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A Role of Swi/snf Complex in Aba-Dependent Drought Responsive Gene Expression in Arabidopsis Thaliana

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Abstract
The survival of plants as sessile organisms depends on their ability to cope with environmental challenges. Of key importance in this regard is the phytohormone abscisic acid (ABA). ABA not only promotes seed dormancy but also triggers growth arrest in postgermination embryos that encounter water stress. This is accompanied by increased desiccation tolerance. Postgermination ABA responses in *Arabidopsis thaliana* are mediated in large part by the ABA-induced basic domain/leucine zipper transcription factor *ABA INSENSITIVE5* (*ABI5*). Here, I show that loss of function of the SWI/SNF chromatin remodeling ATPase BRAHMA (BRM) causes ABA hypersensitivity during postgermination growth arrest. *ABI5* expression was derepressed in *brm* mutants in the absence of exogenous ABA and accumulated to high levels upon ABA sensing. This effect was likely direct; chromatin immunoprecipitation revealed BRM binding to the *ABI5* locus. Moreover, loss of BRM activity led to destabilization of a nucleosome likely to repress *ABI5* transcription. Genetic interaction revealed that the *abi5* null mutant was epistatic to BRM in postgermination growth arrest. In addition, vegetative growth defects typical of *brm* mutants in the absence of ABA treatment could be partially overcome by reduction of ABA responses, and *brm* mutants displayed increased drought tolerance. I propose a role for BRM in the balance between growth or stress responses. Intriguingly, BRM resides at the *ABI5* promoter both in the absence and presence of the stress signal. I found that BRM interacts with the core components of abscisic acid signaling transduction pathway. Moreover, the C-terminus of BRM can be phosphorylated in an ABA dependent manner *in vitro*. It is therefore likely that stress sensing inactivates the BRM complex to allow *ABI5* upregulation.

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ABSTRACT

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The survival of plants as sessile organisms depends on their ability to cope with environmental challenges. Of key importance in this regard is the phytohormone abscisic acid (ABA). ABA not only promotes seed dormancy but also triggers growth arrest in postgermination embryos that encounter water stress. This is accompanied by increased desiccation tolerance. Postgermination ABA responses in *Arabidopsis thaliana* are mediated in large part by the ABA-induced basic domain/leucine zipper transcription factor *ABA INSENSITIVE5 (ABI5)*. Here, I show that loss of function of the SWI/SNF chromatin remodeling ATPase BRAHMA (BRM) causes ABA hypersensitivity during postgermination growth arrest. *ABI5* expression was derepressed in *brm* mutants in the absence of exogenous ABA and accumulated to high levels upon ABA sensing. This effect was likely direct; chromatin immunoprecipitation revealed BRM binding to the *ABI5* locus. Moreover, loss of BRM activity led to destabilization of a nucleosome likely

to repress *ABI5* transcription. Genetic interaction revealed that the *abi5* null mutant was epistatic to BRM in postgermination growth arrest. In addition, vegetative growth defects typical of *brm* mutants in the absence of ABA treatment could be partially overcome by reduction of ABA responses, and *brm* mutants displayed increased drought tolerance. I propose a role for BRM in the balance between growth or stress responses. Intriguingly, BRM resides at the *ABI5* promoter both in the absence and presence of the stress signal. I found that BRM interacts with the core components of abscisic acid signaling transduction pathway. Moreover, the C-terminus of BRM can be phosphorylated in an ABA dependent manner *in vitro*. It is therefore likely that stress sensing inactivates the BRM complex to allow *ABI5* upregulation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. Introduction

(Adapted from Han and Wagner, *Journal of Experimental Botany*, 2014 Jun;65(10):2785-99. doi: 10.1093/jxb/ert403. Epub 2013 Dec 3.)

1.1 Summary

Plant stress can be defined "Any unfavorable condition or substance that affects or blocks a plant's metabolism, growth or development" (Lichtenthaler, 1998). Plants are exposed to a plethora of environmental stresses through their life. Drought attributable to climate change already causes water shortages in large parts of the world (Vorosmarty *et al.*, 2010). Therefore enhanced response to water deficit is an important trait for both crops and wild plant populations. Water is essential for plant metabolism, transport systems and for generating the turgor pressure that allows an upright growth habit in herbaceous plants (Des Marais and Juenger, 2010). It also adversely affects other aspects of plant growth, for example water stress reduces the rate of nitrogen fixation by legumes and their symbionts (Gil-Quintana *et al.*, 2013). Due to their sessile nature, plants cannot escape from a water deficient habitat. They instead need to adopt special strategies to cope with water limitation and to avoid substantial impacts on fitness, growth and development (Cramer *et al.*, 2011; Less *et al.*, 2011). Ability of the plant to display tolerance to water stress depends on transcriptional reprograming (Ahuja *et al.*, 2010; Shinozaki and Yamaguchi-Shinozaki, 2007). For instance, factors involved in regulation of stress signal transduction as well as osmolytes and proteins that protect the cell from damage during water stress are induced in response to water deficit (Shinozaki and Yamaguchi-Shinozaki, 2007).

In plants, water stress triggers the biosynthesis of the phytohormone abscisic acid (ABA) (Nambara and Marion-Poll, 2005; Xiong and Zhu, 2003), this triggers a signal transduction cascade that leads to stomatal closure and transcriptional reprograming (Umezawa *et al.*, 2010). Increasing evidence shows that transcriptional reprograming in stress-responsive gene expression, proper resource allocation to growth versus stress responses, acclimation and long-term stress memory are at least in part attributable to changes in the chromatin organization (Chinnusamy *et al.*, 2008; Gutzat and Mittelsten Scheid, 2012; Mirouze and Paszkowski, 2011). This is not surprising given that chromatin has long been viewed as the interface between the environment and the genome (Badeaux and Shi, 2013; Johnson and Dent, 2013; Suganuma and Workman, 2013).

In this chapter, I will briefly introduce water stress response during postgermination development, abscisic acid signal transduction pathway and key factors in abscisic acid response. I will review in more detail the roles of various mechanisms that affect chromatin organization in water stress responses, explore the link between water stress perception and modulation of chromatin regulator activity, and discuss resource allocation to diverse survival programs by chromatin regulators as well as the role of chromatin in transient or long-term stress memory.

1.2 Water stress response during postgermination development

When seed dormancy is broken by the appropriate environmental and endogenous cues, the radicle penetrates the seed coat during germination (Bewley, 1997). The newly germinated embryo next initiates a series of developmental changes prior to entering the

seedling developmental program (Bewley, 1997; Lopez-Molina *et al.*, 2001). Most notably, the germinated embryo must ensure appropriate food and water supply by switching to autotrophic growth (photosynthesis) and by elongating the root, respectively. These reprogramming events occur during the first 48 h after dormancy is broken in the postgermination embryo and culminate with seedling establishment and onset of vegetative development (Lopez-Molina *et al.*, 2001). During postgermination, the embryo is no longer protected by the seed coat and thus is particularly vulnerable to drought stress. If plants encounter water stress during this developmental window, a growth arrest is triggered that helps protect germinated embryos against water stress–mediated cell and tissue damage (Lopez-Molina *et al*., 2001). The growth arrest and induction of the quiescent state involves similar signaling and response mechanisms to those that operate during seed development to induce desiccation tolerance and dormancy (Bensmihen *et al.*, 2002; Finkelstein *et al.*, 2008; Lopez-Molina *et al.*, 2001; Lopez-Molina *et al.*, 2002). When plants sense water stress, the levels of the stress hormone abscisic acid (ABA) rise (Nambara and Marion-Poll, 2005; Xiong and Zhu, 2003).

1.3 Abscisic acid signaling

Abscisic acid (ABA) plays a major role in adaptive stress response in plants. Upon sensing of water stress, cellular ABA levels increase and lead to adaptive response such as stomatal closure to prevent water loss and to maintain root growth for adequate water supply (Cutler *et al.*, 2010). The signal transduction pathway has recently been elucidated (Ma *et al*., 2009; Park *et al*., 2009). ABA sensing by START domain proteins from the PYR-PYL/RCAR family leads to inhibit of type2 serine/threonine protein phosphatases

(PP2Cs). The PP2Cs are negative regulators of ABA responses and inhibit the SNF1-related protein kinases 2 (SnRK2) to phosphorylate and activate basic domain/ leucine zipper (bZIP) family transcription factors and unknown factors which leads to the upregulation of ABA-responsive element (ABRE)– dependent gene expression (Figure 1-1) and ion channels for stomata closure (Cutler *et al.*, 2010; Fujita *et al.*, 2011; Hubbard *et al.*, 2010; Raghavendra *et al.*, 2010; Umezawa *et al.*, 2010) to enable plant cells to better cope with dehydration (Cutler *et al.*, 2010; Fujita *et al.*, 2011). Exogenous application of ABA often mimics the abiotic stress responses (Fujita *et al.*, 2011).

1.4 Key factors for ABA response during postgermination development

The first components of ABA signaling and response pathway were identified in genetic screens for ABA-insensitive mutants more than a quarter of a century ago (Finkelstein, 1994; Finkelstein and Lynch, 2000; Koornneef *et al.*, 1984; Lopez-Molina and Chua, 2000) and include dominant (constitutively active) clade A PP2C phosphatase mutants (*abi1*, *abi2*) and recessive loss-of-function mutants of transcriptional activators of the ABA response (*abi3*, *abi4* and *abi5*) (Finkelstein, 1994; Finkelstein and Lynch, 2000; Koornneef *et al.*, 1984; Lopez-Molina and Chua, 2000). Conversely, loss of function of these PP2Cs or gain of function of the transcription factors leads to ABA hypersensitivity (Brocard *et al.*, 2002; Gosti, 1999; Kang *et al.*, 2002; Lopez-Molina *et al.*, 2001; Merlot *et al.*, 2001; Parcy and Giraudat, 1997; Rubio *et al.*, 2009; Soderman *et al.*, 2000).

Both ABA and the bZIP transcription factor ABI5 are important for osmotic stress responses during late seed maturation and for execution of the ABA-dependent growth

arrest prior to photosynthetic growth (Bensmihen *et al.*, 2002; Brocard *et al.*, 2002; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Lopez-Molina *et al.*, 2001). *ABA INSENSITIVE5 (ABI5)* is also implicated in control of radicle emergence (germination) (Lopez-Molina *et al*., 2001, 2002). Loss of *ABI5* function causes reduced ABA sensitivity, whereas ectopic expression of ABI5 enhances ABA sensitivity and drought resistance (Lopez-Molina *et al*., 2001; Brocard *et al*., 2002). *ABI5* expression is the most abundant in dry seeds and decreases during postgermination development (Finkelstein *et al.*, 2005; Lopez-Molina and Chua, 2000; Lopez-Molina *et al.*, 2001). Although *ABI5* expression is low after seedling establishment, *ABI5* is induced upon drought sensing also during vegetative development, in an ABA signaling–dependent manner (Brocard *et al.*, 2002; Gonzalez-Guzman *et al.*, 2012; Mizoguchi *et al.*, 2010; Nakashima *et al.*, 2009; Zhu *et al.*, 2007).

Another key transcription factor important for establishment of desiccation tolerance and dormancy is the B3 domain transcription factor ABI3 (Parcy and Giraudat, 1997). ABI3 has also been linked to regulation of germination (Nambara *et al.*, 2000; Parcy *et al.*, 1994). Importantly, ABI3 has a key role in promoting postgermination growth arrest under osmotic stress conditions and acts upstream of *ABI5* in this process (Lopez-Molina *et al*., 2002). ABI3 is abundant in maturing and mature seeds, but *ABI3* mRNA and protein levels become undetectable upon seedling establishment (Parcy *et al.*, 1994; Perruc *et al.*, 2007). ABI3 cannot be induced by ABA during vegetative development (Nakashima *et al.*, 2006).

1.5 Chromatin changes induced by water stress

Altered transcriptional responses to environmental stimuli, such as abiotic stress, have been linked to chromatin regulation (Chinnusamy and Zhu, 2009; Kim *et al.*, 2010a). In the eukaryotic nucleus, the genome is packaged into the fundamental unit of chromatin, the nucleosome, which is comprised of 147 base pairs of DNA wrapped around a histone octamer (Luger *et al.*, 1997). The histone octamer consists of two copies each of histones H2A, H2B, H3 and H4. Nucleosomal arrays are further condensed into higher-order chromatin structures that incorporate the linker histone H1 (Luger *et al.*, 1997). The compaction of the genome in the context of chromatin physically restricts the accessibility of the genomic DNA to regulatory proteins such as transcription factors and RNA polymerase II (Petesch and Lis, 2012). Genomic DNA accessibility in the context of chromatin can be altered by various mechanisms including incorporation of histone variants, posttranslational modifications of the histones or the DNA, or non-covalent alteration of the positioning or occupancy of the nucleosome (Bell *et al.*, 2011).

In following subtopics, I will discuss each of the different mechanisms that increase or decrease the accessibility of the genomic DNA in the context of chromatin as well as the available evidence that links each mechanism to water stress responses.

1.5.1 Histone modifications

Certain amino acids of histones, for example in their N-terminal tails, are frequently posttranslationally modified *via* acetylation, methylation, phosphorylation, ubiquitination, sumoylation, or ADP-ribosylation (Bannister and Kouzarides, 2011; Zentner and Henikoff, 2013). These modifications are dynamically established or erased by

specialized enzymes called ‘writers’ or ‘erasers’, respectively (Bannister and Kouzarides, 2011). The functional outcome of these changes in histone modifications is either alteration of the strength of the DNA histone interaction or recruitment of non-histone proteins, the so called ‘readers’, to the chromatin (Bannister and Kouzarides, 2011; Patel and Wang, 2013; Zentner and Henikoff, 2013).

Typically, histone acetylation is correlated with more open chromatin and hence more active transcription, whereas the converse is true for histone deacetylation (Zentner and Henikoff, 2013). By contrast, histone methylation can affect different transcriptional outcome, depending on the amino acid modified and the degree of modification (mono-, di-, tri- methylation) (Li *et al.*, 2007). For example, H3K4 and H3K36 tri-methylation are found at actively transcribed genes, whereas methylation of H3K27 and H3K9 are well known marks for repressed loci and heterochromatin, respectively (Zentner and Henikoff, 2013). Histone arginine residues can be methylated by protein arginine methyltransferases (PRMTs). Different PRMT family members can catalyze mono-methylarginine, asymmetric di-methylarginine, and symmetric di-methylarginine that direct either gene activation or repression (Ahmad and Cao, 2012).

Several reports in plant have shown that drought sensing or treatment with the stress hormone ABA induce changes in histone modifications (Kim *et al.*, 2010a; Yuan *et al.*, 2013). For example, a short pulse of ABA or salt stress was sufficient to induce global H3S10 phosphorylation and H4K14 acetylation in cultured Arabidopsis and tobacco cells (Sokol *et al.*, 2007). In 15-day-old Arabidopsis seedlings, H3K9, H3K23 and H3K27 acetylation were enriched at coding regions of drought stress-responsive genes after short drought treatment, which was correlated with gene activation.

