TRANSCRIPTIONAL REGULATION OF SEED-SPECIFIC GENE EXPRESSION - FROM PvALF/ ABI3 TO PHASEOLIN

A Dissertation

by

WANG KIT NG

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

August 2005

Major Subject: Biology

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Approved by:

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ABSTRACT

Transcriptional Regulation of Seed-specific Gene Expression - from PvALF/ ABI3 to Phaseolin. (August 2005)

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Chair of Advisory Committee: Dr. Timothy C. Hall

The *phaseolin* (*phas*) promoter drives the copious production of transcripts encoding the protein phaseolin during seed embryogenesis but is silent in vegetative tissues when a nucleosome is positioned over its three phased TATA boxes. Transition from the inactive state in transgenic *Arabidopsis* leaves was accomplished by ectopic expression of the transcription factor PvALF (*Phaseolus vulgaris* ABI3-like factor), and application of abscisic acid (ABA). PvALF belongs to a family of seed-specific transcriptional activators that includes the maize viviparious1 (VP1) and the *Arabidopsis* abscisic acid-insensitive3 (ABI3) proteins. The major goal of the study is to gain insight to the regulation of seed-specific gene expression in three different aspects. First, since ABI3 (homolog of PvALF) is involved in ABA-mediated expression of several seed-specific protein genes in *Arabidopsis*, understanding its transcriptional regulation will provide insight to the mechanism by which PvALF expression is controlled. To achieve this, *ABI3* promoter deletion analysis using either *β-glucuronidase* (*gus*) or *green fluorescent protein* (*gfp*) reporter gene fusions have identified various regulatory regions within the *ABI3* promoter

including two upstream activating sequences and a minimal seed specific expression region. In addition, a 405 bp 5' UTR was shown to play a negative role in *ABI3* expression, possibly through post-transcriptional mechanisms. Second, placement of PvALF expression under control of an estradiol-inducible promoter permitted chronological ChIP analysis of changes in histone modifications, notably increased acetylation of H3-K9, as *phas* chromatin is remodeled (potentiated). A different array of changes (trimethylation of H3-K4) is associated with ABA-mediated activation. In contrast, H3-K14 acetylation decreased upon *phas* potentiation and increased on activation. Whereas decreases in histone H3 and H4 levels were detected during PvALF-mediated remodeling, slight increases occurred following ABA-mediated activation, suggesting the restoration of histone-*phas* interactions or the redeposition of histones in the *phas* chromatin. The observed histone modifications thus provide insight to the factors involved in euchromatinization and activation of a plant gene. Finally, ectopically expressed ABI5 and PvALF renders the activation of *phas* ABA-independent, suggesting ABI5 acts downstream of ABA during *phas* activation.

DEDICATION

This dissertation is dedicated to my parents, my brothers, my friends, my supervisor and his family for their love and support.

ACKNOWLEDGMENTS

I would like to express my gratitude and appreciation to my supervisor, Timothy Hall, for his patience guidance and support during the development of this dissertation. I am indebted to him for his valuable advice, patience and insightful comments. In addition to his academic support, Dr. Hall and his family have provided me a friendly and welcoming home where we enjoyed dinners and shared lots of fun together.

I would like to thank all my committee members, Rodolfo Aramayo, Alan Pepper and Michael Kladde, for all of their advice and ideas. I would also like to thank Mary Bryk and Keith Maggert for their comments and discussion on work presented in chapter III in this dissertation. My gratitude also goes to Mark Zoran, Debby Siegele and Kay Goldman from the Graduate Advisor's Office for their wonderful help in answering my questions concerning my study.

I also thank Nam-Hai Chua for providing the pER8 plasmid and Michael Kladde for providing the pMPY-3xHA plasmid. Rob Martienssen kindly provided the ChIP procedures used in chapter III in this dissertation. In addition, I would like to thank Terry Thomas for providing the ABI5/AtDPBF-1 overexpressing line and garlic 401b *abi5* mutant line used in chapter IV.

This dissertation would not have been finished without all my labmates and student helpers: Mahesh Chandrasekharan, Raul Carrano, Tao Wang, Xiang-yu Shi, Xin Zhou, Yi-ming Jiang, Guo-jun Yang, Rakesh Pancholy, James Townsend, Charlotte White, Joshua Mink, Erin Hanover and Melanie Neely. I am indebted for their valuable discussion, ideas,

help and comments, especially Mahesh who made tremendous contributions towards the idea of the experiments presented in this dissertation.

I am also thankful for all the support I got from personnel in IDMB (Allison Myrick Jennifer Smith, Caitlin Donovan and Charlie Harris), GTL (Steve Boulware and Larry Harris-Haller), Ginger Stuessy for her help in plant care and other people in the Biology Department.

Last but not least, I would like to thank people from Tom Mcknight's Lab (Ketan Petal and Shuxin Ren) and Terry Thomas's Lab (Jung-Im Hur and Mona Damaj) for their suggestions and friendship.

This work was supported by National Science Foundation grant MCB-9974706 and MCB-0346681 including REU supplements.

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CHAPTER I

INTRODUCTION

Transcriptional regulation in eukaryotes

In eukaryotes, temporal and spatial control of gene expression is under complex controls through transcriptional, post-transcriptional and post-translational mechanisms. In addition to various *cis*-elements that reside in the control regions (promoter) of genes (Lewin 2004), transcriptional control of gene expression is achieved through the alteration of chromatin structure and histone modifications (Hsieh and Fischer 2005). Transcript processing, modification, transport, stability and the initiation of translation then are regulated post-transcriptionally (Day and Tuite 1998; McCarthy 1998). Finally, the availability of functional protein is determined by post-translational control via protein modification, transportation and degradation (Liu and Culotta 1999).

Epigenetic control of gene expression through chromatin

Chromatin has emerged as playing an important role in eukaryotic gene regulation in addition to its structural role in DNA packaging (Kornberg and Lorch 1999; Nemeth and Langst 2004). The nucleosome is the fundamental unit of chromatin and consists of 146 bp DNA wrapped around a histone octamer (a histone H3-H4 tetramer and two histone

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This dissertation follows the style of *Genes and Development*.

H2A-H2B dimers). Nucleosomes are connected by 20-60 bp linker DNA to form a 10 nm chromatin fiber. Association with H1 linker histone and various non-histone proteins leads to the formation of a 30 nm chromatin fiber with a 2-start helix arrangement of 6 nucleosomes per turn (Luger et al. 1997; Dorigo et al. 2004). Further condensation of chromatin results in the formation of chromosomes.

Functionally, chromatin can be divided into two domains, heterochromatin and euchromatin. Heterochromatic regions contains highly methylated and silenced DNA with condensed chromatin. Histones in this region are generally hypoacetylated and methylated at H3-K9, H3-K27 and H4-K20 (Craig 2005). In contrast, euchromatin is a region of de-condensed chromatin with actively transcribing genes. Histones are in general hyperacetylated in these regions and methylated at H3-K4, H3-K36 and H3-K79 (Krogan et al. 2003b; Ng et al. 2003a).

The presence of a nucleosome essentially prevents access of general transcription factors and RNA polymerase II to promoter sequences (Lorch et al. 1987; Han and Grunstein 1988). Thus, a key function for transcriptional activators is to initiate the unfolding or remodeling of repressive chromatin structure (heterochromatin) and permit the formation of a preinitiation complex at the control region of gene for transcriptional activation. Two classes of factors are involved in modulating the dynamic status of chromatin (Nemeth and Langst 2004). The first class is ATP-dependent chromatin remodeling factors, such as SWI/SNF (Vignali et al. 2000), that use energy from ATP hydrolysis to alter interactions between histones and DNA. The second class of factors (for examples, histone acetyltransferase and histone methyltransferase) alters chromatin status

through covalent modifications of the N-termini of histones such as acetylation, methylation, phosphorylation, ubiquitination, ribosylation and sumoylation (Turner 2000; Jenuwein and Allis 2001). This constitutes a histone code that determines the structural and functional state of chromatin in gene regulation through interaction with non-histone proteins in a modification-dependent manner (Luger and Richmond 1998; Strahl and Allis 2000).

Promoter and transcription control

In general, promoters of eukaryotic genes consist of a proximal core promoter region (~-40 to +50 bp relative to transcription start site) and modulating regions (activators, repressors, enhancers or silencers) upstream of the core promoter. The core promoter provides sites for the assembly of RNA polymerase II, the general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH) and mediators (Orphanides et al. 1996). The temporal and spatial regulation of genes is controlled through sequence-specific interactions between various *cis*-elements located within the upstream regulatory region and transcriptional activators or repressors (Hampsey 1998). In general, transcriptional activators consist two functional domains, a DNA binding domain that targets the regulator to its cognate site and a transcriptional activation domain that is involved in gene activation through direct interaction with the basal transcription machinery or recruitment of co-activators such as chromatin remodeling factors that facilitate the formation of the preinitiation complex at the core promoter (Johnson and McKnight 1989; Triezenberg 1995; Kuo et al. 2000).

Role of miRNAs in gene regulation

Recently, it has become evident that gene expression can be negatively regulated post-transcriptionally by a family of 21-25 nucleotide non-coding RNAs, termed microRNAs (miRNAs) (Lai 2003; Bartel 2004). miRNAs were first discovered in *Caenorhabditis elegans* as small temporal RNAs (stRNAs) that involve in developmental regulations (Lee et al. 1993; Lau et al. 2001). Primary miRNA transcripts (pri-miRNAs) are processed by an RNase III enzyme, Drosha, in the nucleus to form pre-miRNAs which then are exported to cytoplasm and processed into miRNAs by Dicer (Lee et al. 2003; Nakahara and Carthew 2004). In plants, ~22 ng miRNAs are expressed endogenously and are processed from precursors containing fold-back secondary structure (Reinhart et al. 2002). Post-transcriptional regulation of gene expression by miRNAs can be achieved through either RISC-mediated miRNAs-directed cleavage of target mRNAs (Hutvagner and Zamore 2002; Llave et al. 2002) or translational repression (Zeng et al. 2002; Aukerman and Sakai 2003; Chen 2004).

Plant embryogenesis

In higher plants, embryogenesis marks one of the major morphogenetic changes that occur during plant development. Complex gene expression and interactions are involved in controlling proper development of an embryo. After fertilization, the zygote goes through several developmental stages to finally become mature. These include the globular-, heart-, torpedo-, walking stick- and mature-stage. Structurally, the embryo is differentiated into two organ systems, the axis and cotyledons. While the axis contains the root- and

shoot-meristem that will develop into vegetative tissues after seed germination, the cotyledon is where food reserves (proteins, lipids and carbohydrates) are synthesized (Goldberg et al. 1989). The synthesis of seed storage proteins constitutes one of the major events during embryogenesis and *cis*-elements pivotal in controlling the expression of seed storage protein genes have been extensively characterized (Bustos et al. 1991; Bobb et al. 1997; Ezcurra et al. 1999; Chandrasekharan et al. 2003a). A class of seed-specific transcription activators containing the B3 domain (Suzuki et al. 1997) has been found to be important for spatial regulation of many seed storage protein promoters (McCarty et al. 1991; Giraudat et al. 1992; Bobb et al. 1995; Parcy et al. 1997). However, it remains unknown as to how the seed specificity of these transcriptional activators is regulated. Therefore, understanding the transcriptional regulation of these factors has the potential to provide a deeper insight into the control mechanisms of seed-specific gene regulation. Additionally, it is of interest to understand the mechanisms used by these transcription activators in mediating seed storage protein gene expression.

Transcriptional regulation of ß-phaseolin

The *phas* promoter provides an excellent opportunity for elucidating chromatin remodeling associated with transcriptional gene activation during embryogenesis. Phaseolin is the major seed storage protein found in French bean, *Phaseolus vulgaris*. The transcriptional regulation of the ß-phaseolin gene (*phas*) is constrained both spatially and temporally. Its mRNA accumulation initiates at 12 daf (days after flowering) and declines at 27 daf during seed development (Sun et al. 1978; Frisch et al. 1995). Li et al. (1998) found that the

establishment of a repressive chromatin structure and the presence of a rotationally positioned nucleosome over three phased TATA boxes of *phas* promoter is responsible for the lack of *phas* expression in vegetative tissues. Further observations by Li et al. (1999) suggested that the activation of *phas* is a two-step process that involves the seed-specific transcriptional activator, PvALF (*Phaseolus vulgaris* ABI3-like factor). While the activity of the *phas* promoter is not inducible by ABA in vegetative tissues (Frisch et al. 1995), ectopic expression of PvALF results in expression from the *phas* promoter in the presence of exogenous ABA (Li et al. 1999). Increased nuclease accessibility to the *phas* promoter was observed in the presence of PvALF. This suggested that the regulation of *phas* expression involves chromatin modification by PvALF and subsequent activation by ABA.

A cornucopia of factor-bound *cis*-elements within the proximal 295 bp *phas* promoter were identified by *in vivo* DMS footprinting in transgenic tobacco (Li and Hall 1999). Subsequent site-directed promoter mutagenesis have confirmed that various *cis*-elements combine in controlling the modular nature of expression from the *phas* promoter during embryogenesis (Chandrasekharan et al. 2003a). Among these *cis*-elements, four RY elements and a G-box located within the proximal 295 bp of the *phas* promoter were identified as having important roles in both quantitative and modular expressions during embryogenesis.

The plant hormone - ABA

ABA regulates many important aspects of plant growth development (Leung and Giraudat 1998; Rock 2000; Finkelstein et al. 2002). During the vegetative phase, ABA mediates

responses of plants to environmental stresses such as drought and low temperature. Under drought conditions, ABA induces stomatal closure by altering ion fluxes in guard cells, thereby limiting water loss by transpiration (Assmann and Shimazaki 1999). ABA-biosynthetic mutants showed an increased tendency to wilt or lose water from excised aerial parts (Léon-Kloosterziel et al. 1996a). A decrease in freezing tolerance has also been observed in both ABA-biosynthetic (*aba1*) and ABA-insensitive (*abi1*) mutants (Koornneef et al. 1998). ABA also plays several important roles in seed development, such as the synthesis of storage proteins, the promotion of desiccation tolerance and dormancy as well as the prevention of precocious seed germination. During seed development, endogenous ABA levels increase at mid- and late-embryogenesis before returning to lower levels in dry seeds (Rock and Quatrano 1995). In cotton, a dramatic increase in ABA level occurs 20 days post-anthesis, coincident with the transition from heart to torpedo stage and the initiation of storage protein synthesis (Galau et al. 1987). ABA biosynthetic/ insensitive mutants fail to become dormant and germinate precociously (Nambara et al. 1995; Léon-Kloosterziel et al. 1996b). Seed storage protein regulation and lipid accumulation have been studied and a positive response to exogenous ABA addition was demonstrated (Rock and Quatrano 1995; Ingram and Bartel 1996).

The seed-specific transcriptional regulators - ABI3 and ABI5

Six abscisic acid insensitive loci (*ABI1*, *ABI2*, *ABI3*, *ABI4*, *ABI5* and *ABI8*) have been identified in *Arabidopsis*. Their mutation leads to reduced sensitivity of seed germination to exogenous ABA (Koornneef et al. 1984; Finkelstein 1994; Brocard-Gifford et al. 2004).

The effects of *abi1* and *abi2* mutations are pleiotropic in that ABA sensitivity in both seeds and vegetative tissues is affected (Rodriguez et al. 1998; Gosti et al. 1999). In contrast, *abi3*, *abi4* and *abi5* mutants display defects in seed ABA sensitivity and seed-specific gene expression without affecting vegetative growth (Finkelstein 1994; Söderman et al. 2000; Nambara et al. 2002). Mutation of *abi8* has been shown to result in a dwarf phenotype and affects stomatal regulation as well as sugar sensing and signaling (Brocard-Gifford et al. 2004).

ABI3 was identified in a genetic screen of mutants insensitive to the inhibitory effects of exogenous ABA on seed germination (Koornneef et al. 1984) and it was the first such locus to be cloned (Giraudat et al. 1992). It encodes a transcriptional factor of the B3 domain family that includes homologs such as viviparous-1 (VP1) from maize (McCarty et al. 1991), PvALF from *P. vulgaris* (Bobb et al. 1995), OsVP1 from *Oryza sativa* (Hattori et al. 1994), AfVP1 from *Avena fatua* (Jones et al. 2000), CpVP1 from *Craterostigma plantagineum Hochst* (Chandler and Bartels 1997) and PtABI3 from *Populus trichocarpa* (Rohde et al. 1998). The expression of ABI3 is seed specific; it is the major transcriptional regulator during seed maturation in *Arabidopsis* (Giraudat et al. 1992). ABI3 mRNA starts to accumulate at 6 daf and continues throughout seed development (Parcy et al. 1994). Precocious seed germination was observed in *abi3* mutants (Nambara et al. 1992) and ectopic expression of ABI3 has led to vegetative expression of seed-specific marker genes in response to exogenous ABA (Parcy et al. 1994). These studies suggested that ABI3 is one of the components in the ABA signal transduction pathway and plays an important role in seed maturation and dormancy. Sequence comparison of ABI3, VP1, PvALF and other

orthologs reveals four highly conserved regions: an N-terminal acidic domain, a repressive domain and three basic domains, B1, B2 and B3 (Bobb et al. 1995). In maize VP1, a repressive domain was also identified for its role in negatively regulates the expression of a-amylase gene that normally expresses during germination (Hoecker et al. 1995). Although the DNA binding capability of these B3 factors remains poorly understood, it has been demonstrated that the conserved B3 domains of VP1 and PvALF have a cryptic ability to bind *in vitro* to the Sph element (Suzuki et al. 1997) and the RY element (Carranco et al. 2004) respectively.

ABI5 encodes a member of the basic leucine zipper (bZIP) transcription factor family that includes homologs DCBF-1 (DC3 promoter binding factor) in sunflower (Kim et al. 1997) and TRAB1 (transcription factor responsible for ABA regulation) in rice (Hobo et al. 1999). ABI5 is expressed in both vegetative tissues and seeds and it regulates a subset of *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) genes expression in response to ABA during embryogenesis (Finkelstein and Lynch 2000). It has been shown that ABI5 binds to the abscisic acid response element (ABRE) in seed-specific promoters (Carles et al. 2002) and interacts with the B1 domain of ABI3 (Nakamura et al. 2001). This factor, thus connects the potentiation step with the ABA-mediated activation step in *phas* expression.

PvALF vs. ABI3

Several lines of evidence show that PvALF and ABI3 are functionally orthologous. Both belong to the same family of seed-specific ABA signal transducers and contain the plant-specific B3 domain. Ectopic expression of ABI3 or PvALF in vegetative tissues leads to the

accumulation of mRNAs for several normally seed-specific genes in response to exogenous application of ABA (Parcy et al. 1994; Li et al. 1999). PvALF and ABI3 share 48% protein sequence identity and high sequence homology exists for the N-terminal acidic domain and the three basic domains, B1, B2 and B3 (Bobb et al. 1995).

A major goal in this study is to address the possible mechanisms by which PvALF expression is regulated. However, whereas ABI3 has been cloned (Giraudat et al. 1992) and its promoter sequence is readily available from GenBank (Accession NC-003074), the *PvAlf* promoter sequence is not available. Therefore, given the close functional similarities of PvALF and ABI3, analyses of regulatory regions and *cis*-elements involved in the temporal and spatial regulation of ABI3 are expected to parallel those involved in regulating PvALF expression.

Broader implications of this research

ABI3 was originally described as being expressed during embryogenesis (Giraudat et al. 1992; Parcy et al. 1994). Subsequent research findings indicate that ABI3 expression is not restricted to seed tissues. Rohde et al. (1999) suggested that ABI3 has a role during vegetative quiescence processes since *ABI3*-driven GUS expression was detected in the apex of dark grown seedlings. In addition, GUS activity has been observed in axils and the receptacle of flower bracts, and pedicel axils as well as in the abscission zones of siliques and rosette leaves of older plants. Therefore, delineation of *cis*-elements and *trans*-acting factors involved in ABI3 expression should clarify how ABI3 is regulated. Additionally, elucidation of histone modification changes associated with ABI3/ PvALF-mediated

potentiation and ABA-mediated activation of the *phas* promoter will provide insight to other factors and the mechanisms involved in the regulation of seed-specific promoter expression during plant development.

Research aims

The major goal of this research is to gain insight to the regulation of seed-specific gene expression by studying the mechanisms of PvALF involvement in *phas* gene regulation. Additionally the seed-specific transcriptional regulation of PvALF itself will be addressed through the expression analysis of its ortholog, ABI3, in *Arabidopsis*. Specific objectives included are: 1) To delineate the regulatory region(s) and element(s) responsible for seed-specific expression of the *ABI3* promoter; 2) To investigate PvALF- and ABA-mediated histone modifications at the *phas* chromatin and 3) To examine other factors involve in the regulation of *phas* expression.

CHAPTER II

TRANSCRIPTIONAL REGULATION OF ABI3*

Introduction

Arabidopsis abscisic acid insensitive3 (ABI3), *Phaseolus vulgaris* ABI3-like factor (PvALF) and *Zea mays* viviparious1 (VP1) are orthologous members of the plant-exclusive B3 class of transcription factors. Each is known to play a vital role in the spatial regulation of maturation-specific (*MAT*) genes (McCarty et al. 1991; Giraudat et al. 1992; Parcy et al. 1994; Bobb et al. 1995). Detailed studies on the phaseolin promoter (*phas*), which drives expression of one member of the small gene family that encodes phaseolin (the major seed storage protein of *P. vulgaris*), have established that transcription is rigorously confined to embryogenesis and microsporogenesis (Bustos et al. 1991; Chandrasekharan et al. 2003a). In leaves and other vegetative tissues, a nucleosome is translationally and rotationally positioned over the TATA region of *phas*, making it inaccessible to the transcription machinery (Li et al. 1998). Studies on tobacco (Li et al. 1999) and *Arabidopsis* (Chandrasekharan et al. 2003b) transgenic for a -1470phas-gus construct established that ectopically supplied PvALF mediates remodeling of the repressive chromatin architecture

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*Part of the data reported in this chapter is reprinted from "The 5'UTR negatively regulates quantitative and spatial expression from the *ABI3* promoter" DannyW-K Ng, Mahesh B. Chandrasekharan and Timothy C. Hall, 2004. *Plant Molecular Biology*, 54, 25-38 with kind permission of Springer Science and Business Media. Copyright 2004 by Springer Science and Business Media.

in leaves, potentiating *phas* (and other *MAT* promoters) for transcriptional activation in response to exogenously-supplied abscisic acid (ABA). Similarly, as shown here, in the presence of exogenously-supplied ABA, ectopic expression of ABI3 results in β-glucuronidase (GUS) expression in leaves of *Arabidopsis* transgenic for -1470phas-gus.

The lack of GUS expression in vegetative tissues transgenic for the -1470phas-gus construct, even in the presence of ABA, strongly implies the absence of ABI3 in the case of *Arabidopsis* or its ortholog in the case of tobacco. This situation is in accord with the embryogenesis-restricted expression of PvALF in bean (Bobb et al. 1995) and ABI3 in *Arabidopsis* (Giraudat et al. 1992). Further, expression of seed-specific genes was observed in transgenic *Arabidopsis* leaves ectopically expressing ABI3 only in the presence of ABA (Parcy et al. 1994). It has also been shown that VP1 and ABI3 can repress expression of genes involved in post-germination events. For example, GUS expression from the barley *α-amylase* promoter was detected in *vp1* mutant aleurone cells but not in wild type (Hoecker et al. 1995). Similarly, GUS expression from the *Cab* promoter, that is normally induced by light during germination, was observed during maturation of *abi3* embryos (Nambara et al. 1995). This is in accord with our finding that PvALF mediates chromatin reorganization (Li et al. 1999) and reflects the complex nature of the VP1/ABI3 family of factors, which contain an acidic activation domain, a repressive domain and three basic domains (Holdsworth et al. 1999).

