolism
AT1G07180	256057_at	0.50	ATNDI1	Internal NAD(P)H dehydrogenase	Other metabolism
AT3G56880	246289_at	0.50		VQ motif-containing protein	Unknown
AT5G66440	247049_at	0.50		expressed protein	Unknown
AT5G37480	249625_at	0.50		expressed protein	Unknown
AT4G22530	254318_at	0.50		embryo-abundant protein-related	Embryonic development
AT2G43780	260605_at	0.50		expressed protein	Unknown
AT5G13750	250252_at	0.51		sugar transporter	Transport
AT5G04590	250846_at	0.51	SIR	sulfite reductase / ferredoxin (SIR)	Other metabolism
AT3G49530	252278_at	0.51	ANAC062	no apical meristem (NAM) family protein	Transcriptional regulation
AT2G19580	265935_at	0.51		senescence-associated protein	Development
AT5G01520	251084_at	0.51		zinc finger (C3HC4-type RING finger) protein	Transcriptional regulation
AT4G31270	253543_at	0.51		gt-2-related	Transcriptional regulation
AT3G16720	258436_at	0.51	ATL2	zinc finger (C3HC4-type RING finger) family protein	Transcriptional regulation
AT1G26800	261265_at	0.51		zinc finger (C3HC4-type RING finger) family protein	Transcriptional regulation
AT1G70580	260309_at	0.51	AOAT2	glutamate:glyoxylate aminotransferase 1 (GGT1)	Other metabolism
AT4G27657	253859_at	0.51		expressed protein	Unknown
AT2G41410	266371_at	0.51		calmodulin	Unknown
AT1G74380	260222_at	0.51		galactosyl transferase GMA12/MNN10 protein	Protein metabolism
AT2G46225	266591_at	0.52	ABI1L1	a subunit of the WAVE complex	Cell organization and biogenesis
AT2G38860	266167_at	0.52	YLS5	proteasel (pfpI)-like protein (YLS5)	Unknown
AT5G57050	247957_at	0.52	ABI2	abscisic acid-insensitive 2 (ABI2)	Stress response

Table A-2. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT5G20070	246126_at	0.52		MutT/nudix family protein	Unknown
AT3G18570	256827_at	0.52		glycine-rich protein / oleosin	Lipid metabolism
AT2G38610	266420_at	0.52		KH domain-containing protein	Unknown
AT3G26770	258257_at	0.52		short-chain dehydrogenase/reductase (SDR)	Other metabolism
AT5G02590	251005_at	0.53		chloroplast lumen common family protein	Unknown
AT1G14530	261482_at	0.53		tobamovirus multiplication protein 3	Unknown
AT2G28400	265276_at	0.53		expressed protein	Unknown
AT1G60680	264941_at	0.53		aldo/keto reductase	Unknown
AT3G51860	246302_at	0.53	CAX3	cation exchanger	Transport
AT4G00750	255637_at	0.53		dehydration-responsive family protein	Stress response
AT1G80245	262056_at	0.53		expressed protein	Unknown
AT4G02690	255493_at	0.53		expressed protein	Unknown
AT5G07370	250607_at	0.53	IPK2a	inositol polyphosphate 6-/3-/5-kinase 2a	Cell organization and biogenesis
AT5G06320	250676_at	0.53	NHL3	harpin-responsive family protein	Defense response
AT5G50240	248544_at	0.54	PIMT2	protein-L-isoaspartate O-methyltransferase	Protein metabolism
AT4G38740	252973_s_at	0.54	ROC1	peptidyl-prolyl cis-trans isomerase	Signal transduction
AT1G32550	256468_at	0.54		ferredoxin family protein	Other metabolism
AT3G28430	257845_at	0.54		expressed protein	Unknown
AT1G07910	260677_at	0.55		tRNA ligase	Other metabolism
AT5G64510	247293_at	0.55		expressed protein	Unknown
AT5G65890	247118_at	0.56	ACR1	ACT domain-containing protein (ACR1)	Other metabolism
AT5G01750	251073_at	0.56		expressed protein	Unknown
AT5G17700	250045_at	0.56		MATE efflux family protein	Transport
AT1G21770	262499_at	0.56		expressed protein	Unknown
AT1G75440	261110_at	0.56	UBC16	ubiquitin-conjugating enzyme 16	Protein metabolism
AT2G41720	260523_at	0.56	EMB2654	pentatricopeptide (PPR) repeat-containing protein	Embryonic development
AT5G33290	246682_at	0.56		exostosin family protein	Unknown
AT2G17972	265819_at	0.56		expressed protein	Unknown
AT3G07360	259012_at	0.56		armadillo/beta-catenin repeat family protein	Protein metabolism
AT5G67300	246987_at	0.56	AtMYB44	myb family transcription factor	Transcriptional regulation
AT4G34500	253239_at	0.57		protein kinase family protein	Protein metabolism
AT3G05510	259113_at	0.57		phospholipid/glycerol acyltransferase	Other metabolism
AT1G21780	262495_at	0.57		BTB/POZ domain-containing protein	Unknown
AT3G02570	258483_at	0.57		phosphomannose isomerase type I protein	Carbohydrate metabolism