H3K4me3 enrichment with gene activation was similar to H3K9 acetylation (Kim *et al.*, 2008). Genome-wide analysis in 4-week-old rosette *Arabidopsis* leaves under dehydration stress revealed a modest change in H3K4me2 and H3K4me1 levels at a subset of known stress response genes, but the H3K4me3 abundance over gene bodies changed more dramatically at genes whose transcript levels increased or decreased during dehydration (van Dijk *et al.*, 2010). Recent genome-wide analysis in 25-day-old rice seedling also uncovered a positive correlation between H3K4me3 accumulation and the expression levels of some of drought-responsive genes during dehydration. This correlation could be extended to genes involved in stress-related metabolite and hormone-signaling pathways (Zong *et al.*, 2013). As changes in transcription direct changes in histone modifications (Zentner and Henikoff, 2013), further studies are needed to elucidate whether the observed alterations in posttranslational histone modifications are a cause or consequence of the transcriptional changes triggered by water stress.

1.5.2 Histone (de)acetylases

More direct evidence for a role of histone modifications in water stress responses comes from the studies of mutants lacking histone-modifying enzymes. Several studies from rice and *Arabidopsis* have shown that the expression of histone deacetylases is regulated by drought and/or ABA (Luo *et al.*, 2012; Sridha and Wu, 2006). In *Arabidopsis*, the expression of the plant specific HD2 histone deacetylases is repressed by ABA and NaCl (Luo *et al.*, 2012; Sridha and Wu, 2006). Plants overexpressing AtHD2C exhibited ABA-hyposensitivity (Sridha and Wu, 2006), while *hdc2* mutants display ABA hypersensitivity

during germination (Luo *et al.*, 2012). The gene expression changes reported for these mutants are inconsistent with the phenotypes of mutants lacking the components of ABA signaling pathway (Gosti *et al.*, 1999; Merlot *et al.*, 2001), and may therefore be an indirect consequence thereof. Mutations in either one of the genes coding two RPD3-type histone deacetylases HDA6 and HDA19 in *Arabidopsis* also cause ABA hypersensitivity (Chen *et al.*, 2010b; Chen and Wu, 2010; Zhou *et al.*, 2013). Several embryonic genes including *7S1*, *LEC2*, *2S2*, CRA1, *FUS3* and *LEC1* were de-repressed in *hda19* seedlings (Zhou *et al.*, 2013) in agreement with a role of histone acetylation in activation of these genes (Ng *et al.*, 2006). Similar phenomena were observed in *HDA6*-RNAi lines (Tanaka *et al.*, 2008) and in wild-type plants treated with a histone deacetylase inhibitor (Tanaka *et al.*, 2008). HDA19 associates with the regulatory regions of the above-mentioned embryonic genes (Zhou *et al.*, 2013). It remains to be seen whether failure to directly repress embryonic genes is also observed during germination and whether depression of such genes causes the germination defects and ABA hypersensitivity of germinating *hda19* mutants. Histone acetyltransferases (HAT) complex components were also linked to altered water stress responses. Loss-of-function mutant of ADA2b, a component of the GCN5 containing HAT complex leads to increased drought tolerance (Vlachonasios *et al.*, 2011; Vlachonasios *et al.*, 2003). It is not yet known which gene expression changes are directly triggered by this complex and cause the observed phenotype.

1.5.3 Histone lysine methyltransferases

Loss of function of *Arabidopsis* trithorax-like factor ATX1 that trimethylates histone H3 at lysine 4 (H3K4me3) results in decreased dehydration tolerance compared to wild-type

seedlings. ATX1 directly regulates transcription of *NCED3*, which encodes a key ABA biosynthesis enzyme. Activation of *NCED3* transcription upon dehydration or ABA treatment is greatly reduced in *atx1* mutant, suggesting that ATX1 mediated H3K4 methylation is required for *NCED3* induction and possibly ABA accumulation by water stress (Ding *et al.*, 2011).

Trithorax group proteins act in opposition to Polycomb group proteins (Simon and Kingston, 2013). H3K27me3 marks established by the Polycomb group complex 2 (PRC2) induce a persistent silent state of the transcription of the target locus (Simon and Kingston, 2013). In *Drosophila*, Polycomb repressive complex 1 (PRC1) recognizes H3K27me3 and plays a role in the stable maintenance of gene repression (Simon and Kingston, 2013). While PRC2 complex components are conserved in plants and metazoans, this is not true for PRC1 complex components (Holec and Berger, 2012; Zheng and Chen, 2011). In barley, exogenous ABA application induced expression of components of the PRC2 complex such as HvE(Z) and HvFIE in seedlings (Kapazoglou *et al.*, 2010). In *Arabidopsis*, mutations in the two *EMBRYONIC FLOWER* (*EMF*) genes display strikingly similar developmental defects (Aubert *et al.*, 2001; Yoshida *et al.*, 2001). EMF2 is a homolog of the Su(z)12 component of the metazoan PRC2 complex. It is currently unclear whether EMF1 is associated with PRC1 or PRC2 function (Beh *et al.*, 2012; Kim *et al.*, 2012b). The recent identification of EMF1 as a structural homolog of the *Drosophila* PRC1 complex component PSC, its ability to inhibit remodeling activity of SWI/SNF ATPases (Beh *et al.*, 2012) and its ability to act as a potent repressor of transcription (Calonje *et al.*, 2008) provide support for the idea that EMF1 may be associated with PRC1. Genome-wide expression analysis of the *emf* mutants revealed that

EMFs regulate plant hormone and stress signaling-related genes (Kim *et al.*, 2010b). Both EMF1 and EMF2 directly bind to the promoter of *ABI3* and expression of *ABI3* and its targets are de-repressed in 7 and 14-day-old *emf* mutant seedlings (Kim *et al.*, 2010b). More recently, genome-wide binding studies revealed that genes occupied by EMF1 and marked by H3K27me are significantly enriched for Gene Ontology terms such as "ABA response" and "abiotic stress response" (Kim *et al.*, 2012b). A bypass of the embryo lethality of the single unique PRC2 complex component, FIE, allowed assay of the gene expression defects and postembryonic phenotypes caused by absence of PRC2 function (Bouyer *et al.*, 2011). This revealed germination defects as well as de-repression of embryonic genes and of positive regulators of ABA responses (Bouyer *et al.*, 2011). Further evidence for a PRC-dependent role in water stress-related responses comes from conditional knockdown of EMF1, which led to increased salt tolerance, while removal of a factor with opposing (trithorax group-related) activity had the opposite phenotype (Carles and Fletcher, 2009; Pu *et al.*, 2013). It remains to be determined in the latter two studies, which of the observed changes in gene expression are direct. Moreover, no evidence is available as yet that the observed changes in gene expression contribute to the altered water stress responses.

1.5.4 Histone arginine methyltransferases

Mutants lacking the *Arabidopsis* arginine methyltransferase *PRMT5*/*SKB1* (henceforth referred to as PRTM5 for simplicity) which catalyzes symmetric arginine dimethylation, display salt and ABA hypersensitivity (Schmitz *et al.*, 2008; Wang *et al.*, 2007; Zhang *et al.*, 2011). Low doses of exogenous ABA result in the growth arrest of germinated *prmt5*

but not wild-type embryos (Zhang *et al.*, 2011). The reported gene expression changes in *prmt5* mutants relative to the wild type (Zhang *et al.*, 2011) are inconsistent with the observed hypersensitive phenotype (Merlot *et al.*, 2001; Rubio *et al.*, 2009; Yoshida *et al.*, 2010). Hence the reported changes in gene expression may be an indirect consequence of the mutant phenotype. As PRTM5 activity also regulates mRNA splicing (Deng *et al.*, 2010; Zhang *et al.*, 2011) and circadian gene expression (Hong *et al.*, 2010; Sanchez *et al.*, 2010), it will not be trivial to identify the genes, whose misexpression underlies the ABA hypersensitivity of *prmt5*. Indeed, a genetic screen for Ca2+ underaccumulation (*cau*) mutants identified an allele of *prmt5* that displays increased drought tolerance and stomatal closure (Fu *et al.*, 2013). The drought tolerance is at least in part due to de-repression of the direct PRMT5/H4Rsme2 target and calcium accumulation sensor *CAS* (Fu *et al.*, 2013).

In summary, mounting evidence supports the idea that post-translational modifications of histones are critical for correct water stress responses in plants. One of the biggest remaining challenges is to elucidate the causal defects that underpin the observed water stress-related phenotypes of mutants lacking histone-modifying enzymes. After identification of genes whose expression is altered in a given mutant in a manner consistent with the observed phenotypes, direct association of the histone-modifying enzyme in question with loci of interest should be tested. Coupled with expected changes in the histone modifications at these loci in stress and non-stress conditions in the mutant and wild-type background, this will allow identification of candidate direct targets of the histone-modifying enzyme. Subsequent genetic tests will enable elucidation of the role (if any) of the identified candidate direct targets in the water stress phenotypes observed in

mutants lacking activity of a given histone-modifying enzyme. Since loss-of-function of histone-modifying enzymes and other mutants that affect the chromatin organization are pleiotropic, it cannot be ruled out that the altered stress phenotype of constitutive mutants is due to secondary effects of the altered plant morphology (leaf size, stature). Phenotypic and molecular investigations of chromatin regulators should therefore rely as much as possible on inducible loss-of-function mutants. Tissue specific knockdown of chromatin regulators can minimize pleiotropic defects. Temporally inducible knockdown of a histone-modifying enzyme enables analysis of altered water stress responses shortly after knockdown in wild-type looking plants, significantly reducing the secondary effects typical of constitutive mutants.

1.5.5 Histone variants

In most organisms including *Arabidopsis*, there are multiple genes that code for the highly conserved canonical histones (H3, H4, H2A and H2B), which are mostly expressed during the S phase of the cell cycle (Burgess and Zhang, 2013; Skene and Henikoff, 2013; Talbert and Henikoff, 2010). Other less conserved subtypes of histones called histone variants are expressed throughout the cell cycle (Skene and Henikoff, 2013; Talbert and Henikoff, 2010). The canonical histones are replaced with histone variants independent of DNA replication. Although they generally do not differ much in sequence from the canonical histones, histone variants can impart distinct characteristics to the nucleosomes, such as stronger or weaker association with the genomic DNA and incompatibility with certain post-translational modifications (Skene and Henikoff, 2013; Talbert and Henikoff, 2010). Recent genome-wide studies have revealed the genomic

distribution of a subset of the plant histone variants (Costas *et al.*, 2011; Skene and Henikoff, 2013; Talbert and Henikoff, 2010; Wollmann *et al.*, 2012; Zilberman *et al.*, 2008).

In plants, linker histone (H1) variants have been linked to water stress response. The linker histone variant *HIS1-3* gene in *Arabidopsis* is specifically induced by salt, drought and ABA (Ascenzi and Gantt, 1997; Zhu *et al.*, 2012). Similarly, the tomato linker histone variant *H1-S* gene is also induced by and accumulates in the chromatin in response to water deficit (Scippa *et al.*, 2000). H1-S also accumulates in a drought-tolerant genotype of tomato (Trivedi *et al.*, 2012). Indeed, knockdown of H1-S levels by antisense in transgenic tomato triggered altered physiological response to water loss such as altered stomatal conductance, transpiration and net photosynthetic rate (Scippa *et al.*, 2004). Transgenic plants showed an increased association of the heterochromatin with the nuclear membrane under water stress condition (Scippa *et al.*, 2004), this may trigger increased silencing of these regions (Hubner *et al.*, 2013). Although up-regulation of expression of variants of the linker histone H1 in response to drought is a conserved response in higher plants, detailed mechanistic insight into how this histone variant affects chromatin structure or gene expression during water stress is as yet not available. The H2A variant H2A.Z is largely conserved through evolution (Talbert and Henikoff, 2010). Genome-wide studies revealed that the localization of H2A.Z inversely correlates with DNA methylation in both heterochromatin and in gene bodies of active genes (Zilberman *et al.*, 2008). It has been proposed that the anti-correlation between H2A.Z and DNA methylation is primarily due to the exclusion of H2A.Z from methylated DNA (Coleman-Derr and Zilberman, 2012). Moreover, Gene Ontology terms enriched among

genes up-regulated in *h2a.z* triple mutants include "Response to water deprivation" and "Response to ABA" (Coleman-Derr and Zilberman, 2012). The authors propose that H2A.Z deposition in gene bodies confers higher variability in the expression of inducible genes including those that respond to water stress. By contrast, gene-body DNA methylation may stabilize constitutive expression of housekeeping genes by antagonizing H2A.Z deposition (Coleman-Derr and Zilberman, 2012). It will be of interest to determine the effect of reduced availability or incorporation of these and additional histone variants on water stress responses in plants. Given their widespread roles in chromatin stability, conditional disruption of histone variant availability or incorporation may allow more precise investigation of such phenotypes.

1.5.6 DNA methylation

Methylation on the fifth carbon of cytosine bases is an important epigenetic mark that influences chromatin structure and gene expression (Jones, 2012). In plants, cytosine methylation is found in the context of CG, CHG and CHH (H=A, C or T). Symmetric CG maintenance methylation is catalyzed by DNA Methyltransferase I (MET1), a homolog of the mammalian methyltransferase Dnmt1 (Chan *et al.*, 2005; Goll and Bestor, 2005; Law and Jacobsen, 2010). Symmetric CHG maintenance methylation is catalyzed by Chromomethyltransferase 3 (CMT3), a plant specific methyltransferase. Asymmetric CHH methylation is maintained through *de novo* methylation by Domains Rearranged Methyltransferase 2 (DRM2), a homolog of the mammalian Dnmt3a/b and the RNA-directed DNA methylation (RdDM) pathway (Chan *et al.*, 2005; Goll and Bestor, 2005; Law and Jacobsen, 2010). DDM1 is a SWI/SNF superfamily chromatin remodeler

required for all DNA methylation (CG, CHG, CHH) over long transposable elements (TEs) and in heterochromatin (Vongs *et al.*, 1993). DDM1 was recently shown to cooperate with the CMT2 methyltransferase to mediate CHH DNA methylation in parallel with the RdDM pathway (Zemach *et al.*, 2013).

In *Arabidopsis*, centromeric and pericentromeric regions, repetitive DNA sequences and transposons are heavily methylated. Many genic regions are also highly methylated, this is correlated with high gene expression, whereas promoters are mostly depleted of DNA methylation (Saze *et al.*, 2012; Zhang *et al.*, 2006). In plants, DNA methylation is associated with diverse biological processes including development and environmental responses (Law and Jacobsen, 2010; Sahu *et al.*, 2013; Saze *et al.*, 2012).