However, more recent studies have suggested that *ABI3* expresses and functions outside the seed (Rohde et al. 2000). GUS was found to be expressed from *ABI3-gus* fusion constructs in the apex of dark grown *Arabidopsis* seedlings and it has been suggested that

ABI3 is involved in vegetative quiescence. Additionally, GUS expression from the *ABI3* promoter in vegetative tissues (stipules, abscission zones of siliques) was reported (Rohde et al. 1999). Light has been suggested as one of the factors regulating ABI3 expression, as *abi3* mutants are defective in plastid dedifferentiation when grown in the dark. DET1 (deetiolation1), a negative regulator involved in light-mediated development and gene expression (Pepper et al. 1994), is required for full expression from the *ABI3* promoter during embryogenesis (Rohde et al. 2000). Furthermore, in the presence of exogenous auxin or ABA, Brady et al. (2003) demonstrated the activation of *ABI3* promoter-driven GUS expression in lateral roots of *Arabidopsis*.

All of the ABI3 studies mentioned above were undertaken using a 5.4 kb *ABI3* promoter to drive *gus* expression in *Arabidopsis* (Parcy et al. 1994), and we are unaware of any previous functional analysis of the *ABI3* promoter. Given the importance of the ABI3 transcription factor and its orthologs in plant development, our interest in the mechanisms involved in the spatial regulation of expression from the *phas* promoter (Li et al. 2001), and the need to address the apparently conflicting information on ABI3 expression, we decided to undertake a functional analysis of the *ABI3* promoter.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (Columbia ecotype) seeds were germinated in soil and, following a 2-day 4°C vernalization treatment in the dark, grown at 22°C under a 16/8 h light/dark cycle.

Transformants were selected on Murashige and Skoog (MS) medium (GIBCO™ Invitrogen Corporation) containing 50 mg l^{-1} kanamycin (Sigma), 2% (w/v) sucrose and 0.8% (w/v) agar.

Chimeric gene construction

All primers used in this study are listed in Table 2.1 and a schematic diagram showing the cloning of various gene constructs is shown in Figure. 2.1. Three different primer sets (P7 and 10; P15 and P11; P6 and P2) were used to facilitate amplification from *Arabidopsis* genomic DNA of the 5.15 kb *ABI3* upstream sequence (*-4630/+519ABI3*) that includes a 519 bp 5' UTR (for untranslated region). Polymerase chain reaction (PCR) fragments generated were cloned into pPCR-Script Amp (SK+)® vector (Stratagene) and recombined to give pPCR-4630/+519ABI3 by restriction enzyme sites at positions -3449 (AccI) and -1883 (SpeI). Restriction enzyme sites HindIII and NcoI were incorporated into the P7 forward and P2 reverse primers, respectively. Various 5' promoter deletion fragments were also generated using primers designed to give the desired fragment with flanking HindIII and NcoI restriction enzyme sites. pPCR-3600/+519ABI3 was generated by recombining *-3600/-1801ABI3* and *-2033/+519ABI3* PCR fragments through the common SpeI site at position -1801. The other constructs: pPCR-2033/+519ABI3, pPCR-882/+519ABI3, pPCR-364/+519ABI3 and pPCR-23/+519ABI3 were obtained by cloning corresponding PCR-amplified fragments into pPCR-Script Amp (SK+)® vector. The identity of the cloned fragments was verified by sequencing.

Table 2.1 Oligonucleotide primers.

Primer	Sequence (5'-3')[1]	5' position and direction on *ABI3*[2]
P2	aaccatgGATTGGTCTTTGGTTCTAATAGAACAG	+519 reverse
P3	gcaagcTTCTAGGGTAATAAAATCCGTG	-882 forward
P4	gcaagctTCAAATGCTGAAAAACTGTTAC	-364 forward
P5	gcaagcttTGAAAGAAAGAGAGAGTCTTC	-23 forward
P6	gcaagcttGTATTCCTTGTAGTACGCATAG	-2033 forward
P7	gcaagcttAGTAAAGGACATGATGGAG	-4630 forward
P10	TCGTCGACCTCTTTCTCTCGTG	-3449 reverse
P11	cgccatggTTGACTTGTAATTACGTAC	-1801 reverse
P15	gcaagcttCATGTTAAGTTTTACTAGATACG	-3600 forward
P16	ggggtaccaagcTTCTAGGGTAATAAAATCCG	-882 forward
P17	aaccatggATTGGTCTTTGGTTCTAATAGAACAG	+114 reverse
P18	gctctagaATTTTTCAAAGAAGACAAAAAGCAA	-364 reverse

[1]lower case letters are introduced restriction enzyme site(s) sequences
[2]primer position on *ABI3* upstream sequences and its direction

Full-length and 5' truncated *ABI3* promoters were digested with HindIII and NcoI and inserted into pUC-295phas-gus3' vector (Chandrasekharan et al. 2003a) to obtain pUC-4630/+519ABI3-gus-phas, pUC-3600/+519ABI3-gus-phas, pUC-2033/+519ABI3-gus-phas, pUC-882ABI3/+519-gus-phas, pUC-364/+519ABI3-gus-phas and pUC-23/+519ABI3-gus-phas. For *gfp* reporter fusions, the *gfp* coding region was PCR amplified from pBINm-gfp5-ER (Haseloff et al. 1997) and cloned into pPCR-Script Amp (SK+)® vector with introduced flanking BspHI and XbaI enzyme sites to give pPCR-BspERgfpX. BspHI and XbaI-digested *gfp* fragment was inserted into vectors containing the *ABI3* upstream sequence and *phas* 3' sequence to yield the constructs: pUC-4630/+519ABI3-gfp-phas, pUC-3600/+519ABI3-gfp-phas,

pUC-2033/+519ABI3-gfp-phas, pUC-882/+519ABI3-gfp-phas, pUC-364/+519ABI3-gfp-phas and pUC-23/+519ABI3-gfp-phas. Promoter fragments, *-2033/+114ABI3* and *-882/+114ABI3* (lacking 405 bp of the 5' UR.) were also generated by PCR and cloned into pPCR-Script Amp (SK+)® vector and then into pUC-295phas-gus3' vector to obtained the constructs, pUC-2033/+114ABI3-gus-phas and pUC-882/+114ABI3-gus-phas respectively. Similarly, the pUC-882/-364ABI3/-64/+6CaMV35S-gus-phas construct was obtained by cloning the *-882/-364ABI3* PCR fragment into the p35S-14 (van der Geest and Hall 1996) plasmid containing the *-64/+6CaMV35S* minimal promoter. The plant transformation vector pHM301K is a derivative of pCB301 (Xiang et al. 1999), modified to include a *CaMV35S-nptII-nos3'* cassette and a poly-cloning site in an inverted orientation to that of the parent vector. HindIII and EcoRI-digested fragments of the *ABI3* promoter-reporter-*3'phas* fusion constructs were cloned into pHM301K and transformed into *Agrobacterium tumefaciens* GV3101 by electroporation.

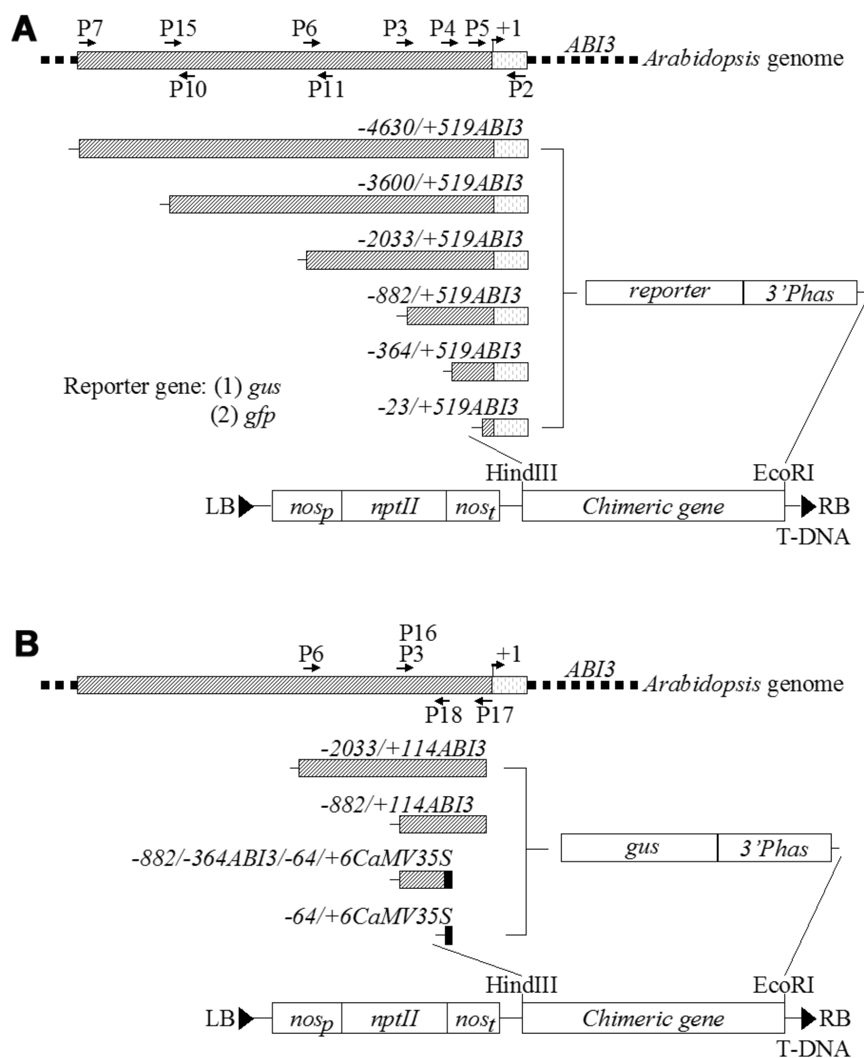

Figure 2.1 Schematic diagram of various chimeric gene constructs. Different primer sets (see Table 2.1) were used to generate various truncated *ABI3* promoter fragments in a PCR using genomic DNA extracted from *Arabidopsis* leaves. (*A*) The amplified 5' truncated *ABI3* promoter fragments were fused to either *gus* or *gfp* reporter gene and inserted to a plant transformation vector through HindIII and EcoRI restriction enzyme sites. Numbers denoted the position of the truncation relative to the transcription start site of *ABI3*. (*B*) *ABI3* promoter fragments (*-2033/+114ABI3* and *-882/+114ABI3*) with deletion at both the upstream region and the 5' UTR region were fused to *gus* and inserted into the plant transformation vector. A *-882/-364ABI3* fragment was fused to a *-64/+6CaMV35S* minimal promoter before cloning into vector containing the *gus* reporter and subsequently into the plant transformation vector.

Plasmid constructs for in vitro *transcription and translation*

The *gus* coding region (1.8 kb) with 114 bp (+1/+114) or 519 bp (+1/+519) of the*ABI3* 5' UTR was PCR amplified from pUC-4630/+519ABI3-gus-phas or pUC-2033/+114ABI3-gus-phas, respectively. The resulting plasmid, pGEM-519UTR-gus-phas or pGEM-114UTR-gus was obtained by cloning the PCR amplified fragment into pGEM-T vector (Promega). The *gus* coding region (1.8 kb) with 519 bp (+1/+519) *ABI3* 5' UTR was amplified from pUC-4630/+519ABI3-gus-phas by PCR using BspHIABI3+1-for (5'-GCATCATGAGTTGGAGTAAACCCAA-3') and Xba-GUSend-rev (5'-CGTCTAGATTCATTGTTTGCCTCCC-3') primers. Purified PCR product was then cloned into pGEM-T (Promega) to yield pGEM-519UTR-gus for *in vitro* transcription. Similarly, pGEM-114UTR-gus was obtained by cloning a PCR fragment amplified from pUC-2033/+114ABI3-gus-phas using the same primer set. For constructs lacking the *ABI3* 5' UTR, primer set Nco-Gus-for (5'-GCCATGGTCCGTCCTGTAGA-3') and Xba-GUSend-rev (5'-CGTCTAGATTCATTGTTTGCCTCCC-3') was used to amplify the 1.8 kb *gus* coding region from pUC-4630/+519ABI3-gus-phas and then mobilized into pGEM-T vector to yield pGEM-gus.

Plant transformation and selection

Wild-type *Arabidopsis* plants (T_0) with a 10-15 cm tall primary inflorescence were used for transformation (Bechtold and Pelletier 1998). Mature seeds from infiltrated plants were harvested, surface sterilized and putative transformants (T_1) were selected on MS medium containing 50 mg l^{-1} kanamycin (Sigma) and 100 mg l^{-1} Timentin (ticarcillin disodium and

clavulanate potassium, SmithKline Beecham Pharmaceuticals).

Inducible ectopic expression of ABI3 *in -1470phas-gus plants*

The ABI3 coding region (2.1 kb) was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR), using total RNA isolated from *Arabidopsis* siliques. Primers were designed to incorporate ApaI (at the 5' end) and SpeI (at the 3' end) sites upon amplification of *ABI3*. The *ABI3* fragment was inserted into pER8 (Zuo et al. 2000) downstream of the *XVE* promoter to yield the estrogen-inducible construct pER8-ABI3. This was supertransformed into an *Arabidopsis* line homozygous for -1470phas-gus. Doubly transformed plants were selected on MS medium containing 50 mg l^{-1} kanamycin, 25 mg l^{-1} hygromycin B (GIBCO™ Invitrogen Corporation) and 100 mg l^{-1} Timentin. Expression of GUS from -1470phas-gus, dependent on the presence of functional ABI3, was induced by overnight (16 h) incubation of -1470phas-gus::XVE-ABI3 leaves in MS liquid medium containing 25 µM 17β-estradiol (Sigma) and 200 µM ABA (GIBCO™ Invitrogen Corporation), followed by histochemical staining.

ABA treatment

Seeds transgenic for the various *ABI3* promoter-*gus* constructs were germinated on MS medium containing 50 mg l^{-1} kanamycin. Seedlings (2-week-old) were transferred to MS agar plates containing various concentrations of ABA (0.1 µM, 0.3 µM, 0.5 µM, 1 µM, 5 µM, 10 µM and 100 µM). Histochemical GUS staining was performed 7 days after the transfer.

Histochemical and fluorometric assays for GUS activity

Histochemical GUS staining was performed for vegetative tissues (leaves, roots, inflorescence) using 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid (X-gluc) as a substrate (Jefferson et al. 1987). Samples were stained for overnight (16 h) at 37°C and chlorophyll was removed by 95% ethanol after staining. For each promoter-reporter fusion construct, mature T_1 embryos from at least 6 independent transformants were dissected from developing siliques (10-15 days after flowering) and at least 30 embryos were subjected to histochemical GUS staining. Fluorometric assays of GUS activity of mature T_2 seeds was performed as described by Jefferson et al. (1987). GUS activity was calculated as pmol 4-MU h^{-1} μg^{-1} (4-MU; 4-methylumbelliferone) of protein and data were analyzed using SPSS 11.0 for Windows software. For each construct, three independent assays were performed unless otherwise specified.

Fluorescence microscopy

Green fluorescent protein (GFP) expression from developing seeds or embryos of plants transgenic for *gfp* reporter was visualized using a Zeiss SV11 stereomicroscope with a 490 nm excitation filter and 500 nm or 525 nm emission filters.

In silico *promoter analysis*

Cis-elements within the full-length *ABI3* promoter were identified using online PlantCARE and PLACE databases (Lescot et al. 2002). For mRNA secondary structure analysis, the stem-loop structure and the free energy of the 405 nt 5' UTR were predicted according to Zuker (2003).

In vitro *transcription and translation*

For *in vitro* transcription, 5 µg plasmid DNA was first linearized with *Sal*I restriction enzyme and purified by phenol:chloroform extraction and ethanol precipitation. Purified and linearized template (2 µg) was used for *in vitro* transcription using MEGAscript® T7 kit (Ambion Inc.) and in a reaction setup as described in the manufacturer manual. After 1 h incubation at 37°C, the quality and quantity of RNA produced as analyzed by 1% agarose/formaldehyde gel. Equal molar RNA produced for different constructs was used for subsequent *in vitro* translation reaction. Rabbit reticulocyte lysate (RRL) and wheat germ extract (WGE) systems (Promega) were used for *in vitro* translation of RNA obtained from *in vitro* transcription reaction. For each reaction, 1.5 pmol and 6 pmol *in vitro* transcribed RNA were used for *in vitro* translation in RRL and WGE system respectively. Brome Mosaic Virus (BMV) transcript (0.5 µg) was used as a control in both systems. A total 25 µl translation was setup according to manufacture manual. Translation reactions were incubated for 2 h at 30°C and 25°C for the RRL and WGE system respectively. The amount of GUS synthesized was analyzed by using 10 µl lysate in a fluorometric analysis.

RNA extraction and RT-PCR

Total RNAs were extracted from developing siliques using TRIzol reagent (Invitrogen). DNase I-digested total RNAs (0.5 µg) were subjected to RT-PCR analysis using a Qiagen one-step RT-PCR kit. Gene-specific primer sets (0.6 µM), Gus-for (5'-GGTGGGAAACGCGTTACAAG-3') and Gus-rev (5'-GTTTACGCGTTGCTTCCGCCA-3'); Abi3-for (5'-GAAAAGCTTGCATGTGGCGG-3') and Abi3-rev (5'-TGTCTAATGGAATCTCCATGG-3') were used to detect the presence of *gus* and *abi3* transcripts, respectively. Primer pair (0.08 µM), ef-for (5'-TGCTGTCCTTATCATTGACTCCACCAC-3') and ef-rev (5'-TTGGAGTACTTGGGGGTAGTGGCATC-3'), targeting the *EF1α* gene was used as an internal control for the RT-PCR. The RT-PCR products were resolved in 1% agarose gel and visualized by ethidium bromide staining.

Results

The full-length ABI3 *promoter drives strong expression of GUS and GFP in embryos but not in leaves*

The *ABI3* promoter was initially isolated as a 5.4 kb fragment from a cosmid clone and it was shown that this region, fused to the *gus* coding sequence, yielded embryo-specific GUS expression in transgenic *Arabidopsis* (Giraudat et al. 1992; Parcy et al. 1994). However, inspection of the genomic sequence (NC-003074) in the current GenBank revealed that its

5' boundary overlaps the stop codon of an upstream annotated hypothetical protein. Inspection of recent EST (expressed sequence tag) sequences in the TIGR database indicated that transcription of ABI3 commences upstream of the site predicted by Giraudat et al. (1992). The transcription start site (position +1) used here corresponds to position 8997399 on chromosome 3 according to GenBank version NC-003074.4 (GI:30698537); this was supported by RT-PCR of RNA from developing siliques (data not shown).

To avoid the overlap with the upstream gene, a 5.15 kb sequence (that includes a 4630 bp "full-length" promoter region and a 519 bp 5' UTR) was obtained by PCR amplification of genomic DNA from the upstream stop codon to the ABI3 translation start site at position +519. This sequence, defined as the *-4630/+519ABI3* promoter, was fused to *gus* or *gfp* and transformed into *Arabidopsis* (Fig. 2.1).

Mature embryos were dissected from developing T_1 seeds and analyzed via histochemical GUS staining or fluorescence microscopy. The spatial pattern of GUS expression from the full-length *ABI3* promoter in developing embryos was similar to that previously reported (Parcy et al. 1994; Devic et al. 1996), except that strong expression was also detected during microsporogenesis (Fig. 2.2A,B). GFP expression from the full-length *ABI3* promoter was also observed during embryogenesis, starting at the heart stage (Fig. 2.2C-F). However, whereas Rohde et al. (1999) detected reporter expression in certain vegetative tissues of the *ABI3-GUS* line established by Parcy et al. (1994), no reporter expression was detected in vegetative tissues including roots, stems, or rosette and cauline leaves from six independent transformants.

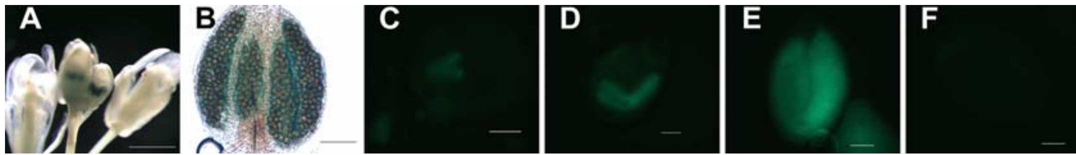

Figure 2.2 Reporter expression from the full-length *ABI3* promoter. GUS-stained anthers are shown (*A*) within or (*B*) excised from the inflorescence. GFP expression is shown for developing seeds containing embryos at various stages: (*C*) heart, (*D*) late torpedo, (*E*) maturation, (*F*) non-transgenic seeds at the maturation stage. Bar = 1 mm (*A*); 100 µm (*B*-*F*).

Although transcription driven by the *phas* promoter is normally stringently constrained to embryogenesis (Frisch et al. 1995), Chandrasekharan et al. (2003b) used a *XVE-PvALF* construct that, in the presence of estradiol and ABA, induced PvALF synthesis and concomitant strong expression of GUS in leaves of *Arabidopsis* transgenic for -1470phas-gus. We conducted a similar experiment using an *XVE-ABI3* construct. In the absence of estradiol, there was no induction of ABI3 and no GUS expression was detected in leaves, even when ABA was supplied (Fig. 2.3A-C). The lack of GUS expression under these conditions reveals the absence of endogenous expression of ABI3 in leaves. As a positive control, incubation of -1470phas-gus::XVE-ABI3 supertransformant leaves in MS medium containing 25 µM estradiol and 200 µM ABA for 16 h in the dark induced ectopic expression of ABI3 and activation of -1470phas-gus transcription (Fig. 2.3D). In addition to demonstrating the seed-specificity of ABI3 expression, these experiments illustrate the parallel functions of ABI3 and PvALF.

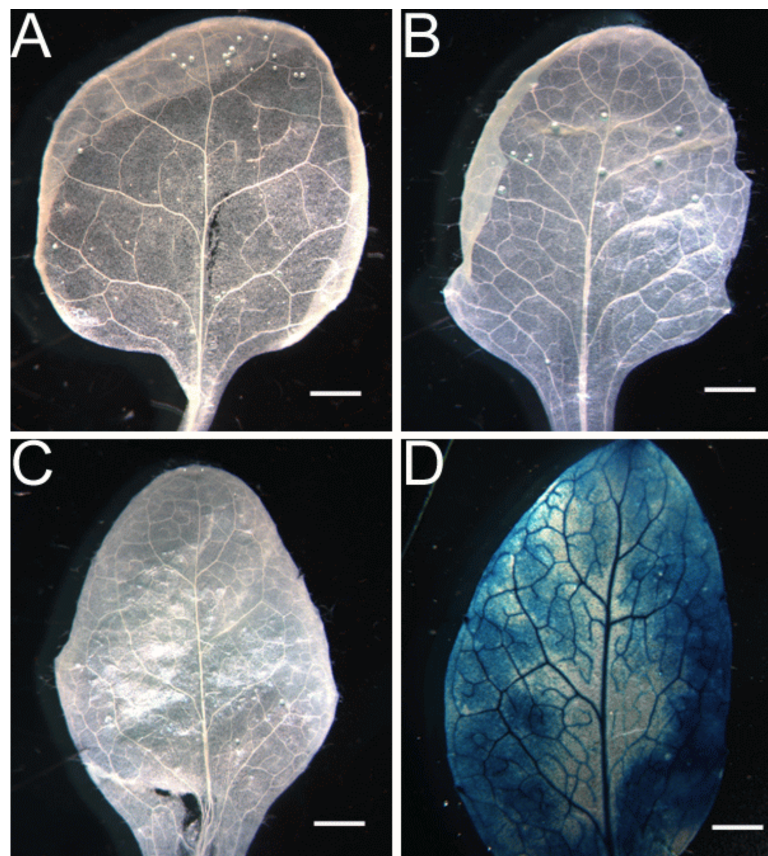

Figure 2.3 Ectopically expressed ABI3-induced *phas-driven* GUS expression. Leaves from -1470phas-gus::XVE-ABI3 plants were subjected to estradiol and ABA treatment, followed by histochemical staining. GUS expression is shown for representative leaves incubated overnight in: (*A*) MS alone (control), (*B*) 25 µM estradiol, (*C*) 200 µM ABA or (*D*) 25 µM estradiol and 200 µM ABA. Bar = 1 mm.