Table A-2. Continued

AGI ID	Systematic	Fold change	Common	Description	GO biological process
AT1G08180	261817_at	0.57		expressed protein	Unknown
AT3G51000	252095_at	0.57		epoxide hydrolase	Unknown
AT1G69890	260411_at	0.58		expressed protein	Unknown
AT4G27530	253870_at	0.58		expressed protein	Unknown
AT1G07430	261077_at	0.58		protein phosphatase 2C	Unknown
AT5G61510	247513_at	0.58		NADP-dependent oxidoreductase	Cell organization and biogenesis
AT5G57790	247883_at	0.59		expressed protein	Unknown
AT3G62420	251237_at	0.59		bZIP transcription factor family protein	Transcriptional regulation
AT4G13180	254759_at	0.59		short-chain dehydrogenase/reductase (SDR)	Other metabolism

Table A-3. Comparison of microarray and qRT-PCR data

AGI	Gene name	RT-PCR	Affymetrix	Agilent	cDNA
At1g01060	LHY	1.58	2.03	1.71	-
At1g01620	PIP1C	1.86	2.53	0.88	0.93
At1g03970	GBF4	2.68	1.02	1.09	-
At1g08230	AtGAT1	2.48	2.51	1.27	-
At1g09960	SUT4	2.34	2.07	1.14	-
At1g11260	STP1	1.17	0.82	1.07	0.70
At1g21970	Lec1	0.17	0.75	1.24	1.59
At1g22710	SUC2	31.33	3.27	1.55	-
At1g31320	LBD4	0.69	0.32	0.38	-
At1g45249	ABF2	4.65	-	-	-
At1g49720	ABF1	0.52	1.53	1.44	0.93
At1g59870	PEN3	1.49	2.13	1.99	-
At1g69490	NAP	7.07	5.39	5.62	-
At1g76180	ERD14	5.34	2.54	4.16	11.28
At1g77450	NAC	0.68	0.45	0.66	0.79
At2g15970	WCOR413	7.76	5.29	4.56	2.28
At2g17770	AtbZIP27	0.59	1.33	1.50	-
At2g28300	Lec2	0.06	0.59	-	-
At2g36270	ABI5	0.001	0.81	0.17	0.54
At2g40170	AtEm6	0.23	0.66	1.01	0.20
At2g40220	ABI4	1.16	1.24	1.99	-
At2g41070	EEL	1.84	2.53	2.12	1.52
At2g43000	NAC	0.46	0.52	1.13	-
At2g46270	GBF3	0.39	0.24	0.36	-
At3g01470	HAT5	5.23	5.27	3.10	2.01
At3g08550	ABI8	0.62	0.92	1.15	-
At3g15670	LEA76	2.61	1.04	1.12	-
At3g19290	ABF4	3.01	1.90	2.24	1.00
At3g22840	ELIP1	0.35	0.41	0.44	-
At3g24650	ABI3	1.60	1.50	1.70	-
At3g26790	Fus3	0.69	1.02	0.66	-
At3g44460	AtDPBF2	5.52	2.28	2.18	1.08
At3g51810	AtEm1	0.95	0.76	1.12	-
At3g53420	PIP2A	2.81	2.98	0.95	1.52
At3g56850	AREB3	0.86	1.21	1.00	1.13
At3g61430	PIP1A	4.72	3.25	1.39	1.47
At4g01120	GBF2	0.28	0.46	0.41	1.02
At4g15910	DI21	2.49	2.31	2.57	-
At4g20260	DREPP	2.73	2.85	6.12	2.23
At4g26080	ABI1	0.65	0.90	1.26	0.64
At4g34000	ABF3	0.69	0.68	1.44	1.17
At4g35770	SEN1	4.00	3.32	3.66	4.17
At4g35900	AtbZIP14	0.79	0.91	-	-
At4g38410	ERD10	2.08	1.82	0.71	-

Table A-3. Continued

AGI	Gene name	RT-PCR	Affymetrix	Agilent	cDNA
At5g05410	DREB2A	1.02	0.98	0.98	0.97
At5g13180	NAC	3.41	3.62	1.13	1.27
At5g13330	RAP2.6	1.09	0.86	1.11	1.27
At5g25610	RD22	0.63	0.39	2.60	-
At5g42910	AtbZIP15	0.51	1.50	1.68	-
At5g44080	AtbZIP13	1.69	1.05	1.28	-
At5g52310	RD29A	2.34	2.69	2.84	1.40
At5g57050	ABI2	0.55	0.50	0.66	0.14
At5g60300	LRK	4.05	2.27	1.30	-

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PEER-REVIEWED PUBLICATIONS

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