Studies from various plant species showed that abiotic stress may trigger hyper- or hypomethylation at different genomic contexts; hypo-methylation of promoters, hyper- or hypo methylation at coding regions and hypo-methylation of transposons (Sahu *et al.*, 2013). For example, genome-wide analysis identified differentially methylated DNA regions in *Arabidopsis* seedlings treated with simulated drought (treatment with Polyethylene glycol). The methylome was widely affected by changes in the water potential, with the most dramatic DNA hypermethylation observed near the TSS (± 500bp) of protein coding genes related to stress responses (Colaneri and Jones, 2013). Moreover, it has been proposed that DNA methylation may contribute to stress adaptation. Mangrove trees grown near a salt march had smaller statures than riverside grown trees and their genomes were globally hypo-methylated (Lira-Medeiros *et al.*, 2010). Likewise, in rice, changes in DNA methylation in response to drought were more pronounced in drought-tolerant genotypes (Wang *et al.*, 2011b). The altered DNA

methylation may contribute to increased differential gene expression upon drought sensing. A subset of the DNA methylation changes induced by drought remained after removal of the stress (Wang *et al.*, 2011b). In *Arabidopsis*, low relative humidity was linked to *de novo* DNA methylation and stable repression of genes involved in stomata development, resulting in lower stomata frequency (Tricker *et al.*, 2012; Tricker *et al.*, 2013). A T-DNA insertion distal to the *AtHKT1* gene, which encodes a sodium transporter, has been identified as a suppressor of *sos3* (*salt overly sensitive 3*). The insertion prevents a distal enhancer element and RdDM from controlling expression of *AtHKT1*, which plays an important role in salt tolerance (Baek *et al.*, 2011). *met1-3* mutants and *met1-3* derived epiRILs show normal germination in non-stress condition, by contrast they fail to germinate in the presence of 150mM NaCl, a concentration that does not impact germination in the wild type (Reinders *et al.*, 2009). Defects in DNA methylation may thus affect phenotypic plasticity (a topic that has received attention from an evolutionary perspective) in response to adverse environmental conditions (Draghi and Whitlock, 2012; Lira-Medeiros *et al.*, 2010; Wang *et al.*, 2011b). It will be critical to identify which of the observed DNA methylation changes contribute to altered water stress response or plasticity.

1.5.7 Non-covalent changes in chromatin state

ATP-dependent chromatin remodeling ATPases alter histone–DNA interactions non-covalently by utilizing the energy derived from ATP hydrolysis to promote changes in nucleosome occupancy, nucleosome positioning or nucleosome composition (Cairns, 2009; Hargreaves and Crabtree, 2011; Narlikar *et al.*, 2013). Chromatin remodeling can

either increase or decrease the accessibility of a given piece of genomic DNA to trans factors and hence facilitate or obstruct transcription, respectively (Cairns, 2009; Hargreaves and Crabtree, 2011; Narlikar *et al.*, 2013). Four well-studied subfamilies of ATP-dependent chromatin remodelers are the SWI/SNF, ISWI, CHD and INO80/SWR1 families. Each subfamily has unique domains, which endow it with specialized function for particular nuclear processes (Cairns, 2009; Hargreaves and Crabtree, 2011; Narlikar *et al.*, 2013). Among these ATP-dependent chromatin remodelers, only the SWI/SNF and CHD subgroups have been implicated in water stress responses in plants.

SWI/SNF ATPases are conserved from yeast to humans and plants (Flaus *et al.*, 2006; Hu *et al.*, 2013; Kwon and Wagner, 2007; Narlikar *et al.*, 2013). Plant genomes contain three types of SWI/SNF subfamily chromatin remodeling ATPases called BRAHMA (BRM), SPLAYED (SYD), and MINUSCULE (MINU) (Jerzmanowski, 2007; Kwon and Wagner, 2007; Sang *et al.*, 2012). The catalytic ATPase subunit forms a core complex together with SWIRM- and SANT- domain proteins (SWI3) and SNF5-domain proteins. Additional accessary proteins, which are frequently tissue- and developmental-stage specific, control targeting and activity of the complex (Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011; Kwon and Wagner, 2007). *In vitro* remodeling activity has not yet been demonstrated for members of this subfamily in plants. In *Arabidopsis*, the BRM complex containing SWI3C and SNF5 (BSH) has been linked to ABA and drought response (Han *et al.*, 2012). Germinating *brm* mutants display ABA hypersensitivity and enhanced growth arrest relative to the wild type. Consistent with the mutant phenotype, derepression of the positive ABA response regulator *ABI5* (Lopez-Molina *et al.*, 2001) was observed (Han *et al.*, 2012). *ABI5* is a direct BRM target

and based on genetic epistasis tests, the *brm* mutant growth arrest is due to the *ABI5* de-repression (Han *et al.*, 2012). BRM repressed *ABI5* expression in the absence of stress by promoting high occupancy of the +1 nucleosome close to the *ABI5* transcription start site (Han *et al.*, 2012). In addition, *brm* mutants displayed increased drought tolerance at multiple stages of development. The molecular underpinnings of this response remain to be elucidated (Han *et al.*, 2012). The MINU1/AtCHR12 ATPase (henceforth referred to as MINU1 for simplicity) has been implicated as a negative regulator of a temporary growth arrest caused by drought and heat stress in adult *Arabidopsis* plants (Mlynarova *et al.*, 2007). Overexpression of MINU1 induces temporary growth arrest under drought as well as salt and heat stress (Mlynarova *et al.*, 2007). Intriguingly, the expression of several stress-inducible dormancy-related genes was reduced in the inflorescence and 4-week-old rosette leaves of MINU1 knock out and increased in MINU1 overexpressing plant. While it is not yet known whether these genes are directly regulated by MINU1 or responsible for the observed phenotypic defects, MINU1 may play a role in the induction of stress response genes upon perception of the stimulus.

The CHD subgroup chromatin remodeler PKL has also been implicated in ABA response. CHD chromatin remodelers have two tandem chromodomains known to bind methylated lysines, these domains were recently shown to couple ATP hydrolysis to remodeling (Hauk *et al.*, 2010). Like SWI/SNF ATPases, CHD remodelers can both promote and repress transcription. The vertebrate Mi2-NuRD complex contains histone deacetylase and methyl CpG-binding domain (MBD) proteins in addition to a CHD domain chromatin remodeler (Clapier and Cairns, 2009). PKL is the best-characterized CHD remodeler in *Arabidopsis* and most closely resembles CHD3. Recently, *in vitro*

chromatin remodeling activity was demonstrated for PKL (Ho *et al.*, 2013). PKL is required for repression of embryonic genes during seedling development and promotes the developmental transition to vegetative growth (Henderson *et al.*, 2004). *pkl* mutants display exaggerated ABA responses during germination, and fail to germinate in conditions where wild type germinates properly (Perruc *et al.*, 2007). The ABA-dependent growth arrest of geminating *pkl* plants is mainly mediated by failure to developmentally repress genes strongly expressed during embryogenesis, including *ABI3* and *ABI5.* Increased expression of *ABI3* and *ABI5* in *pkl* mutants relative to the wild type in the presence of ABA treatment is correlated with reduced levels of two repressive histone modifications, H3K9me2 and H3K27me2 at the promoters of these genes (Perruc *et al.*, 2007). Epistasis test revealed nearly *abi5* like germination and growth responses in *pkl abi5* double mutants, suggesting that the majority of the phenotypic defects can be explained by failure to repress *ABI5*. It is not known whether *ABI5* is directly regulated by PKL. Elucidation of the direct PKL targets is critical, as there is currently evidence for PKL acting both as a trithorax group protein (to counteract Polycomb repression) and as a promoter of Polycomb repression (Aichinger *et al.*, 2009; Jing *et al.*, 2013; Zhang *et al.*, 2012).

1.6 Tradeoffs between growth and water stress responses

Although the underlying mechanisms are largely unknown, growth arrest in adverse environments is thought to be advantageous for plant survival (Achard *et al.*, 2006; Lopez-Molina *et al.*, 2001; Skirycz and Inze, 2010). One hypothesis is that limited resources available to monocarpic (annual) plants in particular can either be allocated to

stress response or to continued growth (Bennett *et al.*, 2012). In support of this idea, ABA and drought stress not only induce expression of stress response genes, but also represses expression of genes linked to growth and metabolism (Chaves *et al.*, 2009; Shinozaki and Yamaguchi-Shinozaki, 2007; Sreenivasulu *et al.*, 2012). In addition, when major drought-responsive transcription factors are overexpressed, transgenic plants display growth retardation in non-drought conditions (Shinozaki and Yamaguchi-Shinozaki, 2007). In conditions when the stress does not threaten survival, growth inhibition may lead to an unnecessary reduction in plant growth and hence productivity and yield (Bennett *et al.*, 2012; Tardieu, 2003).

Consistent with the hypothesized tradeoff between growth and drought response several chromatin regulators have been implicated in stress-mediated temporal growth arrest at different stages of plant development. A highly dehydration sensitive developmental phase in the life of a plant is immediately after germination (Lopez-Molina *et al.*, 2001). Several chromatin regulators act at this stage to trigger water stress-dependent growth arrest, which resembles the growth arrest during late-embryogenesis in seed development. In several cases, the hyperactive stress response is due to a delay or failure to repress the embryonic developmental program (which is geared towards desiccation tolerance and growth arrest) upon germination.

One example of this type of regulator is PKL. Hypersensitive germination response to ABA of *pkl* mutants is due to failure to developmentally repress *ABI3* and *ABI5* accumulation and is restored by removing *ABI5* function (Perruc *et al.*, 2007). Other embryonic genes such as *LEC1*, *LEC2, FUS3* are constitutively de-repressed and cause formation of embryonic structures on adult *pkl* mutant plants (Aichinger *et al.*,

2009; Dean Rider *et al.*, 2003; Henderson *et al.*, 2004). Likewise, a delay in the developmental repression of the embryonic program is observed under conditions of reduced histone deacetylase activity (Tanaka *et al.*, 2008). Double mutants between *pkl* and histone deacetylase *hda6* enhanced persistence of embryonic traits and embryonic gene expression (Tanaka *et al.*, 2008). Polycomb group protein and RETINOBLASTOMA -RELATED protein (RBR) are also required for persistent silencing of late embryonic genes including *ABI3* by increasing their histone H3K27 trimethylation (Bouyer *et al.*, 2011; Gutzat *et al.*, 2011; Kim *et al.*, 2010b; Yang *et al.*, 2013). Although the role of RBR in abiotic stress response has not been investigated, seedlings with reduced RBR function arrest their growth after germination in non-stress conditions; this is accompanied by de-repression of embryonic genes linked to ABA responses including *ABI3* and *ABI5* (Gutzat *et al.*, 2011).

The SWI/SNF ATPase BRM, by contrast, displayed normal developmental down-regulation of embryonic genes (*ABI3, ABI5*) at the onset of autotrophic growth and was instead required for repressing expression of positive regulators of water stress responses in the absence of the stimulus (Han *et al.*, 2012). Moreover, the overall reduced vegetative growth of *brm* mutants under non-stress conditions is partly restored by removing *ABI5* function or by disturbing ABA signaling pathway. However, a role for BRM in repression of the embryonic program cannot be entirely rule out. Several embryonic genes were expressed in mutants lacking BRM and its close homolog SPLAYED based on transcriptome studies (Bezhani *et al.*, 2007). However, the expression of key embryogenesis regulators such as *ABI3*, *LEC1* and *LEC2* was either not changed or only marginally up-regulated (*FUS3*) in adult *brm* hypomorph mutants (Tang

et al., 2008). The SWI/SNF ATPase MINU1 is thought to be required for induction of stress-inducible genes that mediate growth arrest under abiotic stress, although direct targets of MINU1 remain to be identified (Mlynarova *et al.*, 2007).

Taken together, these studies highlight a role for chromatin modifying and remodeling enzymes at the nexus of growth versus stress response pathways, both *via* modulation of developmental programs and *via* enabling proper stimulus–dependent changes in gene expression.

1.7 Links between stress signaling pathways and chromatin modifying or remodeling enzymes.

As outlined above many chromatin changes including a change in histone variant incorporation, histone modifications, nucleosome occupancy or positioning or DNA methylation accompany stress-induced changes in gene expression. A critical question is how chromatin regulator activity is controlled to allow precise stimulus dependent changes in the accessibility of the genome. One way to achieve this may be a direct communication between components of the stress signal transduction pathway and chromatin modifying or remodeling activities.

The question whether histones in the context of chromatin can directly receive and deliver signals from cellular signal transduction cascades to facilitate specific cellular responses has recently received much attention (Badeaux and Shi, 2013; Johnson and Dent, 2013; Suganuma and Workman, 2013). Another intersection between cellular signal transduction and chromatin is indirectly through posttranslational modifications of chromatin modifying or remodeling enzymes (Badeaux and Shi, 2013). Studies in

mammals revealed that histone and DNA methyltransferases are directly phosphorylated by a downstream component of phosphoinositide signaling, the AKT kinase (Cha *et al.*, 2005; Esteve *et al.*, 2011). Likewise SWI/SNF chromatin remodelers have been shown to be phosphorylated by p38 (Simone *et al.*, 2004) as well as acetylated (Bourachot *et al.*, 2003) and SUMOylated upon signal perception (Galisson *et al.*, 2011).

Signaling transduction by SnRK2 kinases and PP2C phosphatases plays an important role in coordinating whole plant water stress responses. Calcium-dependent protein kinases (CDPKs) are also critical for proper water stress response, ABA signaling and reduction of reactive oxygen species (ROS) accumulation (Asano *et al.*, 2012), while the inositol polyphosphate 1-phosphatase FIERY1 acts a negative regulator of ABA and stress signaling (Xiong *et al.*, 2001). Thus far there is no report that links these signaling components directly to the chromatin. However links between other signal transducers and chromatin regulators have been identified. The clade A PP2C phosphatases, Hypersensitive to ABA 1 (HAB1) physically interacts with SWI3B, a core subunit of the putative *Arabidopsis* SWI/SNF complex. HAB1 is recruited to ABA response genes, this recruitment is abrogated upon ABA treatment (Saez *et al.*, 2008). HAB1 may perhaps directly de-phosphorylate SWI/SNF complexes containing SWI3B in an ABA dependent manner. In agreement with this idea, recent phosphoproteomics analyses performed by the Zhu and Shinozaki labs revealed that several chromatin regulators, including the BRM SWI/SNF ATPase, are substrates of SnRK2 type kinases in the ABA response pathway (Umezawa *et al.*, 2013; Wang *et al.*, 2013). Whether the observed phosphorylation/ de-phosphorylation of SWI3B or BRM by SnRK2 kinases/ PP2C phosphatases modulates SWI/SNF complex activity remains unknown. The *Arabidopsis*

Trithorax-like protein and histone H3 lysine 4 methyltransferase ATX1 (Alvarez-Venegas and Avramova, 2005), is involved in dehydration response in both ABA dependent and ABA independent pathways (Ding *et al.*, 2011). Intriguingly, ATX1 also directly interacts with phosphatidylinositol (Ptdlns5P), this negatively influences the ATX1 activity (Ndamukong *et al.*, 2010). Dehydration stress increases accumulation of phosphatidylinositol, a precursor of secondary messengers in stress signaling (Ndamukong *et al.*, 2010). An increase in the cellular levels of Ptdlns5P keeps ATX1 in the cytoplasm thereby diminishing ATX1 binding to target genes linked to proper water stress responses (Ndamukong *et al.*, 2010). The phosphoproteomics studies mentioned above identified additional chromatin regulators as phosphorylated upon dehydration or ABA treatment in a SnRK2 kinase-dependent manner (Umezawa *et al.*, 2013; Wang *et al.*, 2013). Although there was little overlap between the phosphorylated peptides identified in the two studies, chromatin associated proteins identified include putative components of HDAC complexes (eg. SIN3-like 2, HD2B), HAT complexes (eg. SNS1; Eaf7 superfamily), histone methyltransferases (eg. ATXR2, SDG2), chromatin remodeling ATPases (eg. CHR2/BRM, CHR5/CHD1) and Nucleolin like 1, a nucleolar protein linked to rRNA gene methylation and expression (Umezawa *et al.*, 2013; Wang *et al.*, 2013). In addition, the *Arabidopsis* histone acetyltransferase GCN5 was shown to specifically interact with PP2C6.6, a clade E PP2C with no visible mutant phenotype. GCN5 is dephosphorylated by PP2C6.6 *in vitro* and loss of PP2C6.6 activity induces GCN5-mediated histone acetylation (Servet *et al.*, 2008). A possible link to water stress responses is supported by the reported expression of PP2C6.6 in guard cells (Galbiati *et al.*, 2008).