Expression from truncated ABI3 *promoters*

5' truncated promoters *-3600/+519ABI3*, *-2033/+519ABI3*, *-882/+519ABI3*, *-364/+519ABI3* and *-23/+519ABI3* were obtained by PCR-amplification from the *Arabidopsis* genome and

used in fusion constructs with *gus* or *gfp* reporters (Fig. 2.1). Figure 2.4 shows GUS and GFP expression in mature embryos representative of at least six independent lines for each construct. GUS expression was observed in all of the transgenic lines except those harboring *-364/+519ABI3-gus* and *-23/+519ABI3-gus* constructs (Fig. 2.4A). No GUS expression was detected in the radicle region of *-2033/+519ABI3-gus* and *-882/+519ABI3-gus* embryos, suggesting the presence of regulatory element(s) upstream of -2033. As expected, GFP expression was observed in embryos harboring *-4630/+519ABI3-gfp, -3600/+519ABI3-gfp* and *-2033/+519ABI3-gfp* transgenes (Fig. 2.4B). However, GFP expression levels in embryos transgenic for *-882/+519ABI3-gfp* were too low for detection using fluorescence microscopy. In general, reporter expression levels decreased as the length of the promoter was shortened from the -2033 position. In all cases, spatial regulation of the truncated promoter-reporter fusions was confined to microsporogenesis and embryogenesis.

GUS expression in T_2 seeds was further quantified by fluorometric assays (Jefferson et al. 1987). Table 2.2 shows GUS activity relative to the *-4630/+519ABI3-gus* line. Consistent with the histochemical results, GUS activity was seen to decrease as the promoter length was shortened and negligible GUS activity was observed in *-364/+519ABI3-gus* and *-23/+519ABI3-gus* transgenic lines. The levels of expression from *-4630/+519ABI3* and *-3600/+519ABI3* were not significantly different. In contrast, deletion of the 5' 2.6 kb promoter region (-4630 to -2033) led to a significant 3.8-fold decrease in GUS activity. Further deletion from -2033 to -882 resulted in an additional 4-fold decrease in activity and deletion of the -882 to -364 region caused a dramatic 645-fold loss. These

data reveal the presence of at least three positive *cis*-regulatory regions (-3600 to -2033; -2033 to -882 and -882 to -364) in the *ABI3* promoter.

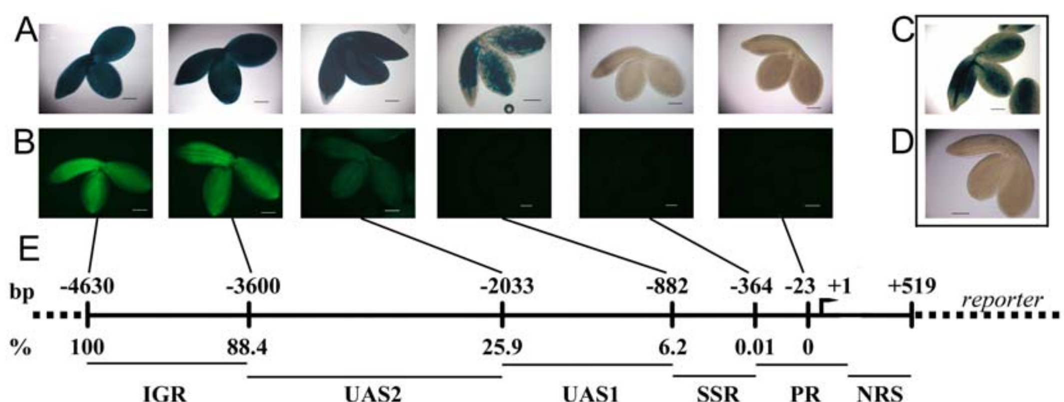

Figure 2.4 Reporter expression in mature *Arabidopsis* embryos transformed with various *ABI3* promoter fusion constructs. The coding region for (*A*) GUS or (*B*) GFP was ligated immediately downstream of the 5' UTR (+519) to the promoter elements indicated in (*E*). *Inset*: GUS expression in mature embryos transgenic for (*C*) *-882/-364ABI3/-64/+6CaMV35S-gus* or (*D*) *-64/+6CaMV35S-gus*. A synopsis of regulatory regions (see Discussion) is shown in (*E*), together their location (in bp relative to the predicted transcription start site) and GUS reporter activity (%) relative to the -4630 construct (see Table 2.2). IGR, intergenic region; UAS1 and UAS2, upstream activation sequences; SSR, seed specific expression region; PR, proximal region; NRS, negative regulatory sequence. Magnification of embryos is indicated in each panel: bar = 100 µm.

The -882 to -364 region confers seed-specific expression

Since GUS expression was observed in *-882/+519ABI3-gus* seeds but not in *-364/+519ABI3-gus* seeds, it follows that the -882 to -364 region contains *cis*-elements conferring seed-specific expression. To verify this, the *-882/-364ABI3* region was fused to the *-64/+6CaMV 35S* minimal promoter (van der Geest and Hall 1996) and the *gus* coding

sequence. GUS expression was observed in developing embryos of plants transformed with *-882/-364ABI3/-64/+6CaMV35S-gus* (Fig. 2.4C) and was similar to that of *-882/+519ABI3-gus* embryos both in its spatial distribution (compare Fig. 2.4A,C) and intensity (Table 2.2). No GUS expression was detected in vegetative tissues for either of these constructs (data not shown). As a result of these findings, the sequence from -882 to -364 was designated as the seed-specific region (SSR) of the *ABI3* promoter.

Table 2.2 Contribution of various regions within the *ABI3* promoter to GUS expression in seeds.

		Natural logarithmic scale[2]				
Construct	N[1]	ln mean	SE	P-value[3]	Mean GUS activity[4]	GUS activity (%)[5]
-4630/+519ABI3-gus	19	9.84	1.05	n.a.	18857.55	100.00
-3600/+519ABI3-gus	20	9.72	1.13	0.547	16675.95	88.43
-2033/+519ABI3-gus	22	8.49	1.28	0.000	4875.56	25.85
-882/+519ABI3-gus	15	7.07	1.43	0.000	1175.50	6.23
-364/+519ABI3-gus	16	0.60	0.89	0.000	1.82	0.01*
-23/+519ABI3-gus	13	-0.51	0.66	0.000	0.60	0.00*
-2033/+114ABI3-gus	20	9.84	0.99	0.593	18677.43	99.04
-882/+114ABI3-gus	21	9.76	1.04	0.357	17372.14	92.12
-882/-364ABI3/-64/+6CaMV35S-gus	21	6.72	1.16	0.000	827.77	4.39
-64/+6CaMV35S-gus	9	0.54	0.69	0.000	1.72	0.01*

(1) Total number of individual transformants analyzed
(2) Transformed mean GUS activity (ln mean) using natural logarithmic (ln) scale and its standard error (SE)
(3) ANOVA P-value at 95% confidence interval respective to *-4630/+519ABI3-gus*, n.a. = not applicable
(4) Mean GUS activity (pmol 4-MU hr^{-1} μg^{-1} of protein) calculated by back-transforming the ln mean
(5) Percentage GUS activity relative to transgenic line *-4630/+519ABI3-gus*
* Results are based on two separate trials

The 5' UTR negatively regulates expression from the ABI3 *promoter*

As found for the 5' UTR of mRNAs encoding proto-oncogenes, growth factors and transcription factors (Kozak 1987, 1991b; Han et al. 2003), the 5' UTR of *ABI3* mRNA is

unusually long. Since these studies have shown that the presence of a long leader sequence can be inhibitory, it appeared important to determine if the extensive 5' UTR of *ABI3* contributed negatively to expression. Inspection of the 5' UTR sequence revealed three upstream open reading frames (uORFs), located at positions +239, +337 and +362 and consisting of 26, 11 and 12 codons, respectively (Fig. 2.5A). As shown in Figure 2.5B, the 5' UTR of *ABI3* has a high degree of predicted secondary structure (Zuker 2003). Two constructs lacking 405 bp of 5' UTR (spanning region +114 to +519), and thereby removing the three uORFs, were made (*-2033/+114ABI3-gus* and *-882/+114ABI3-gus*) and transformed into *Arabidopsis*. Remarkably, strong GUS expression was observed in T_1 embryos (Fig. 2.6A,B). Expression levels were equivalent to that for the full-length *-4630/+519ABI3-gus* construct, representing 4-fold and 15-fold increases over the respective constructs containing the 5' UTR (Fig. 2.6C,D; Table 2.2). These results identify the +114 to +519 region of the 5' UTR as a negative regulator of expression. Moreover, this 5' UTR deletion resulted in alteration of spatial expression since GUS expression was evident in roots of most transformants lacking the *ABI3* 5' UTR (Fig. 2.6E,F).

A

guuggaguaa acccaaacgg uuuuagauua cuuauuagcu guucaucagu ucuuccucuc uaaaagagua aaaccuaaac
aucucucucu guucuauuag aaccaaagac caaucuuugu gaacaaaaca caucucguau acuucagauc uagacucgaa
aauuuuagac cucuuuacaa uuggucuuug uucaucugaa guuggagaaa auaguuagcu uaggucggau cuuuucau**au**
M
gcuuuggauc cuccuucguc ucuuuuguau aauuuuaacc uuaucaagag uucuuuuuga aucucaaaag auuauauagu
L W I L L R L F G I I L T L S R V L F E S Q K I I
aguauagaag guuuau**aug**u au*aug*uauag ccagauaguu u**aug**uuguuu aaagauucg*a* *ug*auagccaa guuggguuaa
M Y M Y S Q I V Y V V
M L F K Q S M I A K L G

cuuucuuuuu ccuugccucc uuacucacau acaaacccua ucuguccgua caaaauacua aaaacccuaa cuuuucucuc
uccaccaauc uaguuuauug uuucauuucc acuucaacga ugaaaagcuu gcauguggcg gccaacgccg gagaucuggc
M K S L H V A A N A G D L A
ugaggauugu ggaauacucg guggagacgc ugau
E D C G I L G G D A D

B

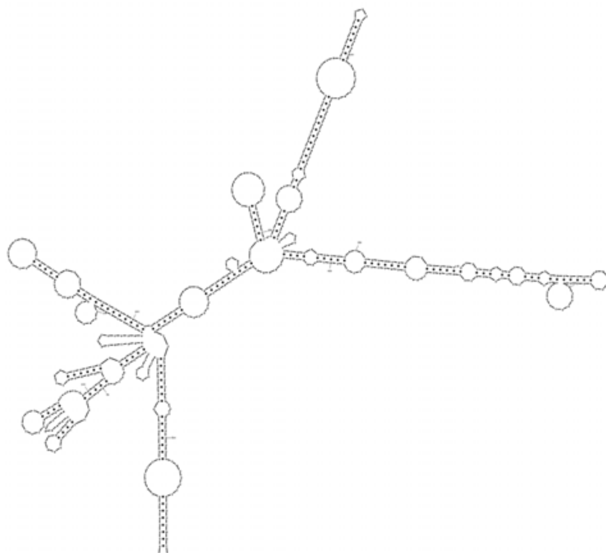

Figure 2.5 uORFs and predicted secondary structure of the 519 nt *ABI3* 5' UTR. (*A*) Nucleotide sequence showing predicted start codons (**aug**) for three short ORFs and two additional aug triplets (*aug*). The predicted start codon and coding region for the first 25 codons of *ABI3* are underlined. (*B*) Predicted secondary structure of the 5' UTR (ΔG = -103.41 kcal/mol).

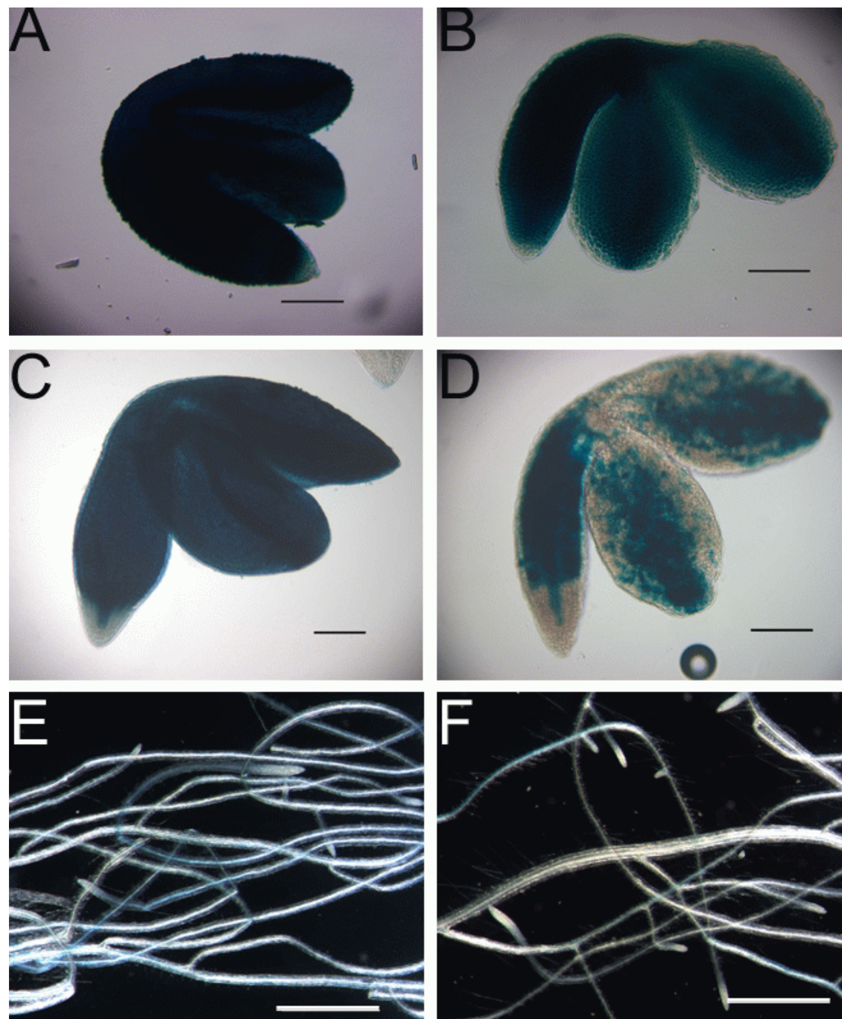

Figure 2.6 Contribution of the 5' UTR to *ABI3* expression. Representative GUS-stained mature embryos are shown from plants transformed with *gus* fusion constructs containing: (*A*) *-2033/+114ABI3* and (*B*) *-882/+114ABI3* that lack the 3' 405 bp of the 519 bp-long *ABI3* 5' UTR; (*C*) *-2033/+519ABI3* and (*D*) *-882/+519ABI3* that contain the *ABI3* 5' UTR; GUS-stained roots of seedlings transgenic for (*E*) *-2033/+114ABI3-gus* and (*F*) *-882/+114ABI3-gus*. Bar = 100 µm (*A-D*); 100 mm (*E-F*).

Negative regulatory role of ABI3 *5' UTR*

Deletion of the 405 bp (+114/+519) 5' UTR from the *ABI3* promoter led to a dramatic increase in reporter (*gus*) activity in transgenic *Arabidopsis* containing either *-2033/+114ABI3-gus* or *-882/+114ABI3-gus* construct (Fig. 2.6). The effect of the 5' UTR could be transcriptional or post-transcriptional (Fig. 2.7). For transcriptional regulation, there could be the presence of a negative regulatory factor which binds to the UTR region and inhibit the transcription. In contrast, post-transcriptional regulation can be achieved at both transcript or protein level. In *trans*, binding of an RNA binding protein to the 5' UTR may increase the rate of mRNA turnover or it may inhibit the translation of protein. In *cis*, the intrinsic structure of the 5' UTR may inhibit translation initiation or efficiency.

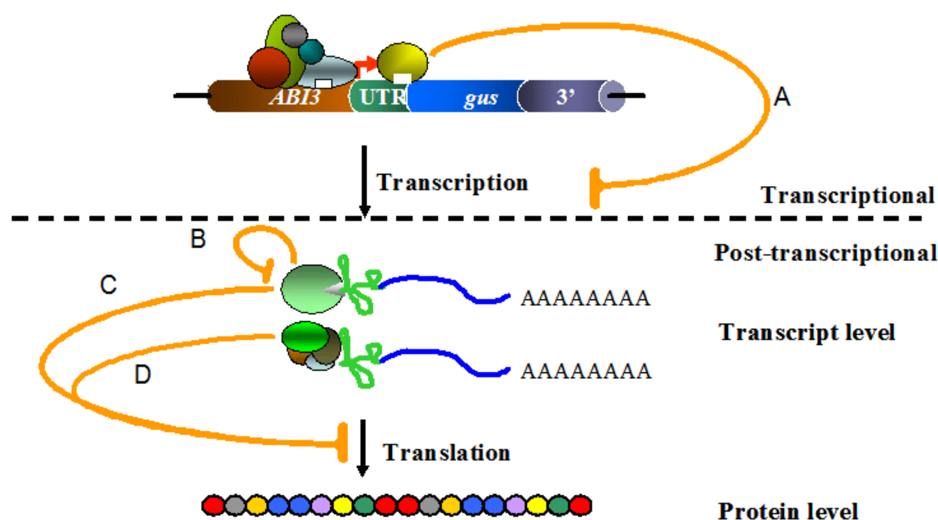

Figure 2.7 Diagram showing possible regulatory role of the *ABI3* 5' UTR. The negative effect of *ABI3* 5' UTR could be the results from repressor binding to the UTR leading to transcriptional repression (*A*). Post-transcriptionally, binding of RNA binding protein to the 5' UTR may increase mRNA turnover (*B*) or inhibit translation initiation (*C*) and the intrinsic structure of the 5' UTR may also inhibit translation initiation and efficiency (*D*).

To further elucidate the effect of the 5' UTR, *in vivo* and *in vitro* approaches were used to determine the involvement of post-transcriptional (at transcript level) versus the translational mechanisms in *ABI3* expression. *In vivo* comparison of transcripts and protein accumulation during embryogenesis was performed by obtaining both total RNA and total seed protein from developing siliques of three different transgenic *Arabidopsis* lines containing constructs with or without the 5' UTR fused to the *gus* coding sequence (*-4630/+519ABI3-gus, -2033/+519ABI3-gus* or *-2033/+114ABI3-gus*). Semi-quantitative RT-PCR and fluorometric GUS analysis (MUG) were used to evaluate the level of *gus* transcript and protein (as reflected by GUS activity) respectively. In transgenic lines (*-4630/+519ABI3-gus* and *-2033/+519ABI3-gus*) containing construct with the 5' UTR, a close correlation between the level of *gus* transcript and GUS activity was evident (Fig. 2.8). In contrast, such correlation was not observed when comparing transgenic line without the 405 bp 5' UTR (*-2033/+114ABI3-gus*) to its corresponding line with the 5' UTR (*-2033/+519ABI3-gus*). Deletion of the 5' UTR led to an increase in GUS expression for similar levels of transcripts detected (Fig. 2.8B, compare lane 4 and 5 to 7). However, a higher transcript level with lower GUS expression was also detected (Fig. 2.8B, compare lane 6 to 8 and 9). It is expected that low transcript level and high GUS activity reflect a translational regulatory role of the 5' UTR whereas high transcript level and low GUS activity reflect a post-transcriptional regulatory mechanism at transcript level. Therefore, while this extends the finding that the 405 bp 5' UTR is involved in post-transcriptional regulation, its control through affecting either transcript stability or protein translation is still unknown.

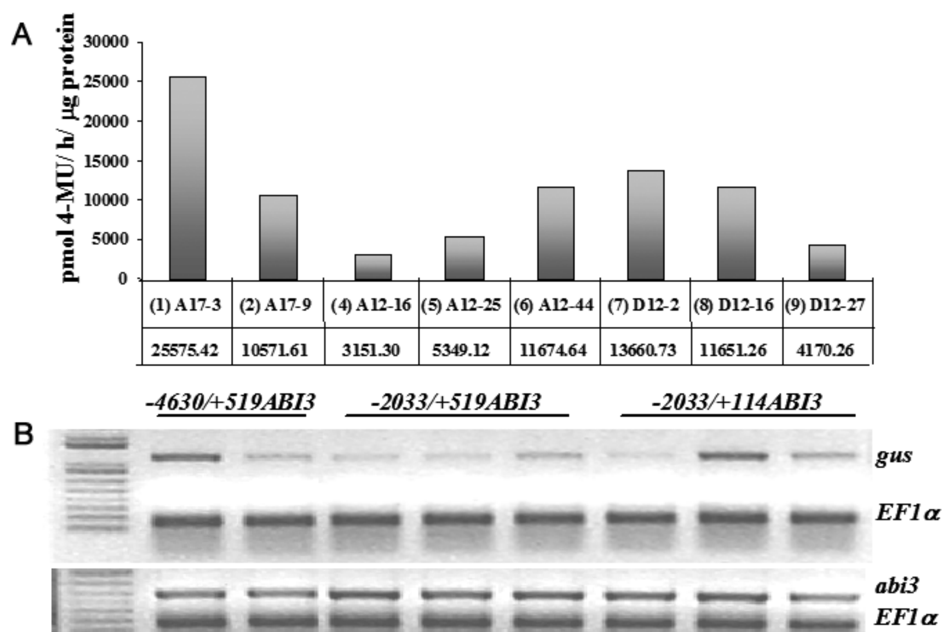

Figure 2.8 Effect of *ABI3* 5' UTR in transcript and protein accumulation *in vivo*. Total RNA and protein were isolated from developing siliques of transgenic plants contain the *-4630/+519ABI3-gus*, *-2033/+519ABI3-gus* and *-2033/+114ABI3-gus* transgenes. GUS activity was quantitated by fluorometric MUG assay (*A*) and semi-quantitative RT-PCR was used to evaluate the *gus* transcript produced, internal control primers targeting *abi3* and *EF1α* transcripts were used in the RT-PCR (*B*).

To determine if the presence of 5' UTR affects the translation of the mRNA, an *in vitro* transcription and translation experiment was performed. Three reporter constructs, with or without the 5' UTR fused with the *gus* coding sequence were used in an *in vitro* transcription reaction (Fig. 2.9A). By providing the same molar amount of *in vitro* synthesized transcripts for *in vitro* translation using either rabbit reticulocyte lysate (RRL) or wheat germ extract (WGE) system, the level of GUS activity after *in vitro* translation was

determined. It is expected that if the 5' UTR does not have an effect on translation, similar GUS activity should be obtained from transcript with or without the 5' UTR. In contrast, variation of GUS activity in the presence/ absence of the 5' UTR would indicate that it affects gene expression translationally. Figure 2.9B shows the results of fluorometric analyses of lysates from *in vitro* translation using either RRL or WGE with the same molar amount of transcripts for the translation. Efficient translation of GUS was only observed for RNA containing the full length 519 bp *ABI3* 5' UTR in both RRL and WGE systems suggesting the present of 5' UTR is important. The lower translation efficiency in WGE without denaturation of RNA suggested the presence of secondary structure in RNA could decrease translation efficiency.