The possibility that chromatin regulator activity is modulated upon stress sensing is intriguing *vis-à-vis* the question how these factors can execute specific roles in the organism. It is furthermore of practical significance. As chromatin regulators broadly alter the stress-inducible transcriptome they may be able to direct tolerance not only to a unique stress but to combinations of stresses that are frequently encountered in the field (Mittler and Blumwald, 2010; Yang *et al.*, 2010). Ability to precisely modulate the activity of chromatin regulators – *via* targeted post-translational modifications for example- should allow utilization of their broad reprograming capacity while minimizing detrimental effects on growth or yield.

1.8 Stress-induced transient or long-term epigenetic memory

In higher plant, stress memory phenomena known as "priming" or "acclimation" have been described (Bruce *et al.*, 2007; Conrath, 2011). Pre-exposure to mild stimuli can make plants more stress resistant and boost responses to recurring stress exposure. Well-known examples of priming are seed priming to enhance germination efficiency and crop yield, temperature acclimation and systemic acquired resistance (Bruce *et al.*, 2007; Conrath, 2011; Gutzat and Mittelsten Scheid, 2012). One mechanism proposed for long-term 'storage' of the stress memory is a mitotically heritable, or epigenetic, change in the chromatin organization. Another could conceivably rely instead on posttranslational modification of chromatin regulators. Epigenetic "Stress memory" could be maintained during subsequent development within the life span of the organism that experienced the priming stress in "somatic memory" or might perhaps even be transmitted to the progeny

across generations in “transgenerational inheritance”, a meiotically heritable change in the chromatin organization.

Unlike the mitotically heritable response to prolonged cold (Song *et al.*, 2012; Zografos and Sung, 2012), the mechanisms underlying long-term somatic stress memory are not well understood. Previous studies have shown that histone tail modifications such as H3 acetylation or H3K4 methylation occur at drought-responsive genes upon drought sensing, and correlate with active transcription of dehydration response genes (Kim *et al.*, 2008). However, drought-induced H3K9Ac marks and RNA polymerase II occupancy rapidly declined upon rehydration (Kim *et al.*, 2012a). By contrast, H3K4me3 decreased much more gradually during a five-hour rehydration period (Kim *et al.*, 2012a), suggesting that H3K4me3 could be a mitotically heritable epigenetic mark for water stress memory. In accordance with this study, another group proposed that H3K4me3 and stalled RNA polymerase II (PolII Ser5P) could function in mitotic stress memory (Ding *et al.*, 2011). Recurrent dehydration induces a higher rate of expression of dehydration response genes such as RD29B and RAB18 than primary dehydration. This is accompanied by higher H3K4me3 and Ser5P PolII accumulation at these loci (Ding *et al.*, 2011). During rehydration, the *RD29B* and *RAB18* transcript levels revert to basal expression, but H3K4me3 and Ser5p PolII association with both loci remain elevated. The observed stress memory endured until 5 days after recovery (Ding *et al.*, 2011). Likewise, H3K4 hypermethylation mediated by the Set1 histone methyltransferase in *Saccharomyces cervisiae* was proposed to provide molecular memory of recent transcriptional events (Ng *et al.*, 2003). It was suggested that elevated H3K4 trimethylation is important for genes to be rapidly switched on and off by environmental

stimuli and that it acts to prevent the associated genes from being silenced (Ng *et al.*, 2003). The combined data suggests presence of a conserved mechanism for stress memory in metazoans.

One of the main difficulties in monitoring epigenetic profiles for long-term stress memory are confounding epigenetic changes caused by altered plant growth and development in stress challenged plants. Another challenge is determining the period for which plants can 'remember' the priming event. Enhanced response to the second treatment shortly after the primary treatment could result from 'left over' proteins and metabolites that were induced by the first stress treatment. Recently, Sani *et al*., 2013 developed an experimental protocol to monitor epigenetic profiles, which aims to avoid these problems. They showed that a mild transient salt treatment of young *Arabidopsis* seedlings establishes long-term somatic memory. This was accompanied by specific changes in the H3K27me3 profile, which remained after a 10-days of subsequent growth, and resulted in drought/ high salt tolerance priming in the pre-treated plants without morphological difference between primed and non-primed adult plants (Sani *et al.*, 2013).

Interestingly, H3K4me3 is generated by a methyltransferase that belongs to the Trithorax Group of proteins (TrxG), while H3K27 is trimethylated by the PRC2 complex of Polycomb Group proteins (PcG). Recently several elegant *in vitro* and *in vivo* studies have shown that mitotic epigenetic inheritance of methylation at H3K4 and H3K27, which have been linked to stress memory in plants (above), may be mediated by the continued presence of TrxG and PcG proteins at the replication fork and on mitotic chromatin (Follmer *et al.*, 2012; Fonseca *et al.*, 2012; Lanzuolo *et al.*, 2011; Lengsfeld *et al.*, 2012; Lo *et al.*, 2012; Petruk *et al.*, 2012).

Naturally occurring DNA methylation based epialleles and epiRILs (Epigenetic Recombinant Inbred Lines) generated in the laboratory are stably inherited for many generations in plants (Becker and Weigel, 2012; Mirouze and Paszkowski, 2011; Paszkowski and Grossniklaus, 2011; Roux *et al.*, 2011; Schmitz *et al.*, 2013; Zhang *et al.*, 2013). Several reports have attempted to demonstrate stress-induced epigenetic states that are inherited by the non-stressed progeny, so -called meiotic or transgenerational epigenetic inheritance (Boyko and Kovalchuk, 2011; Grossniklaus *et al.*, 2013; Gutzat and Mittelsten Scheid, 2012; Hauser *et al.*, 2011; Paszkowski and Grossniklaus, 2011). For example, a recent study reported salt stress-induced epigenetic inheritance of DNA methylation, histone modifications and gene expression (Bilichak *et al.*, 2012). However, clear evidence for stress-induced chromatin modifications that are stably inherited by subsequent generations and contribute to phenotypic plasticity is still lacking in plants (Grossniklaus *et al*., 2013; Mirouze and Paszkowski, 2011; Pecinka and Mittelsten Scheid, 2012). As genetic changes –for example due to transposon activation- are also observed in these lines, careful assessment of the epigenetic nature of the inherited trait is required. Criteria to shore up more unambiguous support for epigenetic transgenerational stress inheritance were recently suggested and include well-controlled stress treatments and phenotypic analyses, a comprehensive or synoptic view of associated chromatin changes, establishment of causality, as well as heritability for more than 2 generations (Grossniklaus *et al*., 2013; Pecinka and Mittelsten Scheid, 2012).

At a time when we face the twin challenges of human population growth and loss of arable land due to climate change, it is critical to understand the molecular mechanisms that regulate water stress tolerance and mitotic inheritance of stress

responses during priming. Evidence is mounting for a role of DNA methylation, histone modifications, and altered nucleosome occupancy, positioning, or composition in both responses. As stresses in nature do not occur in isolation (Mittler and Blumwald, 2010; Yang *et al*., 2010), it is possible that changes in chromatin organization may endow the plants with the ability to survive combinations of stresses and to remain primed for further stress responses. Challenges for the future are: (1) to elucidate which chromatin alterations may be instructive for altered stress responses, rather than a consequence thereof; (2) to understand which chromatin alterations lead to stress tolerance that is mitotically (or meiotically) heritable; and (3) to devise ways to modulate the activity of 'instructive' chromatin regulators in ways that allow enhanced primary or heritable stress tolerance without causing growth or yield trade-offs.

In the following three chapters, I will present the data for my thesis projects. In Chapter 2, I will present the role of SWI/SNF chromatin remodeling complex in water stress and chromatin changes triggered by ABA or loss of BRM. This chapter has been adapted from "The SWI2/SNF2 chromatin remodeling ATPase BRAHMA represses abscisic acid responses in the absence of the stress stimulus in *Arabidopsis*." Han *et al*, The Plant Cell, 2012. In Chapter 3, I will present the data from investigation on the regulation of SWI/SNF complex activity by ABA sensing and signaling. This part is incomplete and substantial experiments are still needed to support proposed hypothesis. I have highlighted the experiments that are in progress to address unanswered questions in Chapter 3. In Chapter 4, I have presented discussion and future direction.

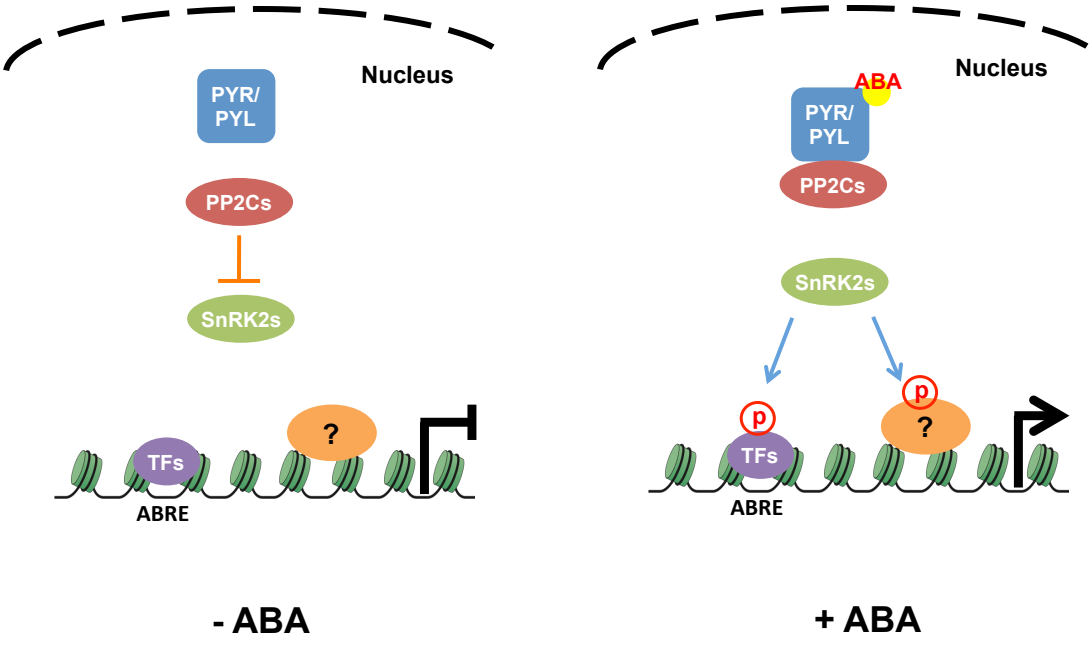


Figure 1-1. Core ABA signal transduction pathway in the nucleus.

ABA receptors (PYR/PYL/RCAR), PP2C and SnRK2 form a core-signaling complex. ABA binds to ABA receptors (PYR/PYL/RCAR) in a ternary complex with the clade A PP2C phosphatases (Ma *et al*., 2009; Park *et al*., 2009), which frees the activity of SnRK2 kinases to phosphorylate downstream targets. ABRE/ABF-type of transcription factors (TFs) that induce ABA responsive gene expression are well known targets of SnRK2 kinases. Other substrates of SnRK2 have not been identified are marked with a question mark.

CHAPTER 2. The role of SWI/SNF chromatin remodeling in ABA dependent drought responses

(Adapted from Han *et al*., *The Plant Cell*, 2012 Dec;24(12):4892-906. doi: 10.1105/tpc.112.105114. Epub 2012 Dec 3.)

2.1 Background

Altered transcriptional responses to environmental stimuli, such as abiotic stress, have been linked to chromatin regulation (Chinnusamy and Zhu, 2009; Kim *et al.*, 2010a). Chromatin mediated control of inducible gene expression is performed by two general types of activities. One mechanism involves enzymes that covalently modify histones and/or the DNA, such as histonemodifying enzymes or DNA (de)methylases (Li *et al.*, 2007). A second general mechanism for chromatin-mediated control of inducible gene expression is noncovalent alteration of the nucleosome position, occupancy, conformation, and composition by chromatin remodeling ATPases. Among the chromatin remodeling ATPases, the SWI/SNF subgroup has been studied extensively (Li *et al*., 2007; Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011).

SWI/SNF subgroup ATPases are conserved from yeast to humans and plants (Flaus *et al*., 2006). Plant genomes contain three types of SWI/SNF subgroup chromatin remodeling ATPases, which are called BRAHMA (BRM), SPLAYED (SYD), and MINUSCULE (MINU) (Flaus *et al.*, 2006; Jerzmanowski, 2007; Kwon and Wagner, 2007; Sang *et al.*, 2012). SWI/SNF ATPases act in large protein complexes that are required for full activity *in vivo* and use the energy derived from ATP hydrolysis to alter histone–DNA interactions (Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011). SWI/SNF complexes can increase or decrease accessibility of the genomic DNA and

hence activate or repress transcription, respectively (Tang *et al*., 2008; Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011). Selectivity of SWI/SNF activity is due to recruitment to target loci by sequence-specific proteins and/or regulation of complex activity by posttranslational modifications or complex composition (Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011).

Constitutive activation of ABA signaling by removing negative regulators of the ABA pathway or by enhancing transcriptional response to ABA causes ABA hypersensitivity and enhanced drought tolerance (Lopez-Molina *et al*., 2001; Kang *et al*., 2002; Fujita *et al*., 2005; Rubio *et al*., 2009). However, it also causes impaired growth under normal growth conditions (Kang *et al*., 2002; Fujita *et al*., 2005). This is because the abiotic stress responses divert resources from normal growth and development (Boyer, 1982; Cramer *et al.*, 2011; Grill and Ziegler, 1998; Less *et al.*, 2011). It is therefore critical that desiccation responses are repressed in non-stress conditions. Here, I describe a role for the *Arabidopsis thaliana* BRM SWI/SNF chromatin remodeling complex components in direct transcriptional repression of *ABI5* during postgermination development.

2.2 Results

2.2.1 ABA responses in the mutant of SWI/SNF chromatin remodeling ATPase

To investigate a possible link between SWI/SNF-dependent chromatin remodeling complexes and postgermination ABA responses, we probed the effect of mutations in each of the four Arabidopsis SWI/SNF ATPases, SYD, BRM, MINU1, and MINU2

(Farrona *et al.*, 2004; Flaus *et al.*, 2006; Sang *et al.*, 2012) on ABA-dependent growth arrest. Of all mutants tested, those in BRM displayed the most dramatic change in ABA sensitivity relative to the wild type (Figures 2-1A and Figure 2-1B). I therefore focused further analyses on the role of the BRM complex in ABA-mediated postgermination growth inhibition. I therefore focused further analyses on the role of the BRM complex in ABA-mediated postgermination growth inhibition.