Surprisingly, for RNA with 114 bp 5' UTR or without 5' UTR, no translation of GUS was detected (Fig. 2.9C,D). Since the quality and quantity of the *in vitro* transcribed RNA were verified prior *in vitro* translation (Fig. 2.9A), the data thus suggested that the presence of the 519 bp 5' UTR is important for the translation of GUS in the *in vitro* system. This is in contrast to its negative regulatory role as suggested by *in vivo* data. Nevertheless, both *in vivo* and *in vitro* analyses suggested that the 5' UTR regulates *ABI3* expression post-transcriptionally rather than transcriptionally.

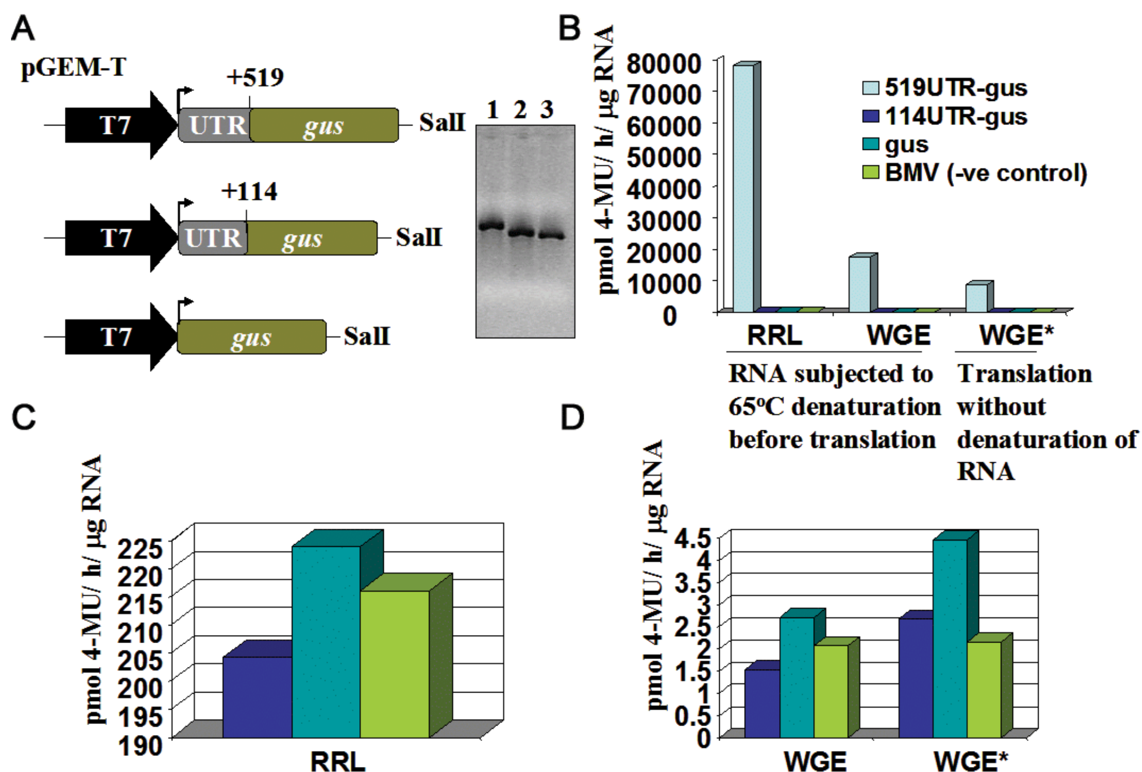

Figure 2.9 Role of *ABI3 5′* UTR in translation *in vitro*. (*A*) Schematic diagram of constructs and 1% agarose/formaldehyde gel showing RNA transcripts from *in vitro* transcription: lane 1, 519UTR-gus; lane 2, 114UTR-gus and lane 3, gus. (*B*) Fluorometric analysis data showing GUS activity of lysate from RRL and WGE after *in vitro* translation reactions. (*C*) and (*D*) Results of (*B*) at a smaller scale showing the GUS activity form *in vitro* translation of 114UTR-gus, gus and BMV RNA. RRL, rabbit reticulocyte lysate; WGE, wheat germ extract.

In silico *analysis of the* ABI3 *promoter*

In silico analysis was performed to examine the profile of *cis*-elements present in the *ABI3* promoter. The *cis*-elements identified were grouped into four categories: seed-specific, light-response, elicitor-response elements and miscellaneous elements (Table 2.3). CCAAT-box elements, involved in enhancing promoter activity (Chaubet et al. 1996), were located within each of the three positive regulatory regions between positions -3714 and -477. T-boxes (AACGTT), known to provide maximal transcriptional activation (Foster et al. 1994), were only found within the -2033 to -364 region. Several *cis*-elements putatively involved in seed-specific expression were identified within the SSR. These include an E-box, an RAV1 binding site, a glutenin-box and a CArG motif. However, these elements were not limited to this region and seed-specific elements (RY-repeat, ABRE/ G-box, A-box, DPBF-core sequence) are also present upstream of this region. Additionally, light-response and elicitor-response elements were found within the three positive regulatory regions (-3600 to -364), suggesting the potential involvement of light and other elicitors such as auxin, ABA or gibberellic acid in the regulation of *ABI3* expression.

Table 2.3 Putative *cis*-elements present in the *ABI3* promoter.

cis-element	Consensus	number present in region -4630/-3600	-3600/-2033	-2033/-882	-882/-364	-364/+1	+1/+519	Function(s)	Reference(s)
Seed-specific elements									
ABRE/ G-box	YACGTGGC	1	1	1	0	0	0	ABA response, seed specificity	(Busk and Pages 1998; Choi et al. 2000)
E-Box	CANNTG	3	5	7	1	3	3	Major motif of napA promoter for high seed-specific expression	(Stalberg et al. 1996)
RAV1 binding site	CAACA	1	1	5	1	0	0	Binding site of RAV1 protein that expresses relatively high in leaves and roots	(Kagaya et al. 1999)
Glutenin box	TGTTAAAGT	0	1	2	1	0	0	Sequence homologous to cereal glutenin gene control element	(Shirsat et al. 1989)
A-box	TACGTA	0	1	1	0	0	0	Binding site for RITA-1 for rice seed protein expression	(Izawa et al. 1994)
CArG-motif	$CC(A/T)_6GG$	0	3	2	1	0	0	MADs domain protein binding	(Tang and Perry 2003)
DPBF-core	ACACNNG	2	2	0	0	1	0	DPBF core binding sequence	(Kim et al. 1997; Finkelstein and Lynch 2000)
AACA-core	AACAAAC	0	0	1	0	1	0	Found in rice glutenin gene, confers endosperm-specific expression	(Wu et al. 2000)
RY-repeat	CATGCA	1	0	1	0	0	0	Important for seed-specific activity of tha *napA* promoter	(Ezcurra et al. 1999)
Skn1-like motif	GTCAT	0	1	2	0	0	0	*cis*-elements requires for endosperm-specific expression	(Washida et al. 1999)
Light-response elements									
GT-1 Consensus	GRWAAW	3	10	9	5	3	2	GT-1 factor binding site found in many light-regulated genes	(Terzaghi and Cashmore 1995)
I-box core	GATAA	2	3	1	2	0	0	Conserved upstream sequence of light-regulated genes	(Terzaghi and Cashmore 1995)
AE-box	AGAAACTT	1	0	0	0	0	0	Part of a light responsive module	(Park et al. 1996)
Box 4	ATTAAT	1	2	1	0	0	0	DNA module associates with light-responsive promoter	(Arguello-Astorga and Herrera-Estrella 1996)
Elicitor-response elements									
ARF binding site	TGTCTC	0	0	0	0	1	0	ARF binding site confers auxin responsiveness of promoter	(Ulmasov et al. 1997)
AURE	CATATG	2	1	0	0	0	1	Auxin response element	(Xu et al. 1997)
ERE	AWTTCAAA	1	0	0	1	0	0	Ethylene responsiveness	(Montgomery et al. 1993)
MYB-GA	TAACAAA	1	1	1	0	0	0	GA-regulated transcription factor binding	(Gubler et al. 1995)
TGACG-motif	TGACG	0	1	0	0	0	0	*cis*-acting regulatory element involvsd in MeJA responsiveness	(Rouster et al. 1997)
LTR	CCGAAA	0	0	1	0	0	0	*cis*-element involves in low temperature responsiveness	(Dunn et al. 1998)
MBS	CAACTG	0	0	1	0	0	0	MYB binding site involves in drought response	(Yamaguchi-Shinozaki and Shinozaki 1994)
Miscellaneous elements									
CAAT-box	CAAT	3	18	13	6	6	3	Common *cis*-acting element in promoter and enhancer regions	(Chaubet et al. 1996)
CCAAT-box	CCAAT	0	3	1	1	1	1	Act with HSE to increase promoter activity	(Rieping and Schoffl 1992)
T-box	AACGTT	0	0	2	1	0	0	b-zip factor binding, for maximal transcription activation	(Foster et al. 1994)

bZip, basic-leucine zipper; HSE, heat shock promoter element; ABRE, abscisic acid response element; napA, napin A; RAV1, related to ABI3/VP1; RITA-1, rice transcription activator-1; DPBF, Dc3 promoter binding factor; ARF, auxin response factor; AURE, auxin response element; ERE, ethylene response element; MeJA, methyl jasmonate; LTR, low temperature response; MBS, Myb binding site

ABA can induce ABI3*-driven expression in vegetative tissues*

ABI3 is one of the major components involved in the ABA signal transduction pathway (Parcy et al. 1994; Giraudat 1995; Meinke 1995; Merlot and Giraudat 1997; Bonetta and McCourt 1998) and *in silico* promoter analysis revealed several ABREs within the *-4630/+519ABI3* upstream sequence (data not shown), prompting an examination of the effect of ABA on *ABI3* expression. Transgenic seeds harboring *-4630/+519ABI3-gus* were germinated for 2 weeks in MS medium, then transferred to MS medium (control) or MS medium containing various concentrations of ABA (0.1 µM, 0.3 µM, 0.5 µM, 1 µM, 5 µM, 10 µM and 100 µM). Histochemical GUS staining was performed 7 days after transfer and, as was recently reported (Brady et al. 2003) for a similar construct, in the presence of exogenous ABA at concentrations as low as 0.3 µM, GUS expression was evident in roots and the intensity of staining increased as the concentration of ABA was increased (Fig. 2.10B-F). However, no GUS expression was detected in vegetative tissues in the absence of ABA (Fig. 2.10A). Similar experiments were conducted using seedlings harboring the *-3600/+519ABI3-gus* and *-2033/+519ABI3-gus* constructs in the presence of 100 µM ABA. Although ABA-induced GUS expression in roots was observed in all cases, no GUS expression was detected in leaves or other vegetative tissues of plants grown in MS medium without ABA (data not shown). In contrast, GUS expression was detected in roots of seedlings transgenic for constructs lacking the 5' UTR region grown on MS medium (Fig. 2.6E,F), and increased GUS expression was detected in the presence of exogenous ABA (data not shown). These results are consistent with the finding that the 5' UTR of *ABI3* plays a negative regulatory role in its expression. No induction of GUS expression by exogenous

application of ABA was found for roots of plants transgenic for the truncated constructs *-882/+519ABI3-gus*, *-364/+519ABI3-gus* or *-23/+519ABI3-gus* (that include the 5' UTR).

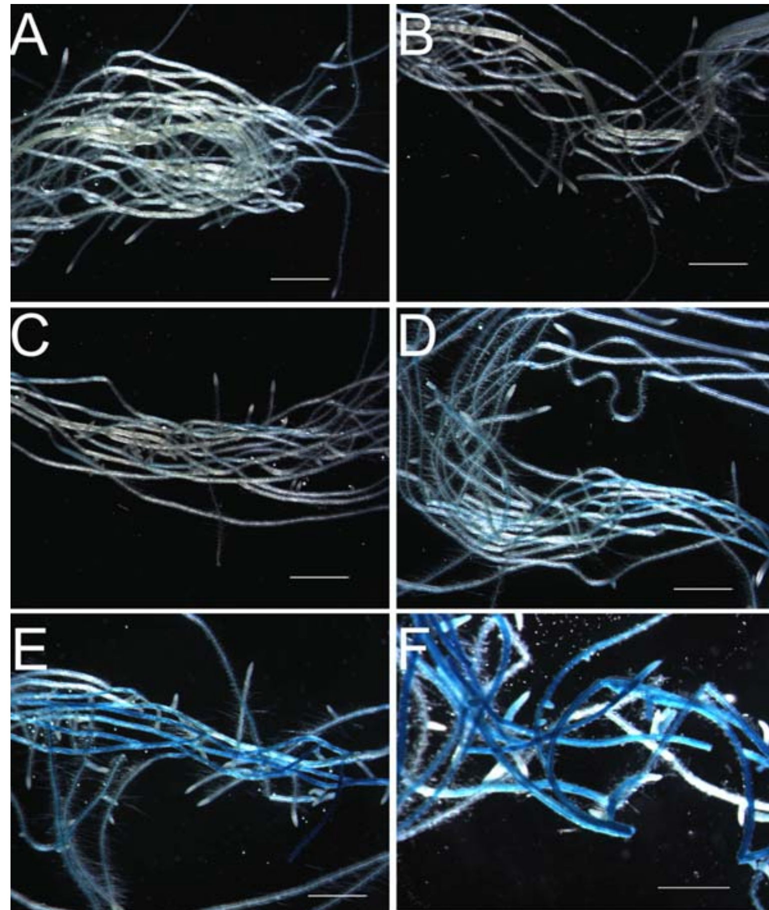

Figure 2.10 Induction of GUS expression in roots of transgenic seedlings by ABA. GUS-stained roots are shown for *-4630/+519ABI3-gus* seedlings grown in MS medium (*A*) lacking or (*B-F*) containing ABA at various concentrations: (*B*) 0.3 µM ABA, (*C*) 1 µM ABA, (*D*) 5 µM ABA, (*E*) 10 µM ABA and (*F*) 100 µM ABA. Bar = 1 mm.

Effect of light/dark treatment on ABI3*-driven expression*

(Rohde et al. 1999) reported *ABI3*-driven GUS expression in shoot apices for 10-15% of 355-377 plants that were germinated in the dark for 10-15 days. No expression of GUS was

observed for plants maintained in the light. Seeds transgenic for the *-4630/+519ABI3-gus* construct were germinated under similar experimental and tested for GUS expression after 15 days culture in the dark. Ten or more plants representing five independent transgenic lines were examined. Although GUS was expressed in all lines during embryogenesis, confirming the functionality of the transgene insert, no *ABI3*-driven GUS expression was detected in shoot or root apices (or other vegetative tissues) of the dark-grown seedlings. Similarly, seedlings grown in the light for 5 days and then transferred to the dark for 15 days failed to exhibit GUS expression in vegetative tissues.

Discussion

Spatial regulation of ABI3 *expression*

Expression of GUS from the full-length *ABI3* promoter was consistent with the results reported by Parcy et al. (1994) in that reporter accumulation started at the globular stage and continued through the heart, torpedo and mature stages of embryogenesis. However, GUS expression was also found to occur during microsporogenesis (Fig. 2.2A,B). This finding is in accord with the observation of *phas* promoter activity (dependent on the presence of PvALF, the bean ortholog of ABI3) during pollen development in *Arabidopsis* transgenic for -1470phas-gus (Chandrasekharan et al. 2003b). Constructs containing the GFP reporter showed similar expression during embryogenesis (Fig. 2.2C-E) to that for GUS. No expression was observed in vegetative tissues for either reporter. However, ectopic expression of ABI3, but only in conjunction with exogenously supplied ABA, led to the

activation of *phas* in leaves, reflecting the functional orthology of ABI3 and PvALF (Fig. 2.3D). Indeed, the absence of GUS expression from the *phas* promoter in leaves, even in the presence of ABA (Fig. 2.3C), provides strong evidence that the *ABI3* promoter is silent in vegetative tissue.

The above results, together with the *ABI3*-driven reporter expression in embryos (Fig. 2.2) support earlier findings (Giraudat et al. 1992; Parcy et al. 1994) that ABI3 is normally a seed-specific factor. However, it has been reported that, under certain experimental regimes, *ABI3*-driven GUS expression can be induced in vegetative tissues. For example, Rohde et al. (1999) observed GUS expression in shoot apices of dark germinated seedlings and Brady et al. (2003) have shown that weak GUS expression was induced in roots of plants transgenic for *ABI3-GUS* (Parcy et al. 1994) by 3 days culture in 0.4 μM ABA. Culture of T_1 seedlings transgenic for *-4630/+519ABI3-gus* for 7 days in the presence ABA at concentrations ranging from 0.3 μM to 100 μM resulted in strong expression of GUS in roots (Fig. 2.10B). A similar observation was obtained for plants transgenic for *-3600/+519ABI3-gus* and *-2033/+519ABI3-gus* constructs but not in those transgenic for *-882/+519ABI3-gus*, *-364/+519ABI3-gus* or *-23/+519ABI3-gus* constructs in the presence of 100 μM ABA. This is in agreement with the presence of an abscisic acid response element in upstream activation sequence UAS1, in UAS2 and in the intergenic region (IGR), but not in the SSR. In contrast, we did not detect *ABI3*-driven GUS expression in seedlings germinated in the dark, as was found by Rohde et al. (1999). Nevertheless, it is evident that *ABI3*-driven GUS expression in roots can be triggered by exogenously supplied ABA despite the fact that ABI3 acts primarily as a transcriptional

activator for seed-specific gene expression.

Functional regions within the ABI3 *promoter*

Reporter fusions to 5' truncations of the *ABI3* promoter revealed similar expression patterns for both GUS and GFP, although GUS proved to be a more sensitive reporter (Fig. 2.4A,B). Several regulatory regions were defined on the basis of fluorometric assays of GUS expression (Table 2.2). Since no augmentation of activity was observed for the -4630 to -3600 region, this was defined as an IGR (Fig. 2.4E), even though it probably includes the 3' terminator of the upstream gene. Promoter lengths of 3600 bp and 2033 bp yielded embryo-specific expression levels that were many-fold greater than that for the -882 truncation and the regions -3600 to -2033 and -2033 to -882 are defined as UAS2 and UAS1, respectively.

The absence of GUS expression in the radicle region of embryos transgenic for *-2033/+519ABI3-gus* and *-882/+519ABI3-gus* (Fig. 2.4A,B) implies the presence of module-specific *cis*-element(s) upstream of position -2033. Module-specific gene expression in plant embryos has been described previously. For example, Perez-Grau and Goldberg (1989) reported modular expression of the *kti3* (Kunitz proteinase inhibitor) gene in soybean embryos and Goldberg et al. (1994) documented differential promoter activity in specific compartments within embryos of transgenic tobacco. The involvement of multiple *cis*-elements within the *phas* promoter in module-specific expression has also been demonstrated (Bustos et al. 1991; Chandrasekharan et al. 2003a).

Truncation of the promoter to position -364 led to a dramatic loss of embryo-specific

GUS expression in construct *-364/+519ABI3-gus*, revealing the presence of important *cis*-elements within the -882 to -364 region. Placement of this region upstream of the minimal *-64/+6CaMV 35S* promoter yielded seed-specific expression (Fig. 2.4C,D), defining it as the SSR. While elements important for spatial regulation are often present in the proximal region of promoters, deletion of the -364 to +1 proximal region (PR) did not affect seed-specificity of expression from the *-882/-364ABI3* promoter, nor did constructs (*-364/+519ABI3-gus*; *-23/+519ABI3-gus*) that include this region yield significant reporter expression in any tissue.

In silico *analysis of the* ABI3 *promoter revealed a plethora of* cis-*elements*

Many *cis*-element motifs were identified using PlantCARE and PLACE software; these were grouped into several functional categories (Table 2.3). The presence of seed-specific elements within the SSR suggests their involvement in conferring seed-specific expression to the *ABI3* promoter. However, it is envisioned that additional seed-specific elements outside the SSR may be required for complete promoter activity, and such elements were identified within the UAS1 and UAS2. Similarly, the presence of light-response and elicitor-response elements in the *ABI3* promoter suggested potential roles for environmental signals (light, temperature and drought) and phytohormones (ABA, auxin, ethylene, gibberellic acid) in its regulation.

Although functional roles can be predicted for the various *cis*-elements identified by *in silico* analysis, experimental evidence is required to determine the actual functional roles of these *cis*-elements in *ABI3* expression. Genomic footprinting has been widely used

to study factor-promoter interactions. Li and Hall (1999) identified multiple factor interactions with the *phas* promoter by *in vivo* DMS footprinting and Kosoy et al. (2002) showed the presence of multiple GAGA binding sites on the *Trl* (trithorax-like) promoter in *Drosophila* via DNase I footprinting. Similar analyses are needed for the *ABI3* promoter, but its 4.7 kb length requires the preliminary identification of regulatory regions and putative functional *cis*-elements undertaken here.

Quantitative and spatial regulatory roles of the 5' UTR

We have established that the 405 bp 5' UTR strongly represses both quantitative and spatial expression from the *ABI3* promoter. For example, higher GUS expression was observed in seeds harboring fusion constructs lacking the 5' UTR than in seeds bearing constructs that include the 5' UTR (Fig. 2.6A-D; Table 2.2) and the normal spatial constraint on *ABI3* expression in roots was lost, even in the absence of ABA (Fig. 2.6E,F). Modulation of spatial constraint via the 5' UTR has been demonstrated for the embryo-specific barley lipoxygenase gene *lox1*: substitution of the 5' UTR of an aleurone-specific gene (*Chi26*) with that of *lox1* yielded expression of *Chi26* in the embryo as well as in the aleurone cells (Rouster et al. 1997).

Brady et al. (2003) showed that *ABI3* promoter-driven GUS expression is increased in the *era1* mutant background and that punctate GUS expression occurred in roots. *Era1* encodes the β subunit of a protein farnesyl transferase and it has been suggested that a farnesylation-dependent negative regulator is involved in ABA signaling (Cutler et al. 1996). The similarity between the effect of the *Era1* mutation in *ABI3* expression and our

observation of the effects of the 5' UTR deletion in *ABI3* expression suggests the existence of a farnesylation-dependent negative regulatory factor that is involved in transcriptional or post-transcriptional regulation of ABI3 expression through its interaction with the 5' UTR. Additionally, it has been suggested that RNA-binding proteins are involved in the ABA signal transduction pathway (Lu and Fedoroff 2000; Hugouvieux et al. 2001; Xiong et al. 2001a; Xiong et al. 2001b; Li et al. 2002). For example, *ABH1* that encodes a subunit of a dimeric *Arabidopsis* nuclear cap-binding complex (CBC) (Hugouvieux et al. 2001), is involved in mRNA processing in yeast and human HeLa cells (Izaurralde et al. 1994; Gorlich et al. 1996; Ishigaki et al. 2001). *abh1* mutants have reduced transcript levels of AtPP2C, a proposed negative regulator of ABA signaling (Sheen 1998; Hugouvieux et al. 2001). Another example of interaction between ABA signal transduction and mRNA metabolism is the *hyl1* mutation which causes ABA hypersensitivity in seed germination and root growth (Lu and Fedoroff 2000). *HYL1* encodes a dsRNA-binding protein and negatively regulates the level of ABI5 expression through the mitogen-activated protein kinase signaling cascade (Lu et al. 2002). Given the involvement of these RNA binding proteins in ABA signal transduction, it is possible that ABI3 expression may also be modulated post-transcriptionally through interaction(s) between these proteins or as yet unidentified RNA-binding protein(s) and its 5' UTR.