2.2.2 Increased ABA sensitivity in *brm* mutants

After germination of *brm-3* hypomorphic (Farrona *et al*., 2007) mutants on agar plates containing submicromolar ABA concentrations, the mutant germinated embryos failed to develop green cotyledons and the first pair of true leaves at the lowest ABA concentration tested (Figures 2-2A and 2-2B). The wild type did not display growth arrest in this condition. Moreover, when I transferred *brm-3* and *brm-1* null (Hurtado *et al.*, 2006) mutants to plates containing ABA, the growth of the primary root of was inhibited by ABA to a greater extent than wild-type roots (Figures 2-2C and 2-2D). Thus, relative to the wild type, *brm* mutants were hypersensitive to ABA.

2.2.3 Mutations in two components of SWI/SNF complex cause ABA hypersensitive phenotype.

The evolutionally conserved SWI/SNF core complex consists of one ATPase, two SWI3 subunits, and one SNF5 complex component (Hargreaves and Crabtree, 2011; Jerzmanowski, 2007; Kwon and Wagner, 2007; Phelan *et al.*, 1999). The Arabidopsis

genome encodes four SWI3 subunit genes (called SWI3A-D) and one SNF5 subunit gene (termed BUSHY) (Brzeski *et al.*, 1999; Sarnowski *et al.*, 2005). The morphological defects observed in *brm* null mutants are very similar to those of *swi3c* null mutants (Archacki *et al.*, 2009; Sarnowski *et al.*, 2005). Moreover, BRM and SWI3C show strong direct physical interaction (Hurtado *et al.*, 2006), suggesting that SWI3C may be a dedicated BRM complex component. Therefore, I next examined the role of SWI3C and BUSHY (BSH) in postgermination ABA responses. Null *swi3c-2* mutants (Sarnowski *et al*., 2005) showed an ABA-hypersensitive phenotype similar to *brm* mutants both with respect to cotyledon greening (Figures 2-3A and 2-3B) and growth of the primary root (Figures 2-3C and 2-3D). Likewise, the hypomorphic *bsh-1* mutant (Tang *et al*., 2008) displayed ABA hypersensitive growth arrest (Figures 2-3E and 2-3F). I next examined seed germination (radicle emergence) in *brm* mutants relative to the wild type using a range of ABA concentrations. In radicle emergence assays (Müller *et al.*, 2006), *brm-3* hypomorphic mutants did not display significantly altered sensitivity to ABA (Figure 2-4A). By contrast, *brm-1* null mutants were significantly more sensitive to low ABA concentrations than the wild type with respect to germination (Figure 2-4B). The combined data suggest that BRM affects germination and postgermination response to ABA with a very prominent role for BRM in cotyledon greening.

2.2.4 Derepression of ABA-responsive genes in the absence of the stress hormone in *brm* mutnats

To gain insight into the molecular underpinnings of the observed *brm* mutant ABA hypersensitivity, I analyzed the expression of ABA-responsive genes in *brm-3* mutant

and wild-type embryos during postgermination development. I employed the hypomorphic *brm-3* allele because, unlike the *brm-1* null mutant, it is fertile and thus facilitates testing of homozygous mutant embryos. I examined expression of the bZIP transcription factor *ABI5* and the B3 transcription factor *ABI3*, key regulators of dormancy and desiccation tolerance in germinated embryos (Finkelstein and Lynch, 2000; Giraudat *et al.*, 1992; Lopez-Molina and Chua, 2000; Lopez-Molina *et al.*, 2001; Lopez-Molina *et al.*, 2002; Parcy and Giraudat, 1997). In addition, I quantified expression of the bZIP transcription factor *ABF3*, which has been shown to act in part in a pathway parallel to *ABI5* (Finkelstein *et al.*, 2005; Kang *et al.*, 2002; Yoshida *et al.*, 2010) and *HY5*, a component of the light signal transduction pathway and direct upstream regulator of *ABI5* (Chen *et al.*, 2008). Gene expression was examined in plants grown in continuous light at day 1.5 and day 2.0 after stratification. These time points were chosen because growth arrest is triggered by ABA only before seedling establishment, in the first 48 h after stratification (Lopez-Molina *et al*., 2001). I observed derepression of *ABI5* expression in *brm-3* relative to the wild type at both time points (4.4-fold and 3.1-fold at days 1.5 and 2, respectively; Figure 2-5A). The level of *ABI5* mRNA was also much higher in *brm-3* mutants relative to the wild type 1 h after ABA sensing; however, the rate of *ABI5* induction by ABA was similar in both genotypes (5.2-fold and 5.1-fold at day 1.5 in the wild type and in *brm-3*, respectively; Figure 2-5A). *ABI3* expression, by contrast, was strongly derepressed only at day 1.5 in *brm-3* mutants relative to the wild type. At day 2, *ABI3* derepression in *brm* mutants and induction by ABA was much less pronounced. Again, there was no increase in the fold induction of *ABI3* expression by ABA in the *brm* mutant relative to the wild type. *ABF3* expression was only marginally

increased in *brm-3* in any condition tested, while *HY5* expression was not at all altered in the *brm-3* mutant (Figure 2-5A). Thus, partial loss of BRM function led to altered expression of select ABA-responsive genes; most notably derepression of *ABI5* and *ABI3* expression in the absence of exogenous ABA application.

2.2.5 BRM directly represses transcription of *ABI5.*

Since SWI/SNF complexes can both activate and repress transcription (Hargreaves and Crabtree, 2011; Kwon *et al.*, 2005; Tang *et al.*, 2008), it is possible that the effect of BRM on *ABI5* and *ABI3* mRNA accumulation in the absence of the stress hormone is direct. The expression of BRM was consistent with a possible role in regulation of gene expression at this stage. BRM was expressed in both 1.5- and 2-d-old germinated embryos (Figure 2-5B). To test for binding of BRM to either the *ABI5* or the *ABI3* locus, I used a green fluorescent protein (GFP)–tagged biologically active version of BRM (ProBRM:BRM-GFP; (Wu *et al.*, 2012)), which fully rescued the morphological defects of the *brm-1* null mutant and displayed wild-type levels of BRM expression (Figure 2-6A and 2-6B). Using the *brm-1* ProBRM:BRM-GFP as a substrate for chromatin immunoprecipitation (ChIP), I detected strong BRM binding to the *ABI5* promoter and to the promoter proximal exon 1 of *ABI5*, but not to exon 2 (Figures 2-6C and 2-6D). In addition, I detected BRM association with the promoter of *ABI3* (Figures 2-6C and 2-6D). To confirm these results, I generated a Hemagglutinin (HA)-tagged version of BRM, which rescued the *brm-1* null mutant and displayed wild-type levels of BRM expression (Figure 2-7A). ChIP using *brm-1* ProBRM:BRM-HA yielded qualitatively similar results as *brm-1* ProBRM:BRM-GFP (Figure 2-7B). The association of BRM

with the *ABI5* and *ABI3* loci, in combination with the observed derepression of *ABI5* and *ABI3* expression in *brm* mutants, supports the hypothesis that BRM directly acts on *ABI5* and *ABI3* expression. Two of the BRM-bound regions (p1 in *ABI5* and p1 in *ABI3*) contain ABREs (Figure 2-6D), *cis*-elements known to be involved in ABA-induced transcriptional responses (Gómez-Porras *et al.*, 2007; Yamaguchi-Shinozaki and Shinozaki, 1994).

I observed high BRM binding at the two loci both in the absence and in the presence of ABA treatment (Figure 2-6C). This finding was surprising, given that the main effect of loss of BRM activity is derepression of *ABI5* and *ABI3* expression in the absence of ABA treatment (Figure 2-5A). The data suggest that BRM is constitutively bound to the *ABI5* and the *ABI3* locus.

2.2.6 ABI5 acts downstream of BRM and is required for *brm* ABA hypersensitivity

Prior molecular and genetic experiments have shown that ABI3 acts upstream of *ABI5* in the ABA-mediated growth arrest of germinated embryos (Lopez-Molina *et al*., 2002). To elucidate the placement of BRM in this genetic pathway, I generated a double mutant between *brm-3* and the *abi5-7* null mutant (Yamagishi *et al.*, 2009). The *brm-3* allele was employed so we could assay the response in homozygous germinated embryos. While *brm-3* was hypersensitive to ABA with respect to inhibition of cotyledon greening, *abi5-7* was not responsive to any of the ABA concentrations tested (Figures 2-8A and 2-8B), consistent with previous reports (Nambara *et al.*, 2002). Interestingly, the *brm-3 abi5-7* double mutant was also not responsive to any of the ABA concentrations tested; like *abi5-7*, it developed green cotyledons even at the highest dose of ABA tested (Figures 2-

8A and 2-8B). The data suggest that, with respect to cotyledon greening, ABI5 is epistatic to BRM. This finding, combined with the observed *ABI5* derepression in *brm* mutants and BRM binding to the *ABI5* locus, support the hypothesis that ABI5 acts downstream of BRM. I also tested the ABA response of *brm-3 abi5-7* double mutant with respect to inhibition of primary root growth. As previously reported (Finkelstein *et al.*, 2005; Miura *et al.*, 2009), the growth of *abi5-7* (nulls) roots is inhibited by ABA (Figures 2-8C and 2-8D), suggesting redundant activities of other ABA-dependent transcription factors in root growth arrest. Nevertheless, *brm-3 abi5-7* roots were significantly less sensitive to 1 or 5 μM ABA than those of *brm-3* (Figures 2-8C and 2-8D). These data suggest that the increased ABA-dependent inhibition of root growth in the *brm-3* mutants is in part attributable to the elevated *ABI5* expression.

2.2.7 Vegetative growth defects of *brm* mutant are partly due to derepressed ABA responses.

In the absence of ABA, *brm* plants are small with short roots (Farrona *et al.*, 2004; Kwon *et al.*, 2006). Given our finding that BRM represses ABA responses in the absence of the stimulus during postgermination development, I wondered whether some of the *brm* mutant vegetative growth defects are attributable to derepressed ABA responses. I therefore monitored root length in double mutants of *brm* and mutants that display reduced ABA sensitivity. Since *ABI5* derepression is only partly responsible for root growth inhibition in *brm* mutants (Figure 2-8C and 2-8D), I employed a genetic background that displays reduced ABA sensitivity, 35S:HAB1 (Saez *et al.*, 2004). *HAB1* encodes for a PP2C phosphatase, a negative regulator of ABA signaling, that prevents

phosphorylation of SnRK2-type kinases (Vlad *et al.*, 2009) and, hence, activation of the ABA-responsive transcription factor *ABI5* (Nakashima *et al.*, 2009). 35S:HAB1 inhibits ABA responses in the absence of ABA treatment because low levels of endogenous ABA are able to partially activate ABA-responsive transcription factors in non-ABA-treated plants (Rodrigues *et al.*, 2009). For these assays, I used the *brm-101* null nonsense allele (Kwon *et al*., 2006) to avoid silencing of the 35S:HAB1 transgene by the T-DNA present in *brm-1* or *brm-3*. As previously reported, the growth of 35S:HAB1 was indistinguishable from the wild type in the absence of applied ABA (Figure 2-9A; Saez *et al*., 2004). However, 35S:HAB1 was able to partly rescue the root growth defects of *brm* mutants under these conditions (Figures 2-9A and 2-9B). In addition, overall growth of *brm-101* 35S:HAB1 was more vigorous than that of *brm-101*. At day 7, the cotyledons of the double mutant were fully expanded, while those of *brm-101* mutants were closed and small (Figure 2-9B). I also measured plant fresh weight in the wild type, *abi5-7* null mutants, *brm-3* mutants, and *brm-3 abi5-7* double mutants (Figure 2-9C). Removal of ABI5 activity from *brm-3* mutants caused a partial but significant rescue of the *brm* mutant vegetative growth defect in the absence of ABA treatment (Figure 2-9C). In combination, the data suggest that the growth defects of *brm* mutants are in part due to the derepressed ABA response. The partial rescue of vegetative growth defects by removal of ABI5 activity suggested that *ABI5* levels may also be elevated in *brm* mutants during vegetative development. I therefore analyzed expression of ABA-responsive genes in 3-week-old soil-grown *brm-1* null mutants and the wild type. *ABI3* expression is repressed after seedling establishment and remains repressed during vegetative development even upon ABA treatment (Nakashima *et al.*, 2006; Perruc *et al.*, 2007;

Tang *et al.*, 2008). Likewise, *ABI3* mRNA was not detectable in the absence or presence of exogenous ABA in the *brm-1* mutant (Figure 2-10A). *ABI5* expression, on the other hand, was derepressed (2.5-fold) in the absence of the stimulus and more strongly induced in response to ABA in the *brm-1* null mutant relative to the wild type (Figure 2-10B). In addition, I tested expression of the bZIP transcription factors, *ABF3* and *AREB1/ABF2*; both genes are strongly induced by ABA, salt, and drought during vegetative development (Fujita *et al.*, 2005). *ABF3* expression was elevated in *brm-1* mutants both in the absence and presence of exogenous ABA, while *AREB1/ABF2* expression was not strongly altered. Thus, BRM is also required for repression of ABA-responsive genes during vegetative development, including that of the bZIP transcription factors *ABI5* and *ABF3*. At least in the case of *ABI5*, the observed effect was direct: BRM associated with the *ABI5* promoter at this stage, based on ChIP (Figure 2-10C).

2.2.8 *brm* Mutants Display Enhanced Drought Tolerance

Mutants with increased sensitivity to ABA, such as *pp2c* mutants or plants overexpressing ABA-responsive transcription factors, display increased dehydration tolerance (Kasuga *et al.*, 1999; Rubio *et al.*, 2009). *brm* mutants were hypersensitive to ABA and showed derepression of ABA/drought-responsive gene expression; hence, I wondered whether *brm* mutants might display increased drought stress tolerance. To test this possibility, 3-weekold *brm* mutants and wild-type plants grown on soil were subjected to drought treatment. After 3 weeks of growth, water was withheld for 15 d (Figure 2-12 and Methods). After water withholding, wild-type and *brm-3* plants looked dehydrated and displayed severe tissue damage, while *brm-1* plants were healthy looking

and maintained greenish leaves. Upon rewatering, both *brm* mutants recovered quickly from the drought stress, while the wild type failed to recover (Figure 2-11). While the drought tolerance of *brm* was remarkable (similar to that described for *pp2c* triple mutants; Figure 2-11), I cannot rule out that it is at least in part attributable to the different morphology of the *brm* mutant leaves, which are curled and smaller than those of the wild type. I therefore challenged younger (2-week-old) seedlings grown on plates with water stress. At this developmental stage, the *brm-3* mutant is morphologically very similar to the wild type (Figure 2-11; (Farrona *et al.*, 2007)). Upon drought treatment, wild-type plants wilted faster than the *brm* mutants; in addition, they did not recover as well from the drought stress (Figure 2-11B). *brm-3* plants again exhibited a significantly higher survival rate than the wild type after drought stress and rewatering (Figures 2-11C and 2-11D). The data are consistent with the hypothesis that *brm* mutant drought tolerance may be due to altered ABA-response gene expression. To further test this hypothesis, I examined whether the drought tolerance of *brm-3* plants was due to elevated *ABI5* expression. Overexpression of ABI5 was previously shown to lead to increased drought tolerance (Lopez-Molina *et al*., 2001). However, *brm-3 abi5-7* plants were as drought tolerant as *brm-3* alone (Figures 2-11E and 2-11F). Thus, either ABI5 does not contribute to the drought tolerance of *brm* mutant or it does so redundantly with other ABA-responsive transcription factors, whose expression is also derepressed in *brm* mutants, such as ABF3 (Figure 2-10).

2.2.9 BRM contributes to placement and occupancy of the transcription start site proximal nucleosome at the *ABI5* locus

To gain insight into the mechanism by which the SWI/SNF chromatin remodeling ATPase BRM might represses *ABI5* expression in the absence of stress hormone treatment, I next examined nucleosome positioning and occupancy at the *ABI5* promoter using high-resolution MNase mapping (Chodavarapu *et al.*, 2010; Rafati *et al.*, 2011). I identified two well-positioned nucleosomes in the *ABI5* promoter region (-2 and -1 nucleosome) upstream of a 150-bp nucleosome-depleted region (2150 to 0 bp) (Figure 2-13). Nucleosome-depleted regions just upstream of the transcription start site (TSS) are common in eukaryote promoters (Yen *et al.*, 2012). A typical nucleosome protects; 147 bp of genomic DNA from MNase digestion (Yen *et al.*, 2012), as was the case for the -2 and -1 nucleosomes at the *ABI5* locus (Figure 2-13). However, the +1 *ABI5* nucleosome just downstream of the TSS protected; 200 bp of DNA, suggesting that this nucleosome may be present in two alternative positions. A nucleosome position prediction program (NuPop; (Xi *et al.*, 2010)) identified nucleosome start sites around position +45, while the MNase mapping revealed start of the +1 nucleosome close to the +1 position. The data suggest that a subset of the +1 nucleosomes are positioned more TSS proximal than predicted. In *brm* mutant plants, I observed derepression of *ABI5* expression in the absence of ABA treatment (Figure 2-5). Consistent with this observation, I reproducibly found a moderate (~ 40%) reduction in nucleosome occupancy at the + 1 position of the *ABI5* locus coupled with a shift away from the TSS in the absence of ABA treatment in *brm* mutant relative to wild-type germinated embryos (Figure 2-13). No BRM-dependent alteration in nucleosome positioning or occupancy was observed at the -2 or -1 nucleosome of the *ABI5* locus (Figure 2-13). Likewise, no strong change in either occupancy or positioning of nucleosomes was observed at a control locus, a gypsy-like

retrotransposon gene (Figure 2-14). Thus, BRM may be required to promote high occupancy and TSS proximity of the +1 nucleosome at the *ABI5* locus.

2.2.10 ABA triggered chromatin changes at *ABI5* regulatory region

In addition, I detected reduced occupancy of all three nucleosomes (-1, -2, +1) in response to ABA treatment (Figure 2-13). The observed ABA-dependent change in nucleosome occupancy was similar in germinated *brm-3* and wild-type embryos, suggesting that this effect was likely BRM independent (Figure 2-13). The reduced nucleosome occupancy in response to ABA was specific to the *ABI5* locus; it was not observed at the control locus (Figure 2-14).

2.2.11 Chromatin changes at *ABI5* regulatory regions during postgermination

Finally, I noted development dependent changes in the -1 nucleosome occupancy at the *ABI5* promoter just prior to seedling establishment. The occupancy of the -1 nucleosome was very low at day 1.5 in both mock-treated wild-type and *brm-3* plants but increased at day 2 (Figure 2-15). I did not detect a strong increase in the occupancy of the -2 and +1 nucleosome between days 1.5 and 2.

2.2.12 Well-positioned nucleosome found at ABA-responsive genes

(This analysis was performed in collaboration with the lab of Dr. Brian Gregory. Dr. Qi Zheng reanalyzed the published dataset.)

Visual inspection of a published nucleosome map for vegetative tissues of *Arabidopsis* (Chodavarapu *et al.*, 2010) revealed a well-positioned nucleosome at the *ABI5* locus, which is located over two consensus (PyACGTGG/TC) ABA response *cis* elements (ABREs) (Fujita *et al*., 2011). To determine whether well-positioned nucleosomes are common in the regulatory regions of ABA-responsive genes, we reanalyzed the published dataset (Chodavarapu *et al.*, 2010). Overall promoters of *Arabidopsis* genes were significantly depleted of nucleosomes relative to the genic regions (Figure 2-16A). A small number of genes (126 out of 27,379) had a well-positioned nucleosome with high occupancy (four-fold genome average) in the proximal promoter region (-250 bp to 0 bp), including *ABI5.* Gene Ontology term (GO) analysis using AgriGo (Du *et al.*, 2010) revealed that the 126 genes were enriched for GO terms linked to ABA responses and to seed development (p value $<1x\ 10^{-3}$, FDR<0.05; Figure 2-16B). These findings support the hypothesis that well-positioned promoter nucleosomes may play a role in the transcriptional response to the stress hormone ABA.

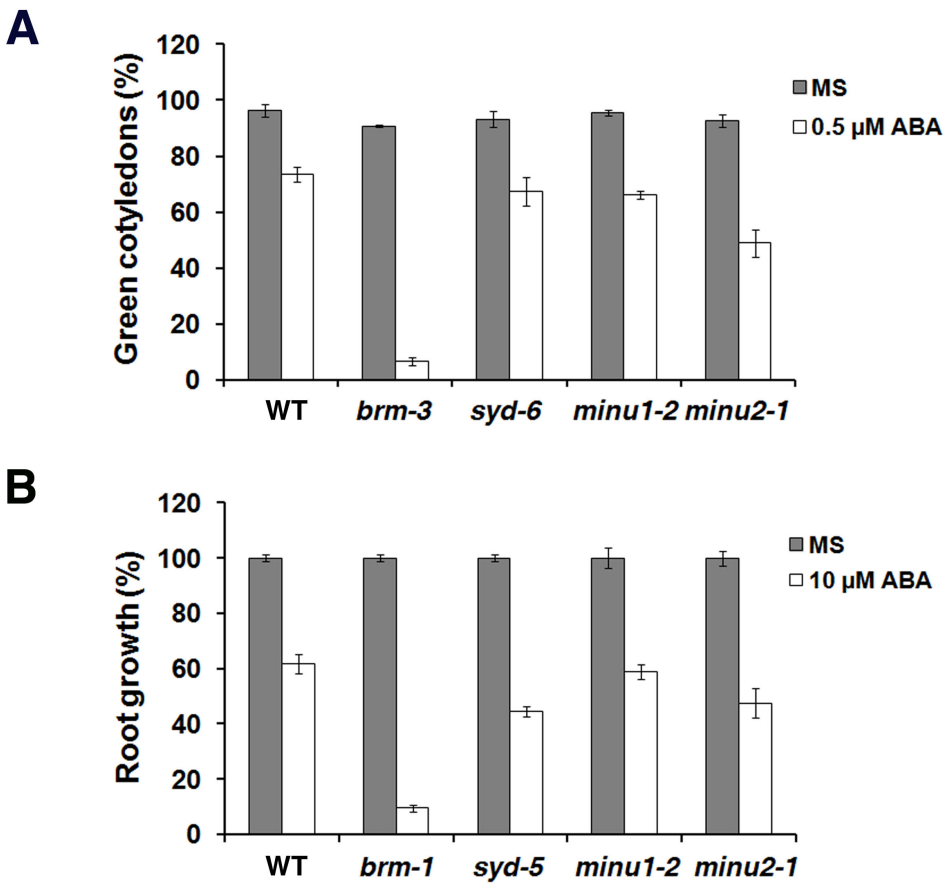


Figure 2-1. ABA responses of SWI/SNF chromatin remodeling mutants

(A) The percentage of germinated embryos that developed green cotyledons in the presence of 0.5 µM ABA in the wild type (WT) and single mutants of the four *Arabidopsis* SWI/SNF subgroup ATPases. The strongest available fertile allele was used for each mutant.

(B) Root growth inhibition of the WT, and null mutants of the four SWI/SNF ATPases in the presence of 10 µM ABA relative to that observed on MS media.

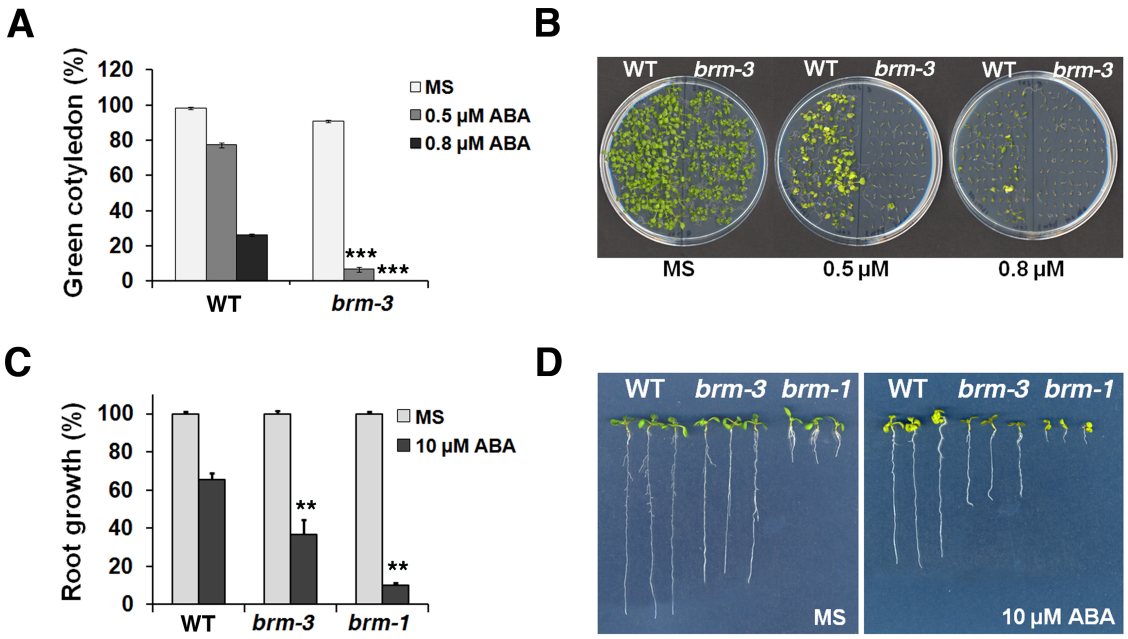


Figure 2-2. *brm* mutants are hypersensitive to ABA.

(A) The percentage of germinated embryos that developed green cotyledons in the presence of 0.5 or 0.8 µM ABA in the wild type (WT) and in the hypomorph *brm-3* mutant. Values are mean ± SEM from three independent experiments. Asterisks indicate statistical significance compared with wildtype values based on $\chi 2$ test ($n = 250$, $P < 1E-10$). (B) Representative pictures for the data shown in (A). Photographs were taken 11 (MS) and 18 (ABA) d after stratification. (C) Root growth inhibition of *brm-1* null and *brm-3* hypomorph mutants. Values are mean ± SEM from two independent experiments. Asterisks indicate statistical significance compared with wild-type values based on one-tailed Student's t test ($n = 10$, $P < 0.001$). (D) Representative pictures for data shown in (C). Photographs were taken 10 d after stratification.

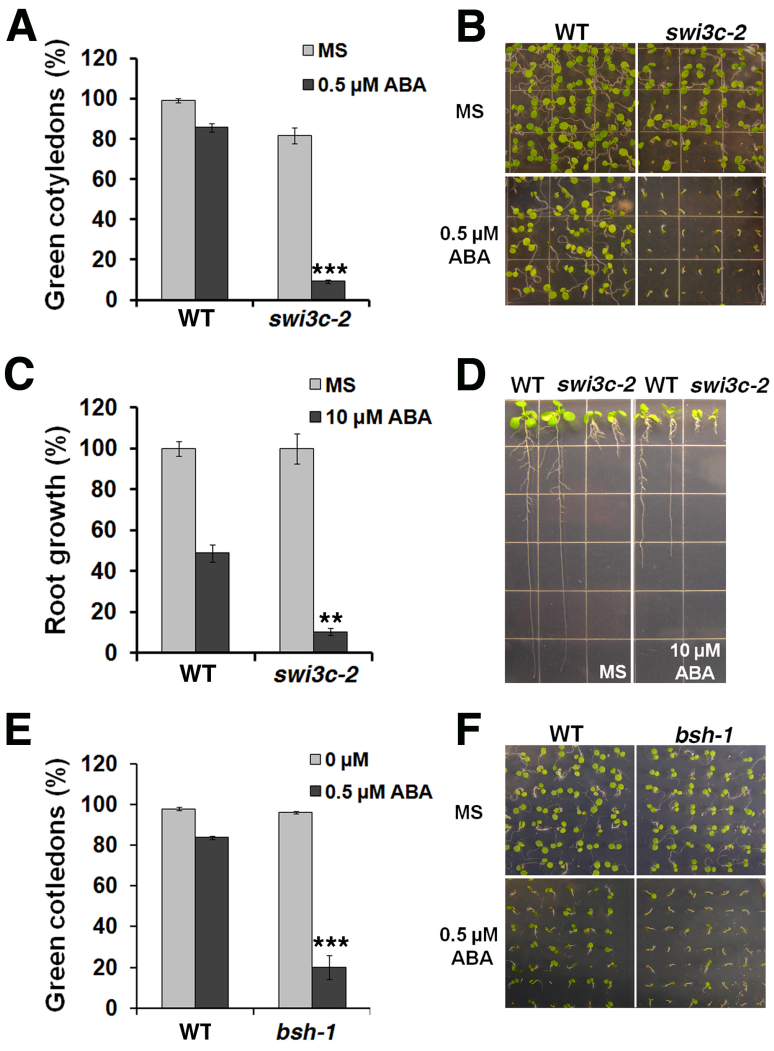

A
Green cotyledons (%)
120
100
80
60
40
20
0
MS
0.5 μM ABA
***
WT
swi3c-2
B
WT
swi3c-2
MS
0.5 μM ABA
C
Root growth (%)
MS
10 μM ABA
**
WT
swi3c-2
D
WT
swi3c-2
WT
swi3c-2
MS
10 μM ABA
E
Green cotledons (%)
0 μM
0.5 μM ABA
***
WT
bsh-1
F
WT
bsh-1
MS
0.5 μM ABA

Figure 2-3. *swi3c-2* and *bsh-1* mutants are hypersensitive to ABA.

(A) The percentage of germinated embryos that developed green cotyledons in the presence of 0.5 μM ABA in the wild type (WT) and in the *swi3c-2* mutant. Asterisks indicate statistical significance based on χ^2 test (n = 100, P < 1E-10).

(B) Representative pictures for data shown in (A) 7 d after stratification.

(C) Root growth inhibition of the wild type and in the *swi3c-2* mutant in the presence of 10 μM ABA relative to that observed on MS media. Asterisks indicate statistical significance based on Student's t test (n =20, P < 0.001).