In addition to the possibility that post-transcriptional mRNA metabolism may be involved in ABI3 expression, as is the case for the 5' UTR of many mRNAs encoding proto-oncogenes, growth factors and transcription factors (Kozak 1987, 1991a), the possibility exists that 5' UTR-regulation of ABI3 expression is translational. The presence

of a long leader can be inhibitory as its sequence or secondary structure may contain features that repress translational initiation of the downstream main ORF (Kozak 1987, 1991a). Han et al. (2003) recently found that the 828 base-long 5' UTR of the mouse tumor suppressor gene *PTEN* severely inhibited translation of both PTEN and firefly luciferase mRNAs. Deletion of a large segment of the *PTEN* 5' UTR greatly enhanced translation efficiency. The presence of three short uORFs in, and the overall high predicted hairpin secondary structure (Fig. 2.5) of, the *ABI3* mRNA leader thus provides an alternate model that their presence may impair translation initiation which in turn affect mRNA stability or protein level. Supporting evidence has been shown in the case of maize *Lc* mRNA that the presence of a 38-codon uORF and a potential hairpin structure represses translation of the downstream ORF (Wang and Wessler 1998, 2001). uORFs in the leader of rice *myb7* mRNAs were also found to inhibit translation *in vivo* and *in vitro* (Locatelli et al. 2002). In addition, the presence of nonsense codons in the 5' UTR may destabilize mRNA through a nonsense-mediated mRNA decay mechanism (Kebaara et al. 2003)). In the yeast *Saccharomyces cerevisiae*, insertion of a small uORF into the 5' UTR of *Cat* mRNA led to a decrease in the translation of the downstream ORF and the stability of the mRNA (Oliveira and McCarthy 1995). Insertion of nonsense codons in the 5' UTR of ferridoxin-1 (*Fed-1*) mRNA decreased its stability under light conditions (Petracek et al. 2000). mRNA secondary structure can also affect cap-dependent translation initiation (Pelletier and Sonenberg 1985). For example, introduction of various stem-loop structures 5' to luciferase mRNA decreased its translation efficacy (Niepel et al. 1999). Comparison of the 5' UTR of ABI3 (519 bp) with orthologous genes from *Chamaecyparis nootkatensis* (accession

AJ131113; 516 bp) revealed the presence of 5 uORFs (of more than 5 amino acids); 2 in *Daucus carota* (accession AB005558; 380 bp), and 6 in *Pisum sativum* (accession AB080195; 761 bp). The presence of long 5' UTRs bearing uORFs in these ABI3 relatives suggests that the presence of a negative regulatory region may be a common feature.

There are many facets to ABA-mediated signal transduction (Rock 2000). Among the complexities lies the fact that some events are stimulatory while others are repressive. The pleiotropic functions of B3 domain-containing factors such as ABI3, VP1 and PvALF, that modulate ABA-induced gene expression, further compound the situation. The recognition in this study of various regulatory domains within the 4.6 kb *ABI3* promoter can facilitate the identification of important *cis*-element(s) or *trans*-acting factor(s) that may involve in the *ABI3* expression. In addition, the potentially high levels of transcription that the *ABI3* promoter can drive, are mitigated by the repressive activity of the 5' UTR that, in turn, presumably reflects interactions with RNA-binding proteins. These findings provide new insight to developmental regulation of plant growth, especially during embryogenesis.

CHAPTER III

DECIPHERING HISTONE CODE IN THE *PHAS* CHROMATIN

Introduction

Although a wealth of information exists concerning the overall regulation of transcription from eukaryotic promoters, much remains to be learned as to how specific promoters are selected for activation. In plants, the *phas* promoter provides an excellent system to explore this challenge as it is silent in all vegetative tissues of the bean (*Phaseolus vulgaris*) plant (van der Geest et al. 1995), but becomes exceptionally transcriptionally active during development of the seed embryo (Hall et al. 1999; Li et al. 2001).

The contrast between the complete failure of *phas*-*gus* constructs to express β-glucuronidase (GUS) in vegetative tissues when stably integrated into the genome and the abundant expression of GUS from the same constructs when transiently inserted into leaves as naked DNA (Frisch et al. 1995), provided compelling circumstantial evidence for the involvement of chromatin in the regulation of *phas* expression. Experimental evidence was obtained by *in vivo* and *in vitro* footprinting studies that the lack of transcriptional expression in vegetative tissues is stringently maintained by a rotationally and translationally positioned nucleosome over the three phased TATA boxes of the *phas* promoter (Li et al. 1998), each of which contributes to its high level of expression (Grace et al. 2004). A significant finding was that, although transcription from the *phas* promoter is not inducible in callus or vegetative tissues by the plant growth regulator abscisic acid

(ABA) alone (Frisch et al. 1995), ectopic expression of a seed-specific transcriptional activator, PvALF (*Phaseolus vulgaris* ABI3-like factor) from the quasi-constitutive *CaMV 35S* promoter (Moravcikova et al. 2004), renders *phas* ABA-inducible in vegetative tissues (Li et al. 1999). Increased DNase I accessibility to the *phas* promoter in isolated nuclei was observed in the presence of PvALF, but the TATA boxes became protected in the presence of both PvALF and ABA, suggesting that chromatin remodeling facilitates occupancy by TATA-binding protein (TBP) under those conditions. These observations provided evidence for a two-step process of *phas* activation in which the first step (potentiation) requires the presence of PvALF and the second step (activation) is achieved by ABA acting through a signal transduction pathway. Placement of PvALF expression under the control of an estradiol-inducible promoter (Zuo et al. 2000) permits analysis of the chromatin status over the *phas* promoter under three discrete conditions. To maintain the *repressed* state, no estradiol is supplied so that PvALF production is uninduced and no ABA is added. The *potentiated* state is attained by supplying estradiol and hence PvALF, but no ABA. When both estradiol and ABA are supplied the *phas* promoter is transcriptionally *active*. This system permits the discrete distinction of events related to remodeling of nucleosome architecture over the promoter from the ABA-motivated recruitment of TBP and initiation of transcription.

Evaluation of covalent histone modifications associated with developmental stages and transcriptional status of eukaryotic promoters has verified the existence of an epigenetic code (Jenuwein and Allis 2001) and rapid advances are being made in deciphering its roles in developmental processes of higher organisms (Margueron et al. 2005). Studies on the

recruitment of specific factors or complexes by specific histone states are providing exciting insights to gene regulation. In plants, elegant studies on vernalization and control of flowering time have revealed that the chromatin status over the *FLOWERING LOCUS C* (*FLC*) is influenced by three regulatory systems and controls downstream flowering-time integrator genes that in turn activate floral meristem-identity genes (Boss et al. 2004; Putterill et al. 2004; He and Amasino 2005). Among animal systems, characterization of histone modifications over the *IFN-β* promoter following infection by Sendai virus is providing novel insight to how transcription is initiated (Agalioti et al. 2002).

A challenging question regarding chromatin dynamics is the fate of the nucleosome during transcriptional activation. Using a novel photochemical method for mapping the contacts of specific histone residues with DNA in the nucleosome before and after remodeling, Kassabov et al. (2003) demonstrated that, in addition to sliding nucleosomes, SWI/SNF displaces DNA off the octamer in a process that remodels 50-bp of DNA within 1 second. This concept appears to be in good accord with histone changes seen here for the *phas* promoter.

In the present work, we show that three discrete conditions of the *phas* promoter are reflected in various arrays of chromatin modifications. In addition to the discrete separation of potentiation from activation, our system allows chronological studies that provide insight to the ordered recruitment of histone modifiers. Insight gained from these studies suggests the existence of close similarities between transcriptional activation of the *phas* and *IFN-β* promoters.

Materials and Methods

Antibodies for ChIP analyses

Antibodies used in ChIP assays were purchased from either Upstate (NY) or Abcam (UK) including: anti-histone H3 *N*-ter (Upstate, #06-755), anti-histone H3 *C*-ter (Abcam, Ab1791), anti-histone H4 *C*-ter (Abcam, Ab10158), anti-acetyl-histone H3 (Upstate, #06-599), anti-acetyl-histone H3-K9 (Upstate, #07-352), anti-acetyl-histone H3-K14 (Upstate, #07-353), anti-dimethyl-histone H3-K4 (Upstate, #07-030), anti-trimethyl-histone H3-K4 (Upstate, #07-473), anti-hyperacetylated histone H3 (Upstate, #06-946), anti-dimethyl-histone H3-K9 (Upstate, #07-441; Abcam, Ab7312), anti-acetyl-histone H4-K5 (Abcam, Ab1758) and anti-dimethyl-histone H4-K20 (Upstate, #07-367).

Plasmid construction

A triple-HA tag sequence (3xHA) was PCR-amplified from pMPY-3xHA (a kind gift from Dr. Michael P. Kladde, Texas A&M University), and cloned into a pGEM-T vector (Promega) to yield pGEM-T/3xHA. The 2.3-kb *PvAlf* coding region without the ATG start codon was amplified by PCR from pXVE-HisSPvAlf (Chandrasekharan et al. 2003b) to incorporate flanking BtsI and PacI restriction enzyme sites and cloned into a pGEM-T vector to yield pGEM-T/PvAlf. The *PvAlf* coding region was then released by BsmI and PacI digestion and fused 3' to the 3xHA sequence in BsmI-PacI-digested pGEM-T/3xHA vector to give pGEM-T/HAPvAlf. The *HAPvAlf* fragment was then cloned into pER8 vector (Zuo et al. 2000) through ApaI and PacI enzyme sites. The resulting construct, pER8/XVE-HAPvAlf, was transformed into *Agrobacterium* strain *GV3101* and used for

Arabidopsis transformation to yield estrogen-inducible expression of HA-PvALF.

Plant transformation

Transgenic *Arabidopsis thaliana* (Columbia ecotype) seeds (line 5'14) containing -1470phas-gus (Chandrasekharan et al. 2003a) were germinated on Murashige and Skoog (MS) agar medium (Sigma) containing 50 mg/L kanamycin (Sigma). Seeds were subjected to vernalization at 4°C for 2 days and grown at 22°C under a 16/8 h light/dark cycle. *Agrobacterium*-mediated transformation with pER8/XVE-HAPvAlf of 4 to 5 week old 5'14 plants was conducted with vacuum infiltration (Bechtold and Pelletier 1998). *Arabidopsis* supertransformants (5'14HAPvAlf) were selected by plating T_0 seeds in MS agar containing hygromycin (25 mg/L) and kanamycin (50 mg/L). Transformants with a single homozygous insertion for both *XVE-HAPvAlf* and *-1470phas-gus* transgenes were obtained through antibiotic selection and genomic blot analyses.

Estradiol and ABA induction conditions

5'14HAPvAlf seeds were germinated in hygromycin (25 mg/L) and kanamycin (50 mg/L) selection media and rosette leaves from 3 to 4 week-old plants were collected and transferred to liquid MS media containing 25 μM 17β-estradiol (Sigma) or 200 μM ABA (Sigma). Four independent treatments were performed: (U) uninduced control without addition of estradiol and ABA; (E) 25 μM estradiol alone treatment; (EA) 25 μM estradiol and 200 μM ABA treatment and (A) 200 μM ABA alone control. Leaves were treated for 8 h with gentle agitation in the light unless specified. For chronological experiments in

which estradiol was removed prior to ABA addition, leaves were exposed to MS media containing 25 μM 17β-estradiol for 1 to 4 h, after which estradiol was removed by rinsing with running distilled water. The leaves were then exposed for the times indicated to MS media containing 200 μM ABA.

Histochemical and fluorometric assays for GUS activity

Histochemical staining and fluorometric analysis of GUS were performed with 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid (X-gluc) or 4-methylumbelliferyl-ß-D-glucuronide (4-MUG), respectively, as substrates (Jefferson et al. 1987). For fluorometric analysis, GUS activity was calculated as pmol 4-MU/h/μg protein.

Protein SDS-PAGE and Western blot analyses

After the specific induction treatment (U, E, EA or A), total protein was extracted from leaves (100 mg) using an extraction buffer containing 200 mM MOPS (4-morpholinepropanesulfonic acid), pH 7.5, 200 mM KCl, 20% glycerol, 1 mM EDTA and 3 mM DTT. Total protein was quantitated by the Bradford assay (BioRad) and samples (40 μg protein) were subjected to 12.5% SDS-PAGE. Benchmark pre-stained protein ladder (Invitrogen) was used as a molecular marker for SDS-PAGE. Two parallel gels were prepared, one for Coomassie blue staining after electrophoresis and the other for Western blot analysis. For Western blot analysis, total leaf proteins separated after SDS-PAGE were transferred to PVDF (BioRad) membrane. Polyclonal HA-antibodies (Covance Research Products) at 1:500 dilution were used for Western blots with chemiluminescent detection

to verify the presence of HA-PvALF in the samples.

RT-PCR analysis

Total RNAs were extracted from leaves after various experimental treatments using TRIzol reagent (Invitrogen). DNase I-digested total RNAs (0.5 μg) were subjected to RT-PCR analysis using a Qiagen one-step RT-PCR kit. Gene-specific primer sets (0.6 μM), alf-rt-for (5'-ATGGAGTGTGAAGTGAAGTTAAAAGGGGG-3') and alf-rt-rev (5'-TGAAAGATTGAGGCAGGATCGAAGAAATCATTG-3'), 1-gus-for (5'-ATGGTCCGTCCTGTAGAAACCC-3') and 1-gus-rev (5'-CGATGGATTCCGGCATAGTTAAAGA-3') were used to detect the presence of *HA-PvAlf* and *gus* transcripts respectively. Primer pair (0.08 μM), ef-for (5'-TGCTGTCCTTATCATTGACTCCACCAC-3') and ef-rev (5'-TTGGAGTACTTGGGGGTAGTGGCATC-3'), targeting the *EF1*α gene were used as an internal control for the RT-PCR. The RT-PCR products were resolved in 1% agarose gel and visualized by ethidium bromide staining.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was modified as briefly described below from Gendrel et al. (2002). In brief, 3 to 4 g of *Arabidopsis* leaves from 3 to 4 week-old 5'14HAPvAlf plants were used for each induction treatment (U, E, EA or A) for 8 h. After 8 h, the samples were subjected to 1% formaldehyde cross-linking in a cross-link buffer (0.4 M sucrose, 10 mM Tris-HCl pH 8, 1 mM EDTA) under vacuum for 10 min. Formaldehyde cross-linking was

stopped by adding glycine to a final concentration of 0.125 M and incubated for 5 min at room temperature. Then leaves were rinsed with water and ground into powder in liquid nitrogen. Nuclei were extracted and lysed with 300 μL lysis buffer [50 mM Tris-HCl pH 8, 10mM EDTA, 1% SDS, 1 mM PMSF, 10 mM Na butyrate, 1 mM benzamidine and 50 μL/mL protease inhibitor cocktail (Sigma)]. The resulting chromatin was subjected to pulse sonication (6 pulses, 95% power output for 8 times) using a Branson sonifier M350 (Branson sonic power company) to obtain DNA fragments with sizes ranging from 500- to 1000-bp. After sonication, a 25 μL aliquot was removed for the total input DNA control and the rest of the chromatin solution was diluted ten times with dilution buffer (1.1% TritonX-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8, 167mM NaCl, 10 mM Na butyrate and 50 μL/mL protease inhibitor cocktail). The diluted chromatin solution was then subjected to 1 h preclearing treatment at 4°C with 40 μL salmon sperm DNA/protein A agarose [50% suspension in dilution buffer (Upstate, #16-157) without Na butyrate and protease inhibitor cocktail] to reduce non-specific interactions between protein-DNA complexes and the agarose beads. Overnight (17 h) immunoprecipitation was performed at 4°C using 600 μL chromatin solution with antibodies typically at a final 1:150 dilution or without antibodies (mock control). Immunoprecipitates were collected following incubation with 40 μL salmon sperm DNA/protein A agarose (50% suspension in dilution buffer) at 4°C for 1 h. The protein A agarose beads bearing immunoprecipitates were then subjected to sequential washes and immunoprecipitates were eluted twice with 300 μL elution buffer (1% SDS and 0.1 M $NaHCO_3$). Samples were then reverse cross-linked at 65°C under high salt (0.2 M NaCl) conditions for 6 h. For the input DNA control (25 μL), 275 μL TE buffer

was added and reverse-cross-linked. After reversing cross-links, protein was removed by proteinase K digestion and DNA was purified by phenol-chloroform extraction and ethanol precipitation. Purified DNA was resuspended in 40 μL TE buffer for PCR analyses.

ChIP PCR and densitometry

Primers targeting the proximal region (-282 to -55) of the *phas* promoter, -282Phas-for (5'-CCGCGTCCATGTATGTCTAAATGC-3'), -55Phas-rev (5'-GGTTGGAACATGCATGGAGATTTGG-3') and the *actin2*, actin-for (5'-CGTTTCGCTTTCCTTAGTGTTAGCT-3'), actin-rev (5'-AGCGAACGGATCTAGAGACTCAC-3'), were used for analysis of the immunoprecipitated DNA in ChIP assays. PCR products were visualized by ethidium bromide staining in 1.5% agarose gels. Densitometry of data obtained from ethidium bromide staining of PCR products for at least two completely independent experiments was performed using ImageJ software (National Institutes of Health). Signals from the *phas* or the *actin2* PCR products were normalized against the mock (no antibody) signal and input DNA control signal, respectively. The signal enrichment for *phas* DNA for each individual treatment (U, E, EA or A) was then normalized with its respective *actin2* control. RE values were calculated for each of the four different treatments.

Results

Inducible potentiation and activation system for phas *expression in vegetative tissues*

The seed-specific activation of transcription from the *phas* promoter is a 2-step process that includes PvALF-mediated remodeling of its chromatin architecture (potentiation) and subsequent activation through an ABA-mediated signaling cascade (Li et al. 1999). In developing seeds, events associated with *phas* potentiation and activation are inseparable as both PvALF and ABA are present. In order to differentiate between these sequential events, an estrogen receptor-based inducible system (Zuo et al. 2000) was used (Fig. 3.1A) to permit ectopic expression of a hemagglutinin (HA) epitope-tagged PvALF (HA-PvALF) in leaves of *Arabidopsis* transgenic for a -1470phas-gus reporter construct (Chandrasekharan et al. 2003a). A triple HA tag sequence was fused 5' to the *PvAlf* coding sequence (*HA-PvAlf*) and inserted downstream of a synthetic promoter containing the LexA operator to yield the inducible construct *XVE-HAPvAlf*. XVE is a chimeric protein that contains a LexA DNA binding domain, a VP16 activation domain and a human estrogen receptor site (Zuo et al. 2000). When leaves of transgenic *Arabidopsis* line 5'14HAPvAlf that contains both -1470phas-gus reporter and XVE-HAPvAlf effector constructs are exposed to estradiol, the XVE transactivator protein is induced and binds to the *LexA* operator, driving ectopic expression of HA-PvALF. The presence of HA-PvALF potentiates the repressed *phas* promoter for transcriptional activation upon the addition of ABA.

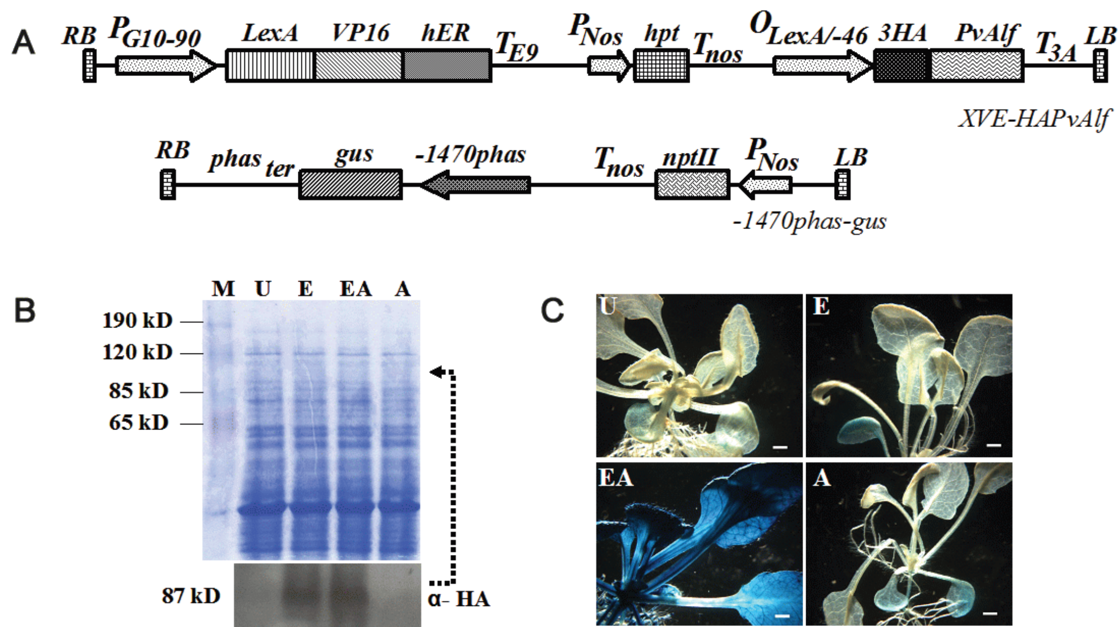

Figure 3.1 Induced ectopic expression of HA-PvALF and *phas* activation in leaves. (*A*) Schematic diagram of the -1470phas-gus reporter construct and XVE-HAPvALF inducible effector construct present in 5'14HAPvAlf *Arabidopsis* supertransformants. (*B*) 12.5% SDS PAGE (*top*) and Western analysis with anti-HA antibody of a parallel gel (*bottom*) of total protein (40 µg/lane) from leaves treated with: 25 µM estradiol (E), 25 µM estradiol + 200 µM ABA (EA) or 200 µM ABA (A). U: uninduced control. M: Protein size markers. (*C*) Histochemical analysis of GUS expression in representative 5'14HAPvAlf plantlets for each treatment. Bar = 1 mm.

To confirm that HA-PvALF expression was dependent on ectopically-supplied estradiol, leaves from 5'14HAPvAlf plants were placed for 8 h in MS medium (see Materials and Methods) containing 25 µM estradiol (E); 25 µM estradiol and 200 µM ABA (EA) or 200 µM ABA (A). Leaves exposed under identical conditions to media lacking estradiol or ABA served as uninduced controls (U). Immunoblot analyses of total protein extracts confirmed that HA-PvALF was only produced in leaves exposed to media containing estradiol (Fig. 3.1B). Histochemical staining for GUS expression confirmed that

transcription from the *phas* promoter in leaves occurred only in the presence of both HA-PvALF and ABA (Fig. 3.1C, panel EA). These experiments confirmed the feasibility of separating the potentiated state of the *phas* promoter (as a result of HA-PvALF expression) from the transcriptionally active state in leaf tissues through the use of an inducible expression system. This permitted detailed analyses of changes in histone modifications associated with each step of transcriptional activation of the *phas* promoter in leaves. Such separation of potentiation from activation is not practicable in seeds because of the presence of both PvALF and ABA and because the very small size of developing *Arabidopsis* embryos greatly limits the amount of material suitable for characterization of chromatin status.

Potentiation and activation of phas *can be temporally separated*

To optimize the induction conditions for HA-PvALF production and ABA activation of the *phas* promoter, *phas*-driven GUS production was followed. Rosette leaves from 3 to 4-week old 5'14HAPvAlf plants were subjected to estradiol (25 μM) and ABA (200 μM) treatment and samples were collected for GUS staining at various time points (Fig. 3.2A). GUS expression was initially detected after 4 h and uniform histochemical staining in the leaves was obtained after 8 h. This indicated that potentiation resulting from HA-PvALF production occurs within 4 h of estradiol addition.

In another set of experiments, leaves of 5'14HAPvAlf plants were exposed to estradiol for 1 to 4 h to induce the production of HA-PvALF, after which the estradiol was removed. ABA was added following the removal of estradiol (Fig. 3.2B). Histochemical

staining of leaves treated with estradiol for 4 h revealed that GUS was produced substantially earlier (1 h) than for leaves treated for only 1 h with estradiol. This suggests that the rate of GUS production is dependent on the amount of HA-PvALF available. After 5 h of exposure to ABA, uniform GUS expression was observed for all treatments and, unless otherwise specified, 8 h was chosen as the standard incubation time for all subsequent analyses.

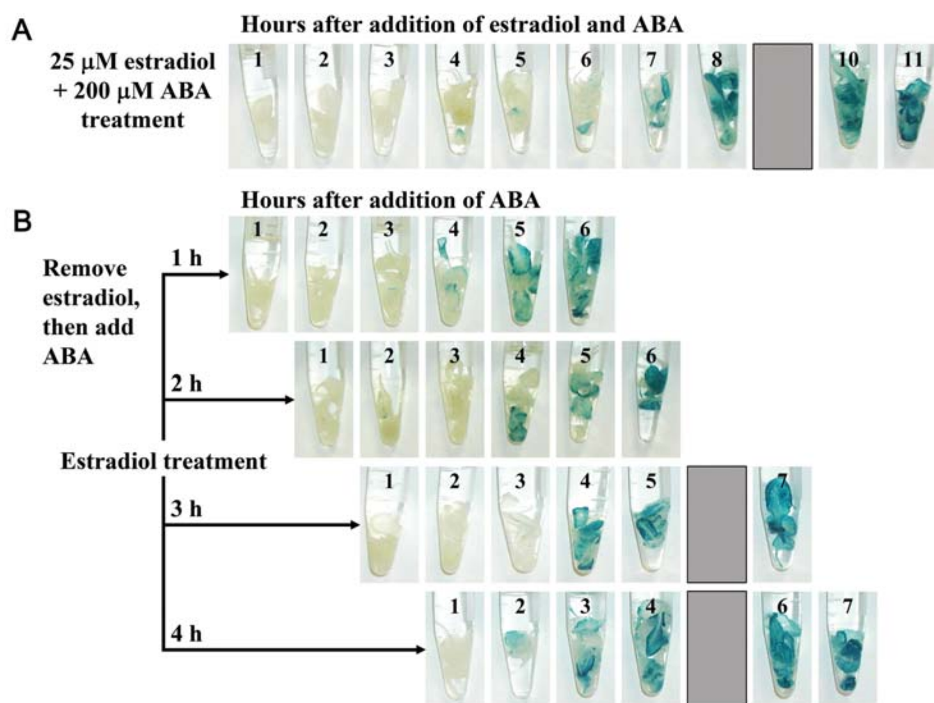

Figure 3.2 Activation of the *phas* promoter. (*A*) Histochemical staining of leaves from 5'14HAPvAlf line at the indicated times following addition of 25 μM estradiol and 200 μM ABA. (*B*) *phas* was potentiated by treatment of leaves from 5'14HAPvAlf line with 25 μM estradiol for 1 to 4 h. ABA was added to a final concentration of 200 μM after estradiol was removed at the indicated times. Histochemical-stained leaves are shown for the indicated times following ABA addition.

Nucleosomal architecture of the phas *promoter during potentiation and activation*

A rotationally positioned nucleosome over the three phased TATA boxes in the *phas* promoter represses activation in vegetative tissue (Li et al. 1998). In transgenic tobacco leaves ectopically expressing PvALF, DNase I hypersensitivity assays coupled with ligation-mediated PCR (LM-PCR) showed an increase in DNase I sensitivity over the TATA region of the *phas* promoter (Li et al. 1999). These assays also revealed a shift in the rotationally positioned nucleosome, commensurate with remodeling of nucleosome association with the *phas* promoter in the presence of PvALF.

In this study, chromatin immunoprecipitation (ChIP) was used to determine the nucleosomal condition of the *phas* promoter upon potentiation and activation in leaves from 5'14HAPvAlf plants under the four experimental regimes (U, E, EA and A) described in the legend to Figure 3.1. DNA purified after immunoprecipitation was evaluated by PCR using primers targeting the proximal region of the *phas* promoter. The targeted 227-bp amplicon includes four RY elements [the binding motif for PvALF: Carranco et al. (2004)] and a G-box [abscisic acid response element: Ezcurra et al. (2000)] that are essential for *phas* expression (Chandrasekharan et al. 2003a). As an internal control, primers targeting the 5' end (160-bp) of *actin 2* (An et al. 1996) were used for PCR. Prior to analyses of the modified histones status associated with the *phas* chromatin at various states, the nucleosome architecture of the *phas* promoter was determined using antibodies targeting the N terminus of histone H3 and C terminus of either histone H3 or H4 in ChIP assays. Upon potentiation, nucleosome association with the proximal region (-282 to -55) of the *phas* promoter is remodeled, as evidenced by the decrease in histones H3 and H4 associated

with the promoter (Fig. 3.3). These data suggest that a decrease in histone-DNA interaction or displacement of histones from the *phas* promoter occurs during *phas* potentiation. A slight increase in the level of histones H3 and H4 was detected when the *phas* promoter is transcriptionally active, suggesting an increase in histone-*phas* interaction or the redeposition of histones in the *phas* chromatin.

Histone acetylation associated with phas *expression*

In general, histone hyperacetylation is correlated with the permissive state of gene expression (Wade et al. 1997; Turner 2000; Lusser et al. 2001). Using anti-acetyl histone H3 antibody in ChIP analyses, an increase in histone acetylation was detected during *phas* potentiation (E) and activation (EA) (Fig. 3.4A). To further characterize histone acetylation at specific lysine residues upon *phas* expression, histone antibodies targeting either H3-K9 or H3-K14 acetylation were used. Interestingly, H3-K9 acetylation was enriched only during *phas* potentiation and decreased when *phas* is actively transcribing (Fig. 3.4B). Quantitation using densitometry with normalization to the *actin2* control (see Materials and Methods) gave relative enhancement (RE) values of 0.7 and 0.18 for the potentiated and activated states compared with 0.04 for the repressed state (Fig. 3.4B, left panel), accentuating the role of these modifications in the early stages of transcriptional expression.

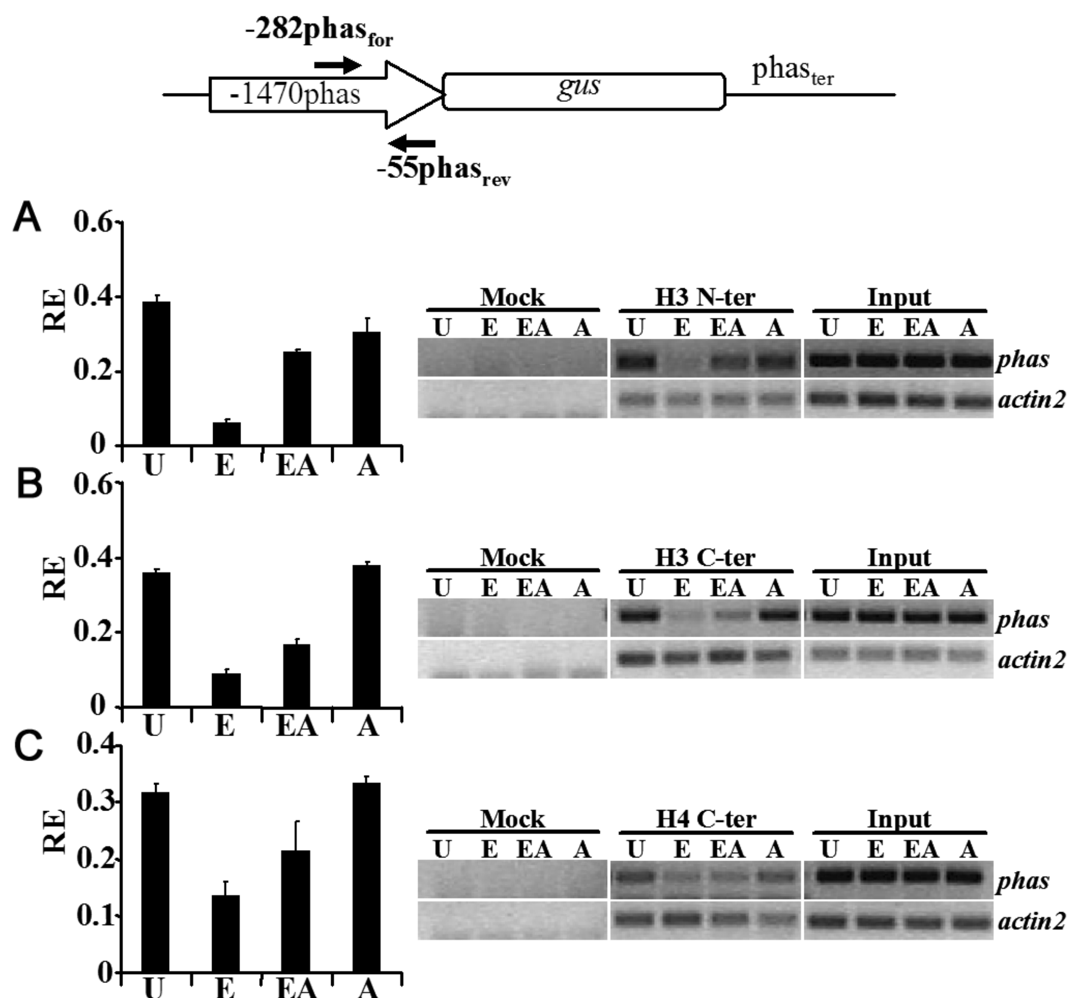

Figure 3.3 Remodeling of *phas* chromatin during potentiation and activation. Leaves from 5'14HAPvAlf plants were subjected to the four regimes described in the legend to Figure 1. ChIP assays were performed with antibodies targeting the N terminus of histone H3 (*A*), the C terminus of histone H3 (*B*) or the C terminus of histone H4 (*C*). Representative ChIP-PCR products for the 227-bp *phas* (-282 to -55, see diagram at *top*)and the 160-bp *actin2* amplicons are shown (*right*). The ethidium bromide-stained products were quantitated by densitometry and the relative enrichment (RE) for each treatment was calculated (*left*). Results shown are averages from at least two independent experiments. Error bars represent standard deviation.

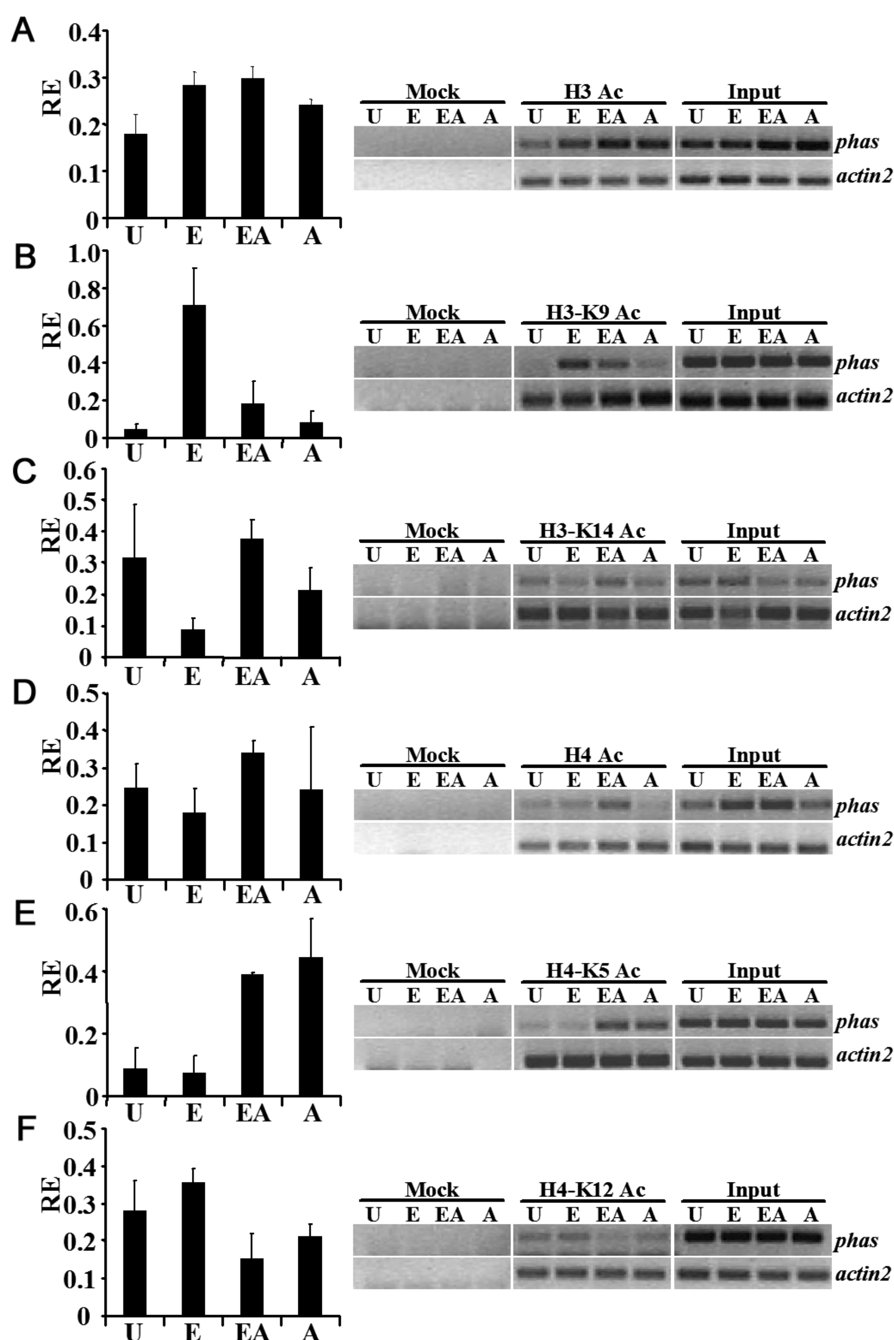

Figure 3.4 Histone acetylation associated with *phas* expression. Representative PCR results (*right*) from ChIP assays using various histone antibodies. (*A*) anti-diacetyl H3 (K9/K14); (*B*) anti-acetyl H3-K9; (*C*) anti-acetyl H3-K14; (*D*) anti-hyperacetylated H4 (K5/K8/K12/K16); (*E*) anti-acetyl H4-K5 and (*F*) anti-acetyl H4-K12. The average relative enrichment (RE) for at least two independent experiments is shown on the *left*. Error bars represent standard deviation.

In contrast to the situation for H3-K9, substantial acetylation was detected in uninduced leaves at H3-K14 and H4-K12 in the repressed *phas* chromatin (RE = 0.32 and 0.28, respectively, Figs. 4C,F; left panels). Further, H3-K14 acetylation decreased upon *phas* potentiation and increased on *phas* activation (Fig. 3.4C). A slight increase in histone H4 hyperacetylation was observed during active transcription from the *phas* promoter (Fig. 3.4D) and a marked enrichment of acetylation of histone H4 on lys5 was detected during *phas* activation but not potentiation. However, results from leaves treated with ABA alone (A) also showed an enrichment of H4-K5 acetylation when compared to the uninduced (U) control (Fig. 3.4E). In contrast, a decrease in acetylation of histone H4 on lys12 was observed in the presence of ABA (Fig. 3.4F). Therefore, by temporally separating *phas* potentiation from activation, we discovered that the lysine residues of histones H3 and H4 are not globally hyperacetylated during *phas* expression. Rather, there is stepwise histone acetylation and deacetylation at specific lysine residues when *phas* is induced to its potentiated state and progresses to its activated state.

Histone methylation status during phas *expression*

Whereas lysine can be mono-, di- or trimethylated, arginine can only be mono- or dimethylated (both asymmetrically and symmetrically) at the histone tails (Lachner and Jenuwein 2002; Bannister and Kouzarides 2004; Tariq and Paszkowski 2004). Unlike histone acetylation, methylation of specific lysine or arginine residues can lead to either repressive or permissive states of gene expression (Kouzarides 2002).

To elucidate the histone methylation status associated with *phas* expression, ChIP

analyses were performed using antibodies targeting methylated lysine residues H3-K4, H3-K9 or H4-K20 under the ascribed experimental treatments (U, E, EA and A) of the 5'14HAPvAlf plants. ChIP using di- or trimethyl H3-K4 antibodies showed that changes in both di- and trimethylation are associated with active transcription from the *phas* promoter (Fig. 3.5A,B). In the presence of ABA alone, *phas* chromatin is dimethylated at H3-K4, acetylated at H4-K5 and deacetylated at H4-K12. This suggests that the involvement of ABA signaling components in recruiting other histone modifiers to the *phas* promoter is PvALF-independent. However, in the absence of PvALF or chromatin remodeling, these modifications were not sufficient to stimulate the transcriptional initiation from the *phas* promoter. Trimethylation of H3-K4 was only detected for the EA regime that fully activates the *phas* promoter (Fig. 3.5B).

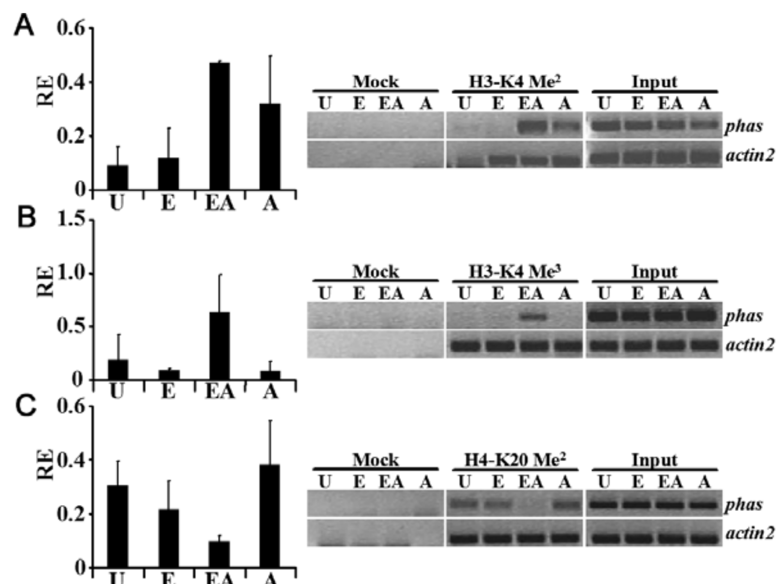

Figure 3.5 Histone methylation associated with *phas* expression. Representative PCR results (*right*) from ChIP assays using various histone antibodies. (*A*) anti-dimethyl H3-K4; (*B*) anti-trimethyl H3-K4 and (*C*) anti-dimethyl H4-K20. The average relative enrichment (RE) for at least two independent experiments is shown on the *left*. Error bars represent standard deviation.

Whereas H4-K20 was dimethylated in uninduced leaves or leaves treated with either estradiol or ABA alone, transcriptionally active *phas* chromatin was found to be devoid of dimethylated H4-K20 (Fig. 3.5C, lane EA). This suggests that dimethylation of histone H4 on lys20 does not impede chromatin remodeling or *phas* potentiation even though it has been imputed to function in general gene silencing (Karachentsev et al. 2005). Methylation of histone H3-K9 is also generally associated with gene silencing and heterochromatin formation through recruitment of HP1 (heterochromatin protein 1)(Bannister et al. 2001; Mutskov and Felsenfeld 2004). It has been shown in mammals (Schotta et al. 2004) that H3-K9 methylation is required for the methylation of H4-K20, we expected to find that histone H3 would be methylated at lys9. However, attempts to analyze the methylation status of histone H3 on lys9 at the *phas* promoter failed to give reproducible results (data not shown). Nevertheless, as methylation and acetylation of H3-K9 are mutually exclusive, the low level of H3-K9 acetylation observed under repressive conditions may imply the presence of methylated H3-K9.

Temporal profile of histone modifications during phas *potentiation and activation*

It is now evident that chromatin architecture is dynamic and our inducible system provides an unusually convenient opportunity to follow chromatin changes associated with promoter activation. Thus, while the data of Figures 3.4 and 3.5 yield the information that, when fully potentiated histone H3 is acetylated at lys9 and that chromatin over the fully activated promoter is trimethylated at H3-K4 and acetylated at H3-K14, the time-course information presented in Figure 3.6 provides insight to the chronological sequence of changes in histone

modification associated with chromatin remodeling and promoter function. The experiments depicted in Figure 3.2A confirmed the functionality of the inducible system. However, as both estradiol and ABA were present at the beginning of the experiment there was no clear separation of potentiation and activation events. By modifying the procedure so that only estradiol was present at the beginning of the experiment, only potentiation (chromatin remodeling) events could occur. As shown in Figure 3.6A, *HA-PvAlf* transcripts were detected by RT-PCR within 1 h after estradiol addition, increased in abundance over the next 3 h, then remained at similar levels for at least 5 h after estradiol was removed. Although HA-PvALF production was only detected 3 h after estradiol induction (Fig. 3.6B), H3-K9 acetylation was evident within 1 h of estradiol addition and was dramatically elevated 1.5 h after the leaves were exposed to estradiol (Fig. 3.6C). This is in accord with the finding that acetylation of H3-K9 is associated with *phas* potentiation (Fig. 3.4B), and probably reflects the presence of HA-PvALF at the early time-point at levels that were too low for detection by the HA antibody used for Western analysis. In marked contrast to the early onset of H3 acetylation at lysine 9, only background (or non-specific interaction) levels were detected for trimethylated H3-K4 throughout the potentiation step. A significant

increase in trimethylated H3-K4 is evident for the 1 h time-point following exposure of the leaves to ABA. At this time, the signal for trimethylated H3-K4 is already strong and intensifies over the following 5 h whereas acetylation at H3-K9 decreases (Fig. 3.6C). After the *phas* chromatin is potentiated from its repressed state in the presence of PvALF but prior to its full activation stimulated by ABA, a transition phase with a gradual decrease in acetylated H3-K9 and an increase in trimethylated H3-K4 was observed over the first 3 h of activation. This suggested that it requires at least 3 h for the potentiated *phas* promoter to attain its fully activated state upon ABA exposure. Therefore, the conditions of this experiment clearly reveal the ordered changes in histone modification associated with, and probably responsible for, transition from architectural remodeling of the promoter to its active participation in RNA transcription. The latter function is evidenced by the appearance of *gus* transcripts within 1 h of ABA addition and their attainment of a steady state level over the following 2 h (Fig. 3.6A). GUS accumulation was detected 3 h after initiation of mRNA production by fluorometric analysis and at 4 h by histochemical analysis (Jefferson et al. 1987) (Fig. 3.6B).