(D) Representative pictures for data shown in (C) 10 d after stratification.

(E) The percentage of germinated wild-type and *bsh-1* embryos that developed green cotyledons in the presence of 0.5 μM ABA. Values are mean ± SEM from two independent experiments. Asterisks indicate statistical significance based on χ^2 test (n = 200, P < 1E-10).

(F) Representative pictures for the data shown in (E) 5 d after stratification.

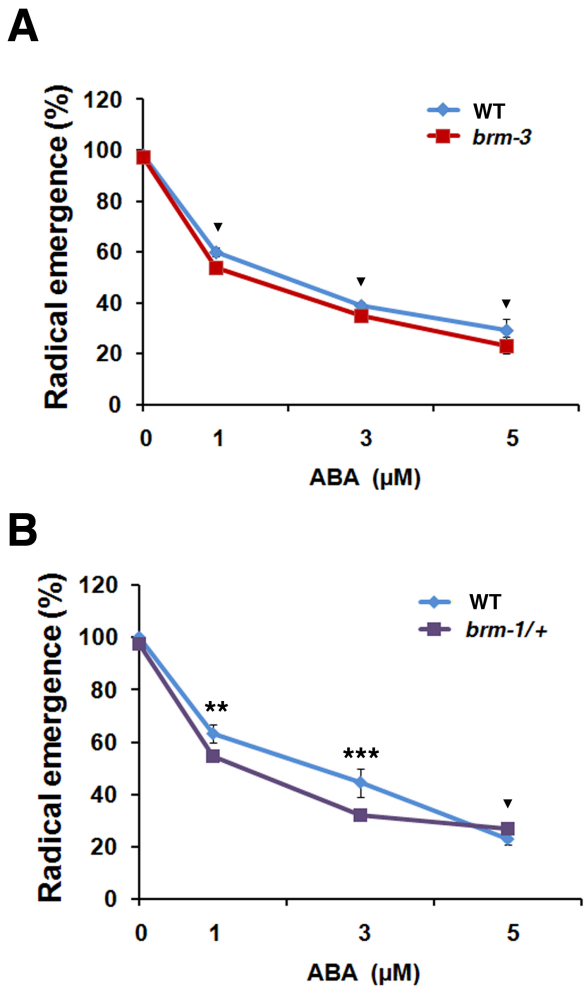


Figure 2-4. Germination assay of *brm* mutants

(A, B) Effect of ABA on radicle emergence in the wild type (WT), in *brm-3* and in *brm-1*/+ progeny 3 days after stratification in the absence and presence of 1, 3, 5 µM ABA. Samples sizes were *brm-3* (n=180) and *brm-1*/+ (n>500) for each ABA concentration. Values are mean ± SEM of 3 independent experiments (Asterisks: **P<0.001, ***P<1E-10, inverted triangles: no statistical significance based on chi-square analysis P>0.01).

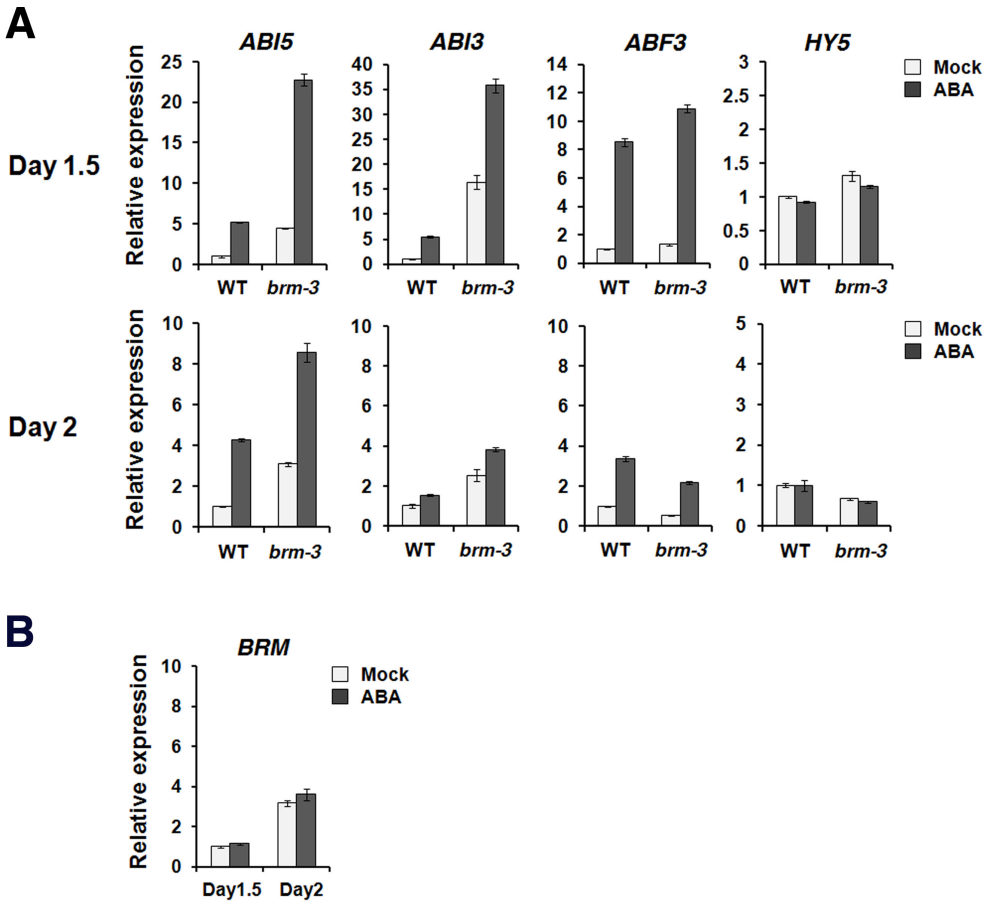


Figure 2-5. BRM represses expression of *ABI5* and *ABI3* during postgermination development.

(A) Quantitative RT-PCR in 1.5- and 2-d-old wild-type (WT) and *brm-3* mutants 1 h after mock or ABA (50 μM) treatment.

(B) Quantitative RT-PCR in 1.5- and 2-d-old wild-type plants 1 h after mock or ABA treatment. Quantitative RT-PCR expression was normalized over that of *EIF4A1*, and expression levels in the mock-treated wild type were set to 1. Values are mean ± SEM of three technical replicates from one representative experiment.

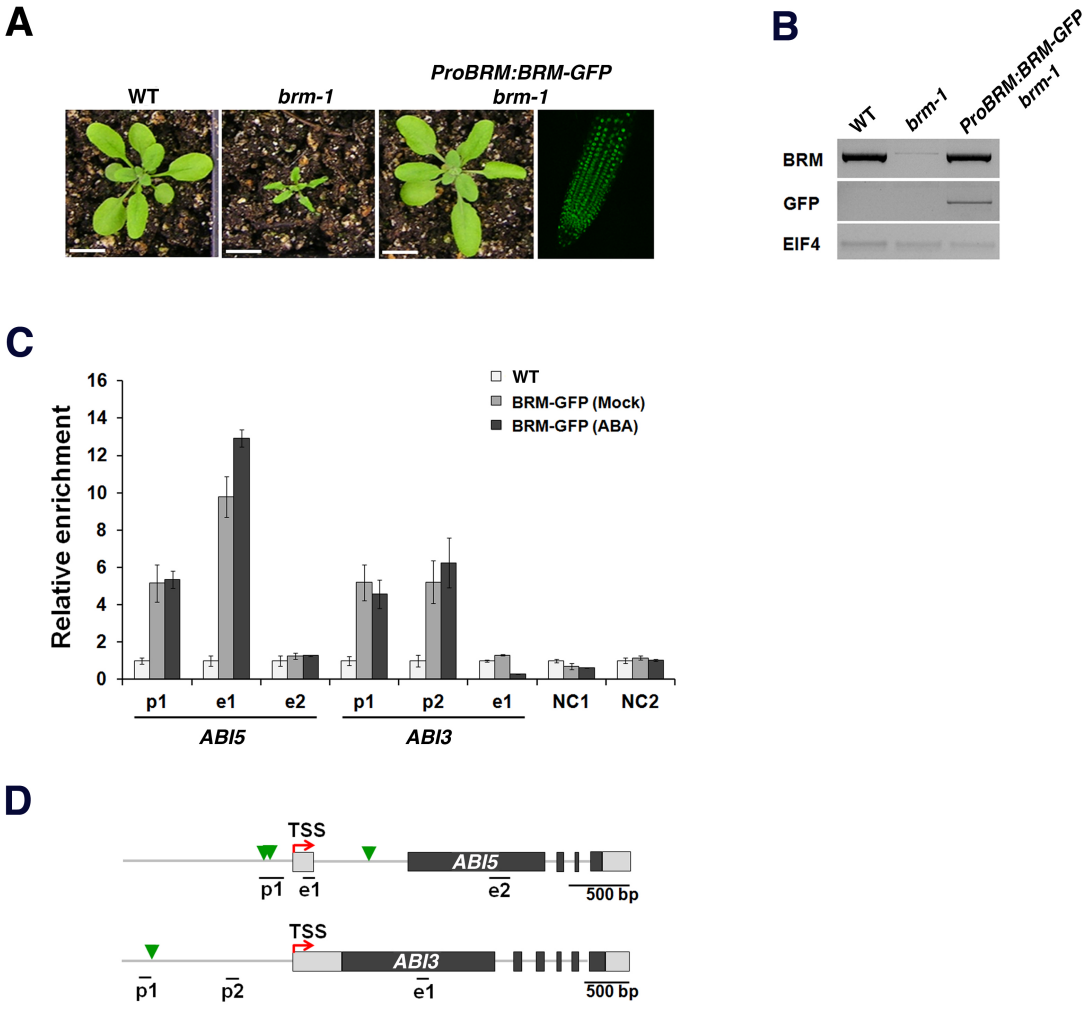

A
WT
brm-1
ProBRM:BRM-GFP
brm-1
B
WT
brm-1
ProBRM:BRM-GFP
brm-1
BRM
GFP
EIF4
C
Relative enrichment
16
14
12
10
8
6
4
2
0
WT
BRM-GFP (Mock)
BRM-GFP (ABA)
p1
e1
e2
p1
p2
e1
NC1
NC2
ABI5
ABI3
D
TSS
ABI5
p1
e1
e2
500 bp
TSS
ABI3
p1
p2
e1
500 bp

Figure 2-6. BRM directly repress *ABI5* and *ABI3* expression during postgermination development.

(A) Left: 3-week-old wild-type, *brm-1*, and *brm-1* ProBRM:BRM-GFP plants. Right: GFP expression monitored by confocal microscopy in 2-d-old *brm-1* ProBRM:BRM-GFP roots.

(B) *BRM* expression (top panel), *GFP* expression (center panel), and *EIF4A1* expression (bottom panel) tested by semiquantitative PCR. Bars = 1cm.

(C) qPCR after anti-GFP ChIP in 1.5-d-old *brm-1* ProBRM:BRM-GFP plants after mock or ABA (50 μM) treatment for 1 h. Relative enrichment is the percentage of input fold change after the percentage of input of the wild type was set to 1. Negative controls: exon regions of the retrotransposon *TA3* (NC1) and of *BRM* (NC2). Values are mean ± SEM of three technical replicates from one representative experiment.

(D) Diagram of the loci tested. Horizontal lines below the schematic, regions amplified by qPCR; green arrowheads, ABREs; gray box, 5' or 3' untranslated region; black box, exon; gray line, intergenic region or intron.

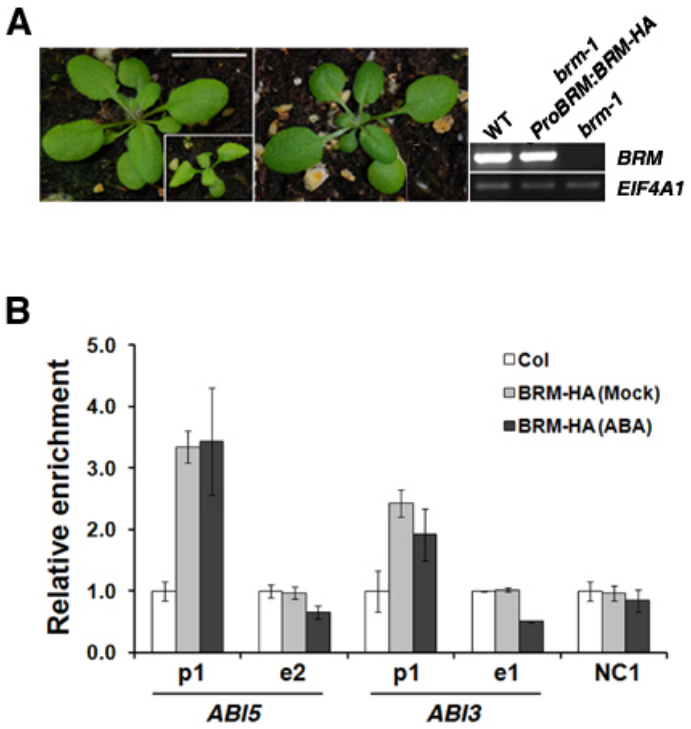


Figure 2-7. Anti HA ChIP in 1.5-day-old *brm-1* ProBRM:BRM-HA plants.

(A) Left: 3-week-old wild type, *brm-1* (inset) and *brm-1* ProBRM:BRM-HA. Right: *BRM* expression (top panel) and *EIF4A1* expression (bottom panel) tested by semiquantitative PCR. Bars = 1cm.

(B) qPCR after Anti-HA ChIP in 1.5-day-old *brm-1* ProBRM:BRM-HA germinated embryos after mock or ABA (50 μM) treatment for 1 hr. Non-transformed wild type (Col) was used as ChIP control.

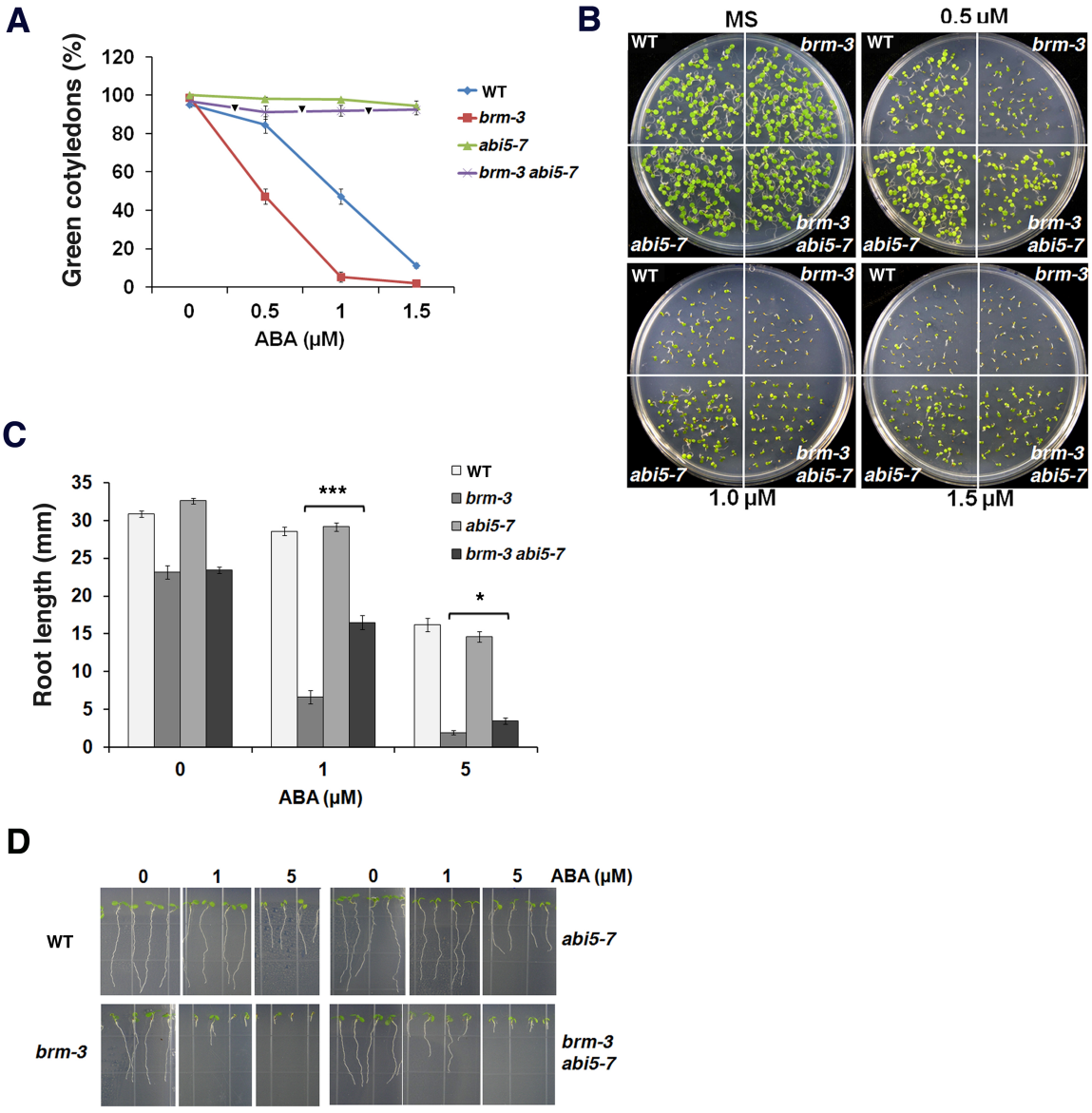

A
Green cotyledons (%)
120
100
80
60
40
20
0
0
0.5
1
1.5
ABA (μM)
WT
brm-3
abi5-7
brm-3 abi5-7
B
MS
0.5 μM
1.0 μM
1.5 μM
WT
brm-3
abi5-7
brm-3 abi5-7
C
Root length (mm)
35
30
25
20
15
10
5
0
***
*
0
1
5
ABA (μM)
WT
brm-3
abi5-7
brm-3 abi5-7
D
0
1
5
0
1
5
ABA (μM)
WT
abi5-7
brm-3
brm-3
abi5-7

Figure 2-8. The hypersensitive *brm* phenotype is due to derepression of *ABI5.*

(A) Percentage of the germinated embryos that developed green cotyledons in the presence of 0.5, 1.0, and 1.5 μM ABA in the *brm-3 abi5-7* double mutants compared with *abi5-7*, *brm-3*, and the wild type (WT) 7 d after stratification. Values are mean ± SEM from three independent experiments. Inverted triangles: no statistical significance compared with wild-type values ($n > 100$, $P > 0.01$).

(B) Representative pictures for the data shown in (A).

(C) Root length in the absence or presence of ABA (1 and 5 μM) in *brm-3*, *abi5-7,* and *brm-3 abi5-7* double mutant plants compared with the wild type.

Two-day-old plants were transferred to MS media containing ABA, and roots were measured at day 7. Asterisks: statistical significance based on one tailed Student's t test ($n > 36$, *$P < 0.01$, ***$P < 1E-10$).

(D) Representative pictures for the data shown in (C).

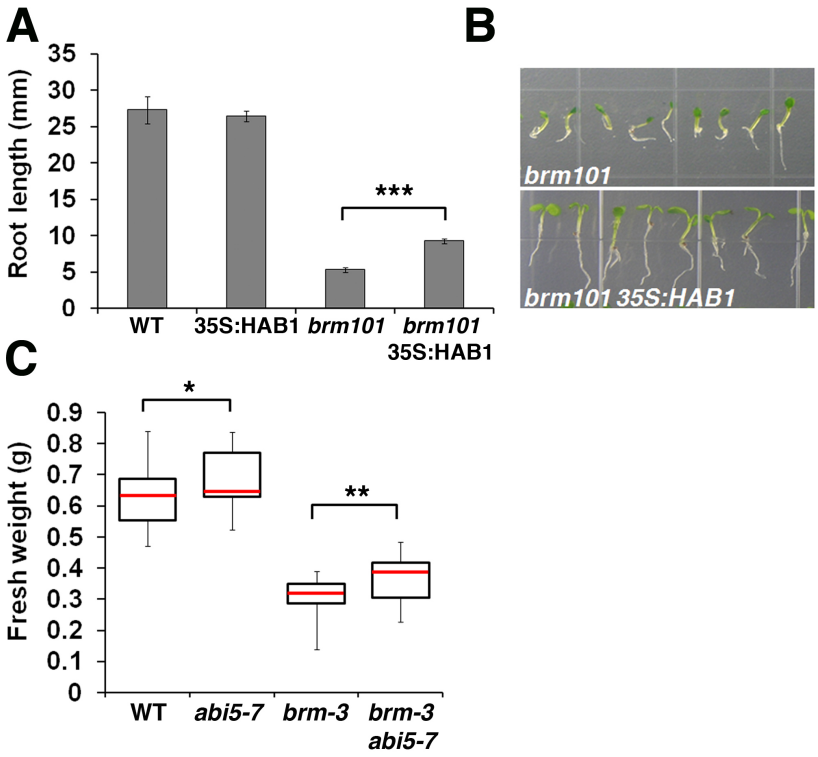


Figure 2-9. The growth defects of the *brm* mutant are partially due to *ABI5* derepression and enhanced ABA response.

(A) Root growth of the *brm* mutant in an ABA-insensitive mutant background (35S:HAB1). The root length of the wild type (WT), 35S:HAB1, *brm-101*, and *brm-101* 35S:HAB1 double mutant was measured 7 d after stratification. Values are mean ± SEM. Sample size was as follows: the wild type (n = 28), 35S:HAB1 (n = 27), *brm-101* (n = 49), and *brm-101* 35S:HAB1 (n = 75). Asterisks indicate statistical significance based on one-tailed Student's t test (P <1E-10).

(B) Representative pictures of data shown in (A).

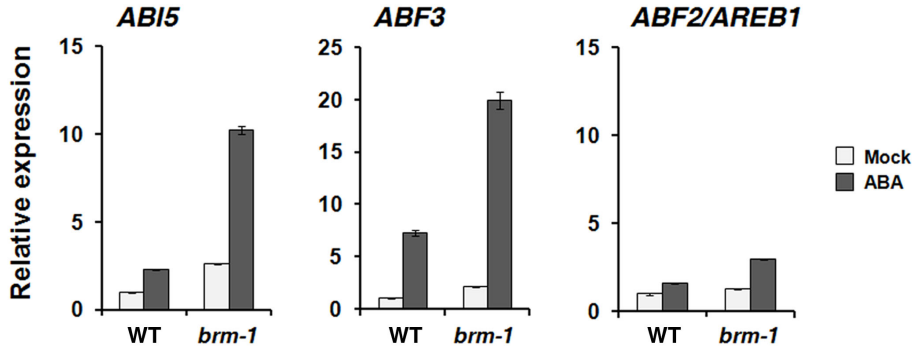
(C) Fresh weight of 4-week-old wild type, *abi5-7*, *brm-3*, and *brm-3 abi5-7* double mutants grown in soil with sufficient water. n > 22 from three independent experiments. Asterisks indicate statistical significance (*P < 0.01 and **P < 0.001).

A

ABI3 expression	Mock	ABA
WT (d15)	ND*	ND
brm-1 (d15)	ND	ND
WT (d21)	ND	ND
brm-1 (d21)	ND	ND

*ND: not detectable (expression level was not detected or low (10e-6 to 10e-8)).

B



C

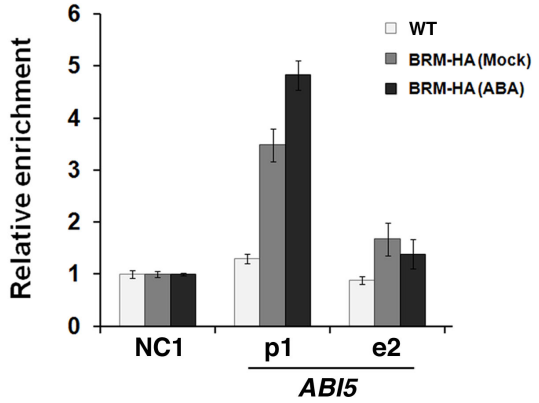


Figure 2-10. BRM directly represses *ABI5* expression during vegetative development.

(A) *ABI3* expression was not detectable by qRT-PCR in 15-day-old or 21-day-old wild type (WT) or *brm-1* mutants in the absence and presence of ABA.

(B) qRT-PCR expression analysis of three ABF/AREB transcription factors in 21-day-old *brm-1* and WT plants grown in soil. Plants were mock or ABA (100 μM) treated for 1 hour. Expression was normalized over that of *EIF4A1* and expression of untreated WT was set to 1.

(C) qPCR after Anti-HA ChIP in 21-day-old *brm-1* ProBRM:BRM-HA plants grown in soil after mock or ABA (100 μM) treatment for 1 hr. The percent input of the immunoprecipitated DNA was normalized over that of the retrotransposon *TA3* (NC1). ChIP was also performed in WT plants to monitor IP background. Values indicate mean ± SEM of three technical replicates from one representative experiment. For a diagram of the *ABI5* locus see Figure 2-6D.

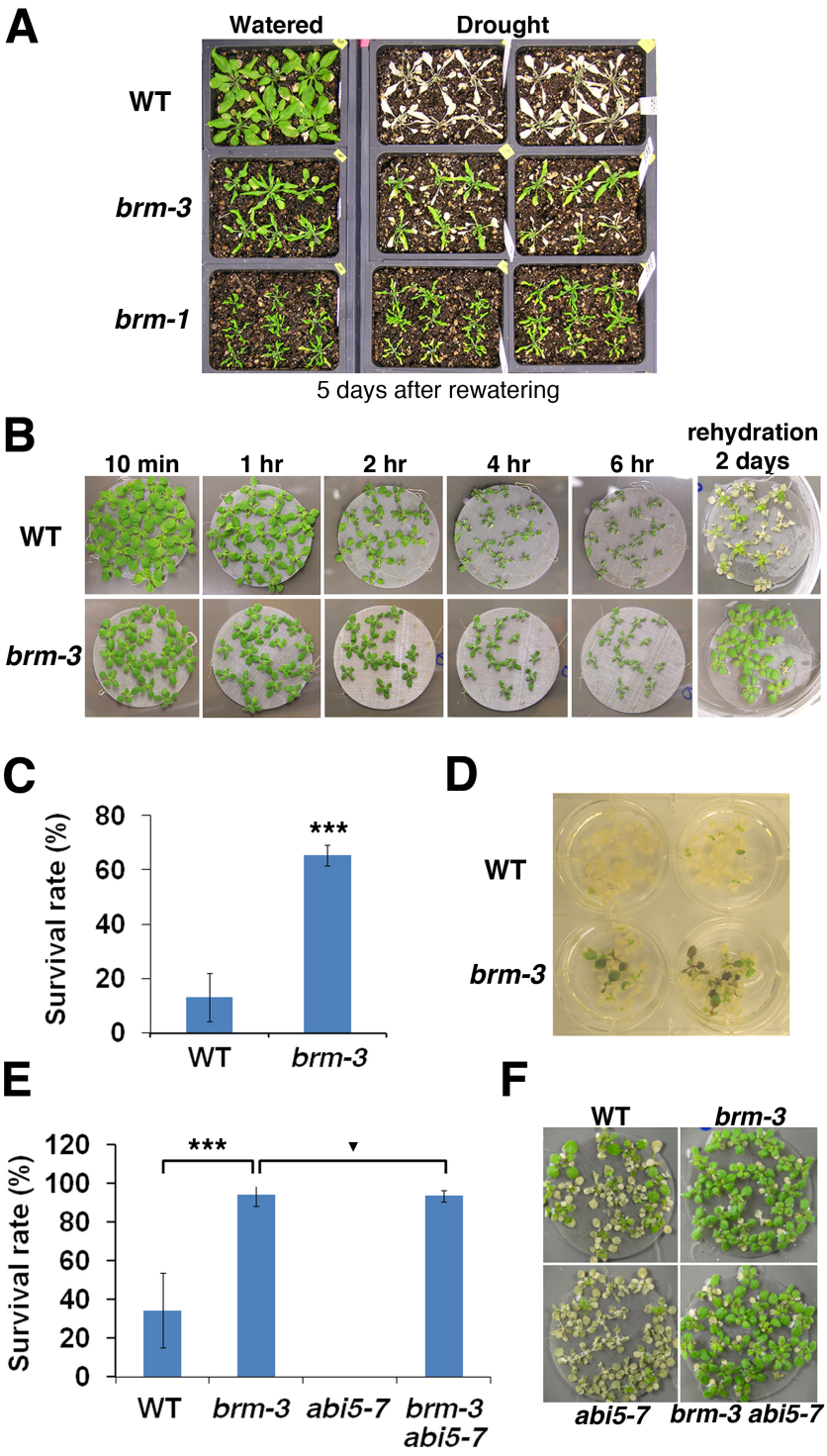

A
Watered
Drought
WT
brm-3
brm-1
5 days after rewatering
B
10 min
1 hr
2 hr
4 hr
6 hr
rehydration
2 days
WT
brm-3
C
Survival rate (%)
80
60
40
20
0
***
WT
brm-3
D
WT
brm-3
E
Survival rate (%)
120
100
80
60
40
20
0
***
▾
WT
brm-3
abi5-7
brm-3
abi5-7
F
WT
brm-3
abi5-7
brm-3 abi5-7

Figure 2-11. *brm* mutants have increased dehydration tolerance.

(A) Wild-type (WT), weak *brm-3*, and null *brm-1* mutant plants grown in soil for 3 weeks followed by continued watering (left) or after drought treatment and rewatering (right).

(B) The effect of dehydration on 2-week-old plate-grown plants. The wild type and *brm-3* mutant during and after drought treatment. The pictures farthest to the right were taken 2 d after rehydration.

(C) Survival rate (%) of 2-week-old wild-type and *brm-3* seedlings after dehydration for 3 h under air flow. Values are mean ± SEM from four experiments (n = 42). Asterisks indicate statistical significance compared with wild-type values (P < 1E-10).

(D) Representative pictures for data shown in (C).

(E) Survival rate (%) of 2-week-old wild type, *brm-3*, *abi5