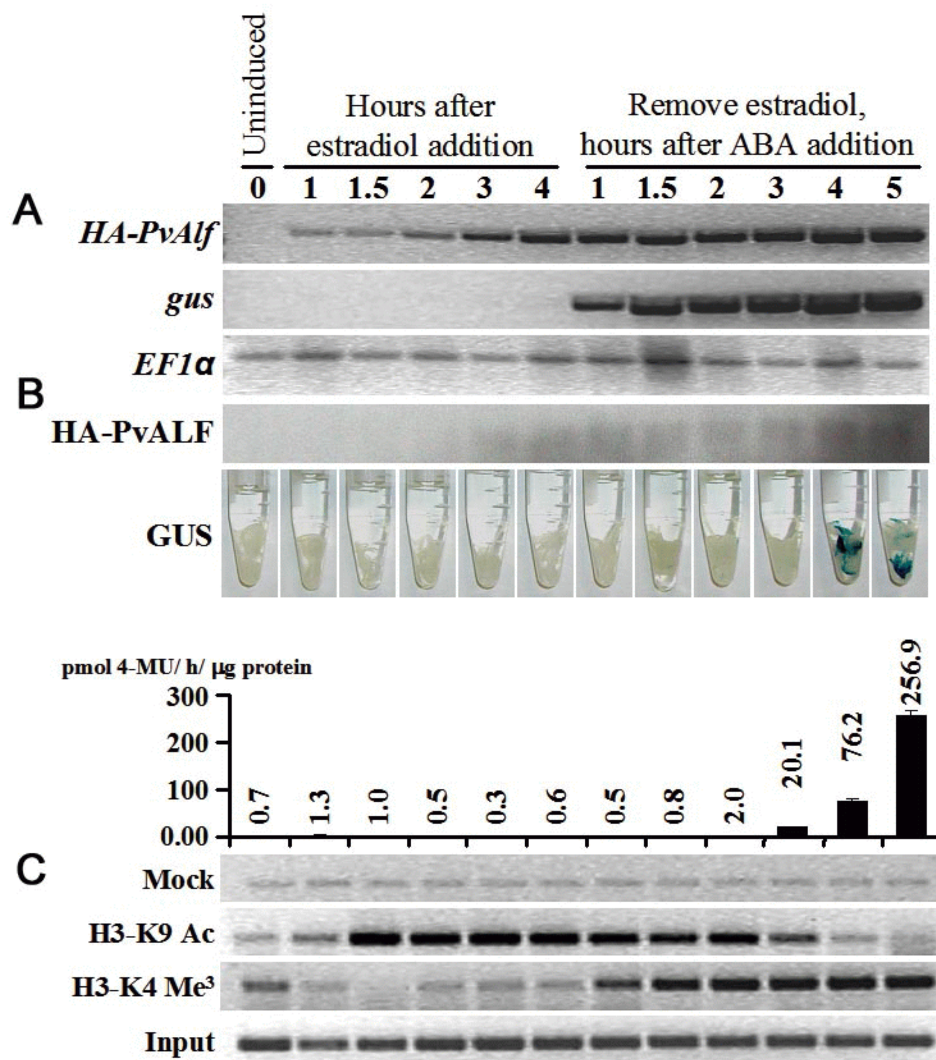

Figure 3.6 Temporal changes in histone modification during potentiation and activation. Leaves from 5'14HAPvAlf plants were induced for 4 h with 25 µM estradiol alone. After washing the leaves with water to remove estradiol, they were placed in medium containing 200 µM ABA. (*A*) RT-PCR analyses of *HA-PvAlf*, *gus* and *EF1α* transcripts. (*B*) Recombinant HA-PvALF detection using anti-HA antibody in a Western blot (*top*) and histochemical and fluorometric analyses for GUS expression (*bottom*). (*C*) *phas* PCR products from ChIP assays using antibodies against acetylated H3-K9 and trimethylated H3-K4.

Discussion

Epigenetic control via histone modifications is now known to play a significant role in gene expression and development (Schneider et al. 2004; Margueron et al. 2005). It has been shown in many systems that histone H3 and H4 hyperacetylation, H3-K9 acetylation, H3-K4 methylation and H3-S10 phosphorylation are involved in active gene expression (Loidl 2004) whereas hypoacetylated histones as well as H3-K9, H3-K27 and H4-K20 methylation are markers of gene silencing (Bender 2004; Craig 2005). In contrast to the wealth of information from animal and yeast systems, the relationship between chromatin dynamics and development has been investigated for only a few plant genes (Hsieh and Fischer 2005).

The *phas* promoter, together with the GUS reporter, provides an excellent system for studying the dynamic changes in histone modification that accompany the transition of a higher eukaryotic promoter from a transcriptionally silent to a highly active state. The establishment of estradiol-inducible production of HA-PvALF in *Arabidopsis* leaves allows precise analysis of these changes in chromatin status for the *phas* promoter as it is remodeled from its repressed form and is poised (potentiated) for transcriptional activation. Exposure of the potentiated promoter to ABA induces the second set of changes in nucleosomal architecture that result in transcriptional activation. Elucidation of the dynamic histone modification changes at the *phas* chromatin will thus provide insight to the identification of potential interactions between various chromatin modifiers and PvALF as well as other components in the ABA signaling cascade throughout seed maturation in plant development.

Histone status of repressed phas *chromatin*

Histone H4 is dimethylated at lys20 when the *phas* chromatin is repressed from transcriptional activation, in accord with the finding that H4-K20 methylation functions in gene repression (Sarg et al. 2004; Karachentsev et al. 2005). Although we were unable to obtain decisive evidence concerning the methylation status of H3-K9 associated with *phas*, H3-K9 is likely to be methylated as it is required for the methylation of H4-K20 (Schotta et al. 2004). The absence of acetylated H3-K9 in the repressed state is in accord with this assumption.

In contrast to the situation for H3-K9, substantial acetylation was detected in uninduced leaves at H3-K14 and H4-K12 in repressed *phas* chromatin. In *Drosophila*, newly synthesized histones H3 and H4 are acetylated at K14/K23 and K5/K12, respectively, before their deposition into chromatin (Sobel et al. 1995). In yeast, identical acetylated histone isoforms were found in the replication-coupling assembly factor (RCAF) subunits that are involved in nucleosome reformation during DNA replication or repair (Tyler et al. 1999). The absence of H4-K5 acetylation in repressed *phas* chromatin may reflect a higher turnover of the acetyl group as opposed that for acetyl groups in H3-K14 and H4-K12 after the deposition of histones into the chromatin (Pesis and Matthews 1986; Sobel et al. 1994). This suggests that these modifications may have a structural role in *phas* chromatin formation but their presence is not sufficient to weaken histone-DNA interaction and establish a permissive state for *phas* expression.

Remodeling of phas *chromatin during potentiation*

In plants, the onset of intense seed storage protein accumulation requires the presence of a B3 domain transcription factor such as PvALF (in *Phaseolus vulgaris*), ABI3 (in *Arabidopsis*) or VP1 (in *Zea mays*). These B3 factors are generally described as being plant-specific, but are now known to include a region that has substantial similarity to the structure of the noncatalytic DNA binding domain of the restriction enzyme EcoRII (Yamasaki et al. 2004). We have previously shown that ectopic expression of PvALF in tobacco leaves potentiates the normally stringently seed-specific *phas* promoter for transcriptional expression (Li and Hall 1999). Similarly, ectopic expression of ABI3 in leaves, together with ABA, permits strong expression from the *phas* promoter in vegetative tissues of *Arabidopsis* (Ng et al. 2004). It thus appears that these factors are capable of recognizing the *phas* promoter in its nucleosomal architecture, and of recruiting remodeling complexes that yield transcriptionally active chromatin over this promoter.

The inducible system used here, together with the increasing array of specific antibodies available for ChIP assays, permits identification of the changes and coordination in histone modifications associated with *phas* remodeling and can be expected to yield insight to the protein complexes involved. The decrease in histones H3 and H4 associated with the promoter, as detected by ChIP analyses, are consistent with histone displacement from the promoter, but could alternatively reflect a decrease in histone-DNA interaction leading to less efficient cross-linking in the ChIP assay. Evidence favoring both possibilities exists. The loss of histone contact with the activated *PHO5* promoter following hyperacetylation of histones in *Saccharomyces cerevisiae* has been reported (Reinke and

Horz 2003; Boeger et al. 2004). In an impressive study in yeast, Lee et al. 2004 showed a depletion in nucleosome occupancy at transcriptionally active regions throughout the genome. However, in a similarly impressive study, Kassabov et al. (2003) showed that SWI/SNF is engaged in a directional unwrapping of DNA from the edge of the nucleosome, resulting in the formation of a more accessible DNA loop during gene activation. This, and earlier work showing co-occupancy of transcriptional activators and histones for the *HIV-1* enhancer (Steger and Workman 1997), provide evidence that histones are not completely displaced when chromatin is being remodeled.

Histone modifications associated with phas *activation*

In our experiments, an increase in histones H3 and H4 was detected concomitant with the onset of active transcription. Since a decrease in histone-DNA interaction or histone displacement from the promoter was detected during *phas* potentiation, this may reflect a simple restoration of histone-DNA interaction or a redeposition of histones; alternatively, a coupled histone displacement and redeposition event could occur during *phas* activation. Histone displacement (during or after potentiation of *phas* chromatin) could account for the loss of H4-K20 dimethylation seen upon transcriptional activation of the *phas* promoter. An alternative explanation would be through enzymatic demethylation. However, such an enzyme has only recently been reported (Shi et al. 2004). This lysine-specific demethylase 1 (LSD1) targets exclusively to mono- or dimethylated H3-K4, and enzymatic reactions capable of removing other epigenetic methylation marks (methylated H3-K9, H3-K27 and H4-K20) remain unknown (Sarma and Reinberg 2005).

As the PvALF-potentiated *phas* chromatin progresses to its activated state in the presence of ABA, a transition phase can be discerned. This is characterized by a gradual decrease in acetylated H3-K9 and an increase in trimethylated H3-K4. In addition to the lost of dimethylation at H4-K20 during *phas* activation, these reflect displacement of histone H3 from the potentiated *phas* chromatin and their replacement by histone H3.3 bearing trimethylated lysine 4, possibly through a replication-independent nucleosome assembly pathway (Ahmad and Henikoff 2002; Workman and Abmayr 2004). In *Drosophila*, chromatin associated with transcriptionally active loci is enriched in histone H3.3 with modifications specifying the active state of transcription (McKittrick et al. 2004). In yeast, phosphate metabolism is regulated by the PHO system. Following hyperacetylation of histones, nucleosomes completely unfold at the transcriptionally active *PHO5* promoter (Reinke and Horz 2003). By analogy, these findings strongly support the notion that potentiation of the *phas* promoter through specific histone modifications and chromatin remodeling leads to histone displacement and redeposition when the *phas* promoter is actively transcribed in the presence of ABA.

Similarities in histone code functions among eukaryotic systems

In agreement with the general observation that histone hyperacetylation is coupled with the permissive state of gene expression (Wade et al. 1997; Turner 2000; Lusser et al. 2001), our results revealed an increase in histone H3 diacetylation (at lys9 and lys14) when *phas* is potentiated and activated. However, an insignificant change in H4 acetylation (hyperacetylation, or specific acetylation at lysines 5 and 12) and H4-K20 dimethylation

was observed when compared to the repressed *phas* chromatin, suggesting that the presence of these histone H4 modifications does not affect PvALF-mediated *phas* remodeling. Analysis of H3 lys9- or lys14-specific acetylation revealed that H3-K9 acetylation is primarily associated with *phas* potentiation rather than activation. In contrast, H3-K14 was found to be deacetylated during *phas* potentiation and acetylated upon activation. A similar order of histone modifications is associated with the initiation of transcription from the *IFN-β* promoter following infection by Sendai virus, where H3-K9 acetylation commences 3 to 5 h post infection and persists throughout the time course of virus infection (Agalioti et al. 2002). In the *IFN-β* system, an early step in transcriptional activation is the formation of an enhanceosome (Merika and Thanos 2001). This complex contains three transcription factors (NF-κB, IRFs and ATF-2/c-Jun) and HMG I(Y), an architectural protein that binds to specific sites in the nucleosome-free enhancer DNA, altering its topology and lowering the free energy for activator binding. In the *phas* system (Falvo et al. 1995), PvALF is known to be required for nucleosome remodeling (Li et al. 1998; Li et al. 1999) and binds to RY motifs (Carranco et al. 2004) present within a 68-bp seed-specific enhancer sequence (van der Geest and Hall 1996); thus, it is functionally equivalent to the architectural protein of the human *IFN-β* system. In their silent state, the TATA regions of both the *phas* and *IFN-β* promoter are masked by a nucleosome, preventing access by basal transcription factors. Targeting of PvALF to the RY motifs within the *phas* promoter leads to the formation of an enhanceosome and results in recruitment of chromatin modifier(s) that contain histone acetyltransferase activities to the *phas* chromatin. Acetylation of H3-K9 is an early event in transcriptional activation of the *phas* promoter and it is logical to speculate that, as for

IFN-β, this event facilitates recruitment by the enhanceosome of SWI/SNF. Subsequent remodeling of the nucleosome over the *phas* TATA region through interaction of bromodomains with the acetylated histone N-termini (Agalioti et al. 2002), permits access by TFIID (Li et al. 1998), and sets the stage for establishment of the basal transcription complex. In the *IFN-β* system, onset of H3-K14 acetylation correlates precisely with TBP recruitment and initiation of transcription (Agalioti et al. 2002). Activation of the *phas* promoter is dependent on the addition of ABA and, as for the *IFN-β* promoter, is commensurate with acetylation of H3-K14. In addition to H3-K14 acetylation, activated *phas* chromatin is enriched with trimethylated H3-K4.

In *Saccharomyces cerevisiae,* dimethylated H3-K4 is associated with both active and inactive euchromatic genes while H3-K4 trimethylation results in active transcription (Santos-Rosa et al. 2002). The yeast SET1 complex is the first H3-K4 methyltransferase to be identified (Briggs et al. 2001). It targets the 5' portion of active mRNA coding regions through interactions with the PolII associated factor 1 (PAF1) complex and the RNA polymerase II complex (Krogan et al. 2003a; Ng et al. 2003b). The yeast PAF1 complex consists of Paf1, Ctr9, Leo1, Cdc73 and Rtf1 (Krogan et al. 2002; Squazzo et al. 2002). *Arabidopsis* relatives of Paf1, Ctr9 and Leo1 were identified as ELF7, ELF8 and VIP4, respectively (Zhang and van Nocker 2002; He et al. 2004). These loci are involved in the *FRIGIDA* (*FRI*)-mediated trimethylation of H3-K4 at the *FLC* chromatin in the winter-annual habit of *Arabidopsis* (He et al. 2004). These studies showed that members of the PAF1 complex in both yeast and plants share similar components in target gene regulation. By analogy, members of the PAF1 complex and H3-K4 methyltransferase [*EFS* in

Arabidopsis; He and Amasino (2005)] may be recruited to the potentiated *phas* promoter through interaction with RNA PolII. Such recruitment could initiate transcription, accompanied by changes in *phas* nucleosome architecture and histone modification.

Model for histone modification changes associated with phas *expression*

A model for events associated with the activation of transcription from the *phas* promoter proposed in Figure 3.7 was derived summarizing the results obtained from ChIP analyses conducted in this study. It is known that during vegetative growth, a rotationally positioned nucleosome is present over the three phased TATA boxes of the *phas* promoter (Li et al. 1998). Dimethylation of histone H4 on lysine 20 contributes to the establishment of heterochromatic *phas* chromatin whereas acetylation at H3-K14 and H4-K12 represent the native modifications present in the histone isoforms that are being deposited in the *phas* chromatin during DNA replication. The interaction of the transcription factor PvALF (normally confined to developing embryos, but supplied ectopically in the leaf system described here) with RY-elements of the *phas* promoter (Carranco et al. 2004) leads to the recruitment of histone acetyltransferase through its acidic activation domain. Acetylation of H3-K9 thus weakens the histone-DNA interactions at the *phas* promoter and constitutes a histone code for the recruitment of a chromatin remodeling complex such as SWI/SNF. This, as a consequence, renders *phas* chromatin more accessible for the assembly of other factors and binding of a preinitiation complex as they are recruited through the ABA signaling cascade.

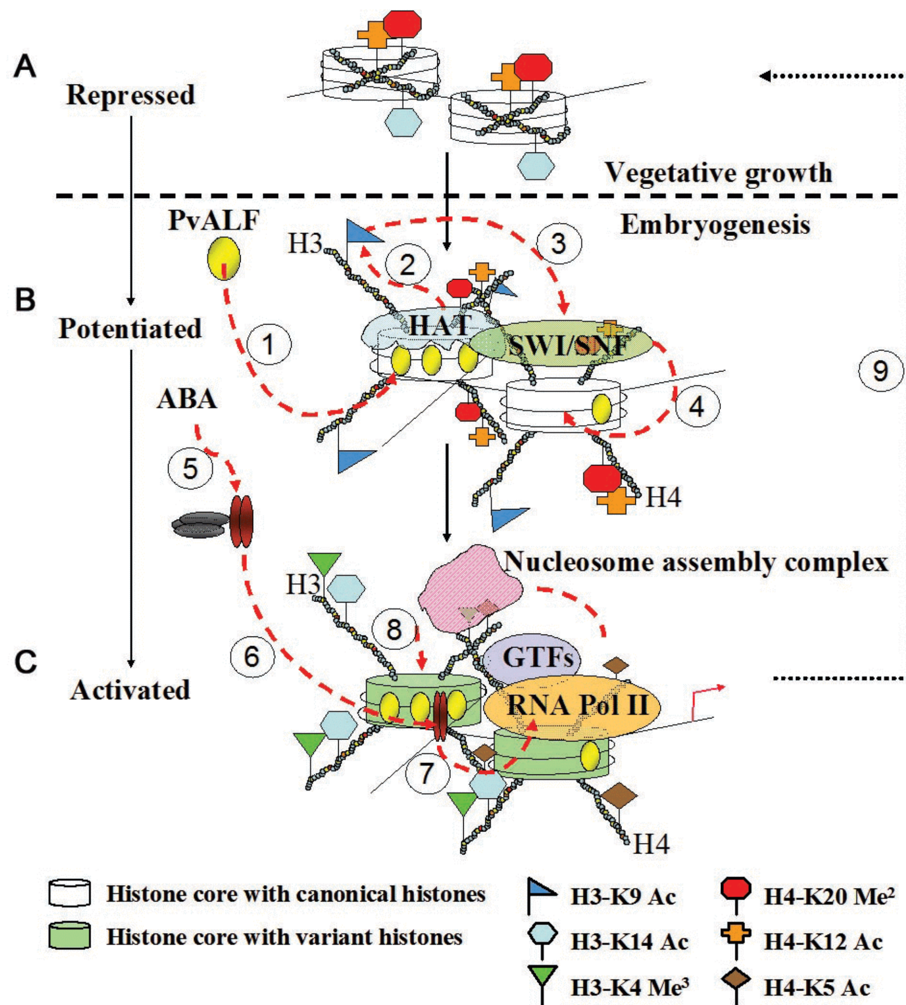

Figure 3.7 Model depicting the sequential events and ordered modification of chromatin over the *phas* promoter during potentiation and activation. (*A*) In the repressed state during vegetative growth, the promoter is envisaged for being heterochromatic, with nucleosomes bearing dimethylated H4-K20. (*B*) PvALF-mediated potentiation of *phas* (1) through recruitment of a complex with histone acetyltransferase (HAT) activity, H3-K9 is acetylated (2). Modifications of histones may recruit a chromatin remodeler such as SWI/SNF (3) resulting in a decrease in histone-DNA interactions (4). (*C*) In the presence of ABA, this triggers components in the ABA signaling cascade (for example, ABI5) (5) that interact with the ABRE within the *phas* promoter (6) with the formation of a preinitiation complex (7). Histone displacement and redeposition of variant histones during *phas* activation incorporates new histone code modifications (H3-K4 trimethylation, H3-K14 and H4-K5 acetylation) at the actively transcribed *phas* chromatin (8). The original repressive chromatin status of *phas* is restored through DNA replication (during seed germination and vegetative growth) (9).

The addition of ABA, or the onset of its presence in embryos, initiates progression from potentiation to active transcription. Cooperative binding and interactions of PvALF and ABA-induced factors, such as ABI5 [a bZIP transcription factor that binds ABRE; Nakamura et al. (2001)], to the RY and ABRE motifs in the *phas* promoter thus permit the assembly of GTFs and RNA PolII at the TATA regions. Upon transcription initiation, histones are displaced from the *phas* chromatin and the repressive methylation mark at H4-K20 is removed. The initiation of replication-independent nucleosome assembly and recruitment of H3-K4 methyltransferase (EFS in *Arabidopsis*) to RNA PolII through the PAF1 complex thus leads to the deposition in *phas* chromatin of replacement histone H3.3 bearing modifications typically associated with active genes (trimethylated H3-K4, acetylated H3-K14 and acetylated H4-K5). The repressive *phas* chromatin state is restored once the level of PvALF and ABA decline at seed maturation. Conversely, on seed germination, histone H3.3 at the *phas* chromatin will then be replaced by canonical histone H3, together with modifications specifying repressed *phas* chromatin through a replication-dependent nucleosome assembly pathway.

CHAPTER IV

ROLE OF ABI5 IN *PHAS* EXPRESSION

Introduction

Within the -248/-243 region of the *phas* promoter is the G-box (CACGTG) that is similar to the consensus sequence ${}^{C}_{T}$ACGTGGC predicted (Shen and Ho 1995; Busk and Pagès 1997) for a strong abscisic acid response element (ABRE). Mutation of this G-box in the *-295phas* promoter dramatically reduces the ability of the *phas* promoter to drive GUS expression in developing seeds (3% activity compare to the wild type 295 bp proximal *phas* promoter) (Chandrasekharan et al. 2003a). Studies have shown that ABI5, a basic leucine zipper (bZIP) class transcription factor, is a positive ABA signal effector for seed protein accumulation (Finkelstein and Lynch 2000). It is induced by exogenous ABA through interaction with ABREs (Busk and Pages 1998) and with ABI3 (Nakamura et al. 2001). Like *abi3*, mutation of *abi5* in *Arabidopsis* causes a decrease in seed ABA sensitivity and seed-specific gene expression (Finkelstein 1994; Carles et al. 2002). In a transient assay, ABI5 was capable of *trans*-activating *phas* promoter-driven GUS expression (Gampala et al. 2002). In this section, the role of ABI5 in *phas* expression is evaluated.

Materials and Methods

Plant materials

Arabidopsis seeds were germinated under conditions described in the Materials and Methods section (p. 14) of chapter II. The ABI5/AtDPBF-1 overexpressing line (Jianzhong Ma, Texas A&M University) was kindly provided by Terry Thomas (Texas A&M University). An *abi5* T-DNA insertion mutant (Garlic 401b F08) from Syngenta Research and Technology was obtained through Terry Thomas (Texas A&M University). Another *abi5-1* mutant (CS8015) and *abi5* T-DNA insertion mutant (Salk_013163) were obtained from *Arabidopsis* Biological Research Center.

Induction treatment

Estradiol and ABA induction treatments were performed as described in the Materials and Methods section (p. 54) of chapter III.

Fluorescence microscopy

Green fluorescent protein (GFP) expression from developing seeds or embryos of plants transgenic for *gfp* reporter was visualized using a Zeiss SV11 stereomicroscope with a 490 nm excitation filter and 500 nm or 525 nm emission filters.

Histochemical staining and fluorometric analysis of GUS activity

Histochemical staining of embryos and fluorometric analysis of GUS activity in transgenic T_2 seeds was performed as described in the Materials and Methods section (p. 21) of chapter

II.

Results

ABA is dispensable for phas *activation in the presence of both PvALF and ABI5*

To evaluate the role of ABI5 in *phas* expression, an ABI5/AtDPBF-1 overexpressing line (kindly provided by Dr. Terry Thomas, Texas A&M University) was doubly transformed with a pER/HisSPvALF effector construct (Chandrasekharan et al. 2003b) and a pHM301/-1470phas-gfp reporter construct. The resulting triple transformants (35S-ABI5::XVE-HisSPvALF::phas-gfp) expressed ABI5 in leaves constitutively whereas expression of HisSPvALF is controlled by estrogen application. Under non-inducing conditions where only ABI5 is ectopically expressed in triple transformants, *phas* is transcriptionally silent in leaves, as revealed by the lack of GFP expression (Fig. 4.1A,B). This showed that the presence of ABI5 alone is not sufficient to activate the *phas* promoter. When leaves from 35S-ABI5::XVE-HisSPvALF::phas-gfp plants were exposed to MS medium containing either estradiol (25 µM) alone or estradiol (25 µM) and ABA (200 µM), GFP fluorescence was observed 8 h after treatment. In the presence of ectopically induced HisSPvALF through exposure to estradiol alone, it was expected that the *phas* promoter would be potentiated but that no active transcription would ensue. However, overexpressing ABI5 in leaves relieved the ABA requirement for *phas* activation in the presence of ectopically expressed PvALF (Fig. 4.1C,D). In the presence of both HisSPvALF and ABI5, ABA had a synergistic effect on *phas* activation (Fig. 4.1E,F). Similar results were observed

in the roots of triple transformants (Fig. 4.2).

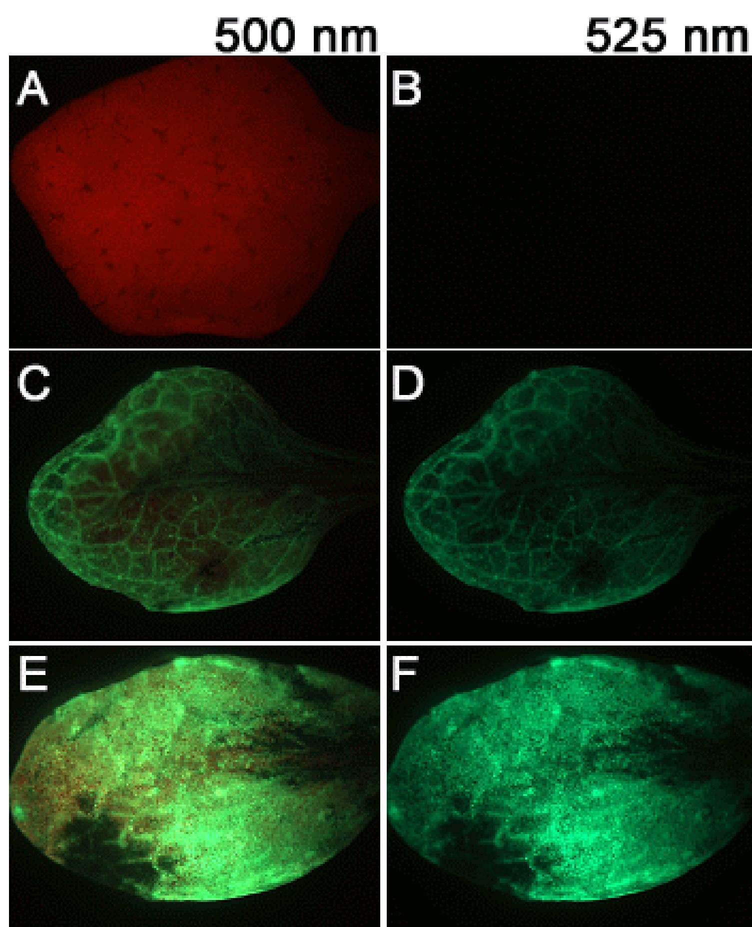

Figure 4.1 Fluorescence microscopy of leaves from *Arabidopsis* triple transformants (35S-ABI5::XVE-HisSPvALF::-1470phas-gfp). Fluorescence microscopy with different emission filters, 500 nm (*left* panel) and 525 nm (*right* panel). Figure shown: (*A,B*) untreated leaves; (*C,D*) 25 µM estradiol-treated leaves and (*E,F*) 25 µM estradiol and 200 µM ABA-treated leaves.

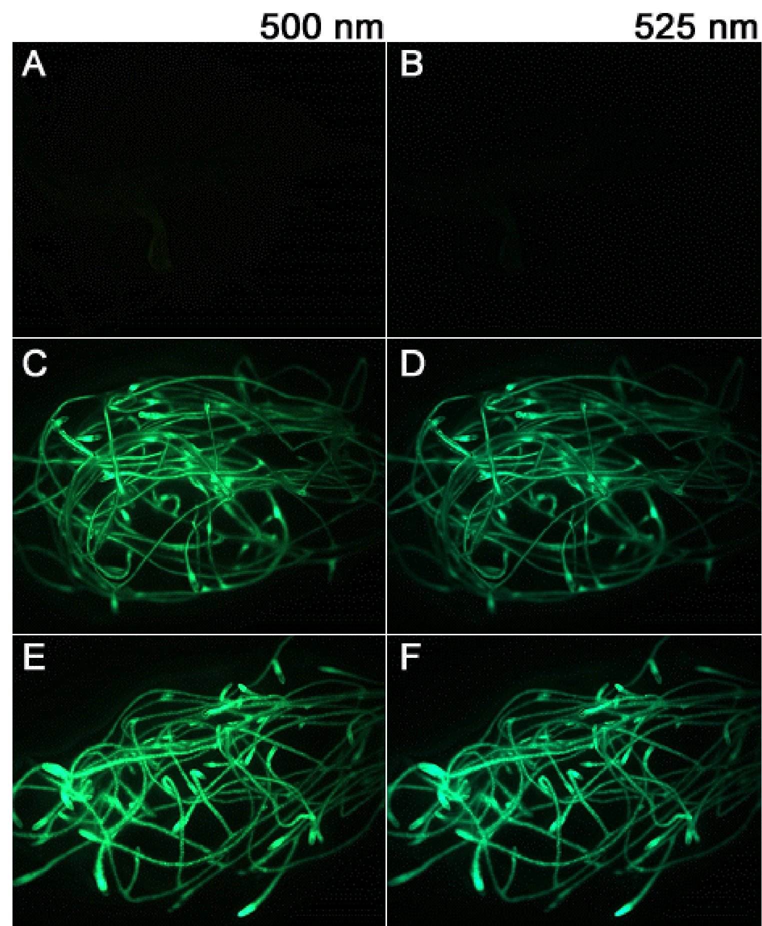

Figure 4.2 Fluorescence microscopy of roots from *Arabidopsis* triple transformants (35S-ABI5::XVE-HisSPvALF::-1470phas-gfp). Fluorescence microscopy with different emission filters, 500 nm (*left* panel) and 525 nm (*right* panel). Figure shown: (*A,B*) untreated roots; (*C,D*) 25 μM estradiol-treated roots and (*E,F*) 25 μM estradiol and 200 μM ABA-treated roots.

Effect of ABI5 *mutation on* phas *expression*

The role of ABI5 in *phas* expression was also evaluated in a reciprocal experiment by transforming pHM301/-1470phas-gus or pHM301/-1470phas-gfp reporter construct into

three different T-DNA insertion mutants of *abi5*. These include an *Arabidopsis abi5* line (Garlic 401b F08, Syngenta Research and Technology) containing two T-DNA insertions in the *ABI5* coding region upstream of the bZIP DNA binding domain, *abi5-1* (CS8105, ABRC) (Finkelstein 1994) and Salk_013163 (ABRC) lines that contain T-DNA insertions in the *ABI5* coding sequence and promoter region, respectively. *Arabidopsis* transformants garlic401-phas-gus, garlic-phas-gfp, abi5-phas-gus, abi5-phas-gfp, salk-phas-gus and salk-phas-gfp were selected for subsequent analyses. In all *abi5* mutants, developing embryos were obtained from developing siliques and *phas*-driven GFP expression was evident as revealed by fluorescence microscopy (Fig. 4.3C-E). Whereas similar expression pattern was observed for the *phas*-driven gus expression in both wild type and *abi5* background (Fig. 4.3G,H), alteration of *phas*-driven gfp expression was observed in the absence of function ABI5 (Fig. 4.3B-E) and a significant decrease in GFP expression was evident in the hypocotyl region of developing embryos. Fluorometric assays for GUS activity were performed for garlic401-phas-gus T_2 seeds and results revealed no significant difference in *phas*-driven GUS expression for either wild type (Col-0) or *abi5* mutant (garlic 401b) plant (Fig. 4.4). The contrasting results obtained from the two reporters may reflect their difference in nature as GUS expression is manifested through histochemical staining whereas evaluation of GFP expression is more direct via the use of fluorescent microscope. Therefore, although no significant change in *phas*-driven GUS expression was observed in all *abi5* mutants tested, the change in GFP expression pattern suggested that ABI5 may play a role in modulating *phas* expression within different regions of the embryo.

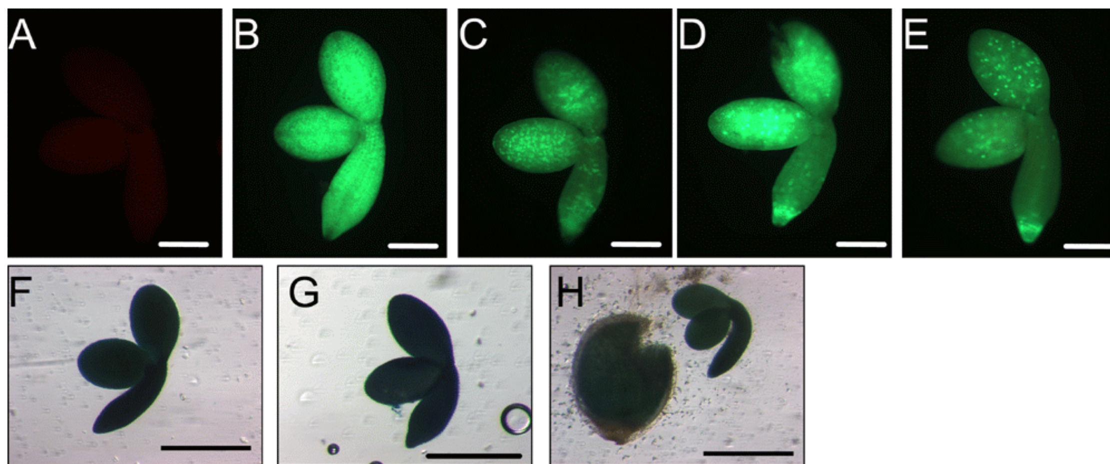

Figure 4.3 *phas* expression in *abi5* mutants. Representative embryos showing *phas* expression in different *abi5* mutant lines revealed by either fluorescence microscopy (with a 500 nm emisson filter) or histochemical staining. (*A*) Wild type (Col-0) *Arabidopsis* embryos under fluorescence microscopy. GFP expression from the *phas* promoter in (*B*) wild type Col-0 (Col0-phas-gfp), (*C*) *abi5* mutant (garlic401-phas-gfp), (*D*) *abi5-1* mutant (abi5-phas-gfp) and (*E*) salk_013163 *abi5* mutant (salk-phas-gfp) embryos. *phas*-driven GUS expression in (*F*) wild type Col-0 (Col0-phas-gus), (*G*) *abi5-1* mutant (abi5-phas-gus) and (*H*) salk_013163 *abi5* mutant (salk-phas-gus) embryos. Bar = 0.25 mm (*A*-*E*); 0.5 mm (*F*-*H*).

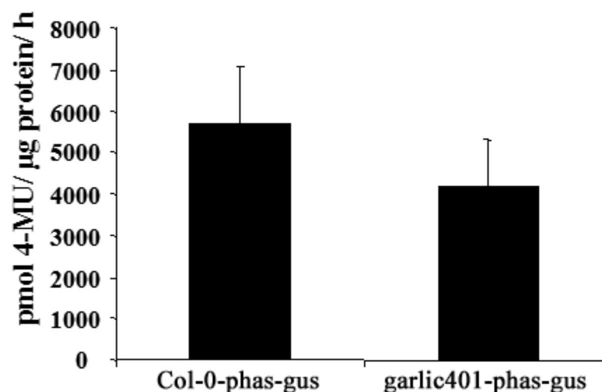

Figure 4.4 Fluorometric analysis of GUS activity in transgenic *Arabidopsis* seeds. GUS activity was measured in transgenic *Arabidopsis* seeds transgenic for *-1470phas-gus* in either wild type (Col-0-phas-gus) or *abi5* (garlic401-phas-gus) background.

Discussion

ABI5 is involved in phas activation

In *Arabidopsis*, ABI5 regulates expression of a subset of late embryogenesis abundant (*LEA*) genes (*AtEm1*, *AtEm6*) in response to ABA (Finkelstein and Lynch 2000; Carles et al. 2002). By exposing 35S-ABI5::XVE-HisSPvALF::phas-gfp triple transformants to estradiol, it showed clearly that overexpression of ABI5 renders *phas* activation ABA-independent in the presence of ectopically expressed PvALF in leaves. This provides the first direct evidence that ABI5 functions downstream of ABA during *phas* expression. In *Arabidopsis*, ABI5 expression is induced by ABA and is cross-regulated by ABI3 that acts synergistically with ABA (Finkelstein and Lynch 2000). Thus, the requirement for ABA in *phas* activation may reflect its role in inducing ABI5 for further recruitment of

factors to the *phas* promoter.

The contrasting results observed between *phas*-driven expression in the reciprocal GUS and GFP experiments using various *abi5* mutants reflect the difference in nature of these two reporter systems. Although the *phas* promoter can still be activated during embryogenesis in the *abi5* mutant, a change in the pattern of *phas*-driven GFP expression was observed that revealed a modular regulatory role of ABI5 and the involvement of additional bZIP transcription factors in *phas* activation. In *Arabidopsis*, approximately 80 genes encoding bZIP transcription factors have been identified (Riechmann et al. 2000). These are divided into ten bZIP groups, each having a similar basic region and motifs (Jakoby et al. 2002). Group A, is annotated as being involved in ABA signaling; ABI5 is one of the 13 genes found in this group. In a yeast two-hybrid assay, Lara et al. (2003) showed that AtbZIP10 and AtbZIP25 can interact *in vivo* with ABI3 in a yeast two-hybrid assay. Additionally, synergistic activation of the *At2S* promoter by ABI3 together with either AtbZIP10 or AtbZIP25 was observed in transient expression assays. Therefore, the observed *phas* expression in *abi5* mutants may reflect an involvement of additional bZIP factors in the activation of the *phas* promoter.

Although *in vitro* studies have provided evidence for a direct binding to Sph and RY elements by the B3 domain of VP1 (Suzuki et al. 1997) and PvALF (Carranco et al. 2004), respectively, no binding of the full length B3 factor to either Sph or RY elements was observed. It has been suggested by Ezcurra et al., (2000) that ABI3 binds to the RY elements in the *napin* (*napA*) promoter and interacts (through the B2 domain) with a TRAB1-type bZIP factor that binds ABREs. However, an alternative model is that ABI5

functions in tethering ABI3 to the *At2S3* promoter through its interactions with a G-box whereas other B3 domain containing transcription factors (FUS3 and LEC2) bind to the flanking RY motives (Kroj et al. 2003). Therefore, it will be of interest to determine if the RY elements within the *phas* promoter are the direct binding sites for PvALF or if ABI5 or other bZIP factors are involved in mediating the interaction between PvALF and the *phas* promoter. This can be achieved through the use of the established 5'14HAPvAlf line (as described in chapter III). Anti-HA antibody can be used to pull down the HA-PvALF-bound DNA by ChIP assay and the consensus sequence of the immunoprecipitated DNA could be obtained by cloning the DNA into vector for sequencing. It is also of interest to determine the role of other B3 domain-containing transcription factors (such as FUS3 or LEC2) in the regulation of *phas* expression.

While, the data presented here show the involvement of ABI5 in *phas* expression, additional molecular analyses are required to verify the presence or absence of abi5 transcripts in the *Arabidopsis* transformants. Further, having established that H3-K4 methylation and H3-K14 acetylation are both associated with *phas* activation (chapter III), it is of interest to evaluate ABI5-mediated change in histone modification at the *phas* chromatin by establishing an estradiol-inducible system for ectopic expression of ABI5 in leaves of transgenic plants containing the -1470phas-gus reporter construct.

CHAPTER V

CONCLUSIONS

Summary

The seed-specific *phas* promoter provides an excellent model for studying various mechanisms involve in the temporal and spatial regulation of eukaryotic gene expression in plants, ranging from the involvement of the transcriptional activator, PvALF, to the chromatin remodeling through histone modifications in the *phas* chromatin.

The seed-specific transcriptional activator, PvALF is important in mediating the alternation of the *phas* chromatin architecture for transcriptional activation in the presence of ABA (Li et al. 1999). In order to understand the spatial control of PvALF expression, transcription regulation of its homolog (ABI3) in *Arabidopsis* was investigated through promoter deletion analyses. The full length 5.1 kb *ABI3* promoter (includes a 4.6 kb promoter region and 519 bp 5' UTR) was PCR amplified from the *Arabidopsis* genome and fused to a *gus* reporter gene. Transgenic *Arabidopsis* containing the *-4630/+519ABI3-gus* transgene shows seed-specific expression from the *ABI3* promoter started as early as the globular stage of embryo development. 5' and 3' *ABI3* promoter deletion and reporter fusion analyses have revealed various regulatory regions within the *ABI3* promoter. These include an intergenic region (-4630/-3600), two upstream regulatory sequences (-3600/-2033; -2033/-882), a seed specific expression region (-882/-364), a proximal region (-364/+114). The 5' UTR (+114/+519) of *ABI3* was found to play a negative regulatory role in *ABI3*

expression. It is likely that post-transcriptional mechanisms are involved in the regulation of the ABI3 expression.

Another aspect of this research is to elucidate the mechanisms involved in PvALF-mediated potentiation and ABA-mediated activation of the *phas* promoter. Through the establishment of a stable inducible system for PvALF expression in leaves of transgenic plants transformed with -1470phas-gus construct, it allowed the separation of the potentiation state of *phas* promoter from its activated state during the course of *phas* expression. Using ChIP analyses with various histone antibodies targeting specific modification on histones H3 and H4, histone modification changes associated with three states of *phas* expression (repressed, potentiated and activated states) were revealed. In general histone H3 or H4 are acetylated and lost contact with *the phas* promoter when the *phas* chromatin is in its permissive state (potentiated or activated). Specific H3-K9 acetylation is associated with potentiated *phas* promoter while H3-K14 is deacetylated upon *phas* potentiation and enriched during *phas* activation. H4-K5 acetylation of the *phas* chromatin was found to be associated with the ABA signaling transduction cascade in mediating the *phas* expression while H4-K12 acetylation was detected in the absent of ABA or when *phas* is either repressed or potentiated. Trimethylation at H3-K4 and lost of H4-K20 dimethylation are associated with activated but not potentiated *phas* chromatin. Dimethylation of H3-K4 was detected in the presence of ABA supporting the involvement of ABA signaling in *phas* activation, however, dimethylation of H3-K4 is not sufficient to cause the activation of *phas*. The diverse histone modifications associated with the *phas* expression reported thus provides new insights to the epigenetic regulation of the *phas*

promoter. A model was proposed for the transcriptional regulation of the *phas* promoter and this provides a basis in subsequent identification of factors involved in the *phas* regulation through the powerful *Arabidopsis* genetics with readily available mutant lines and the wealth of information regarding histone modifications and gene expression in other model systems in yeasts and animals.

Finally, the role of ABI5 in ABA-mediated *phas* activation was investigated. Supertransformation of both estrogen inducible PvALF construct and -1470phas-gus construct into *Arabidopsis* overexpressing ABI5 in leaves render the activation of *phas* ABA-independent in the presence of both ectopically expressed PvALF and ABI5 in leaves. Results suggested ABA functions in activating ABI5 through either transcriptionally or post-transcriptionally mechanisms. This, in turn, lead to *phas* activation in the presence of PvALF. In contrast, *phas*-driven reporter (GUS or GFP) expression is not completely abolished in the absence of ABI5 suggesting the presence of redundant bZIP factors capable of activating *phas* expression.

In conclusion, the findings reported here have provided a more detailed investigation of different mechanisms employed in the developmental regulation of the seed-specific *phas* promoter.

Future Directions

How does PvALF gain access to the phas *chromatin?*

In the regulation of human *IFN-β* promoter, an architectural protein, HMG I(Y) is involved in recruiting three activators (NF-κb, IRFs and ATF-2/c-jun) to the enhancer DNA with the

formation of enhanceosome (Falvo et al. 1995; Yie et al. 1999; Merika and Thanos 2001) for transcriptional activation. In the case of *phas* expression, it is thought that PvALF interacts with the RY motifs in the *phas* promoter through its B3 DNA binding domain. However, while *in vitro* studies have provided evidence for direct binding to Sph and RY elements by the B3 domain of VP1 (Suzuki et al. 1997) and PvALF (Carranco et al. 2004), no binding has been observed for either of the full length B3 factors. Therefore, it is of interest to investigate how PvALF gains access to the repressed *phas* chromatin during its potentiation and to determine if additional factors are involved in PvALF-mediated potentiation of the *phas* chromatin. One approach to achieve this is to use antibody against HA tag to immunoprecipitate the HA-PvALF and its associated proteins from leaves of 5'14HAPvALF plants after estradiol or estradiol and ABA treatment. Protein complex isolated can then be purified, digested into smaller peptides and used in a matrix-assisted laser desorption/ionization time of flying mass spectrometry (MALDI-TOF-MS) analysis. Peptide mass fingerprints obtained can be used to search against SWISS-PROT database with the PeptIdent software.

Nucleosomal architecture of the phas *chromatin over the 68-bp seed-specific enhancer region of the* phas *promoter*

In ChIP assays, a decrease in histone-DNA interaction during *phas* potentiation and activation was observed. Therefore, it is of interest to determine the nucleosomal architecture of the *phas* promoter over the 68-bp seed-specific enhancer region (SSE, -295/-227) where three RY repeat elements and the G-box important for *phas* expression

are located. This can be achieved by comparing DNase I footprints of the *phas* promoter region at its repressed, potentiated and activated states in leaves using the established inducible system. Results obtained will reveal if the 68-bp SSE is positioned in a nucleosome or it is located at the linker DNA region between nucleosomes when *phas* is repressed. In addition, this may provide insight to the nucleosomal architecture of the *phas* chromatin during potentiation and activation.

Temporal histone modification changes in the phas *chromatin during* phas *expression*

The detection of the temporal changes in H3-K9 acetylation and H3-K4 trimethylation during *phas* potentiation and activation demonstrated the feasibility to investigate the gradual histone modification changes in the *phas* chromatin when it is potentiated from its repressed state in the presence of PvALF (induced by estradiol) and activated upon ABA exposure. A more thorough investigation of the temporal histone modification changes using various histone antibodies (e.g. anti-histone H3, anti-acetyl H3-K14, anti-acetyl H4-K5 and anti-acetyl H4-K12) is expected to reveal sequential modifications that occur during the potentiation and the activation of the *phas* chromatin. This thus will permit a more precise analysis of events that occur during *phas* expression.

Mechanisms of ABI5-mediated phas expression

ABI5 was found to play a modular regulatory role in *phas* expression and its presence relieves the ABA requirement for *phas* expression in leaves with ectopically expressed PvALF. Analysis of its role in mediating histone modification changes in the *phas*

chromatin will thus provide more insights to its regulation of *phas* expression. This can be achieved by subjecting ABI5 under the control of the inducible promoter in the estrogen inducible system (Zuo et al., 2000) in this study, and by inducing the expression of ABI5 in leaves of transgenic *Arabidopsis* containing the -1470phas-gus construct, it will permit the analysis of the histone status in the *phas* chromatin in the presence or absence of ectopically expressed ABI5.

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Publications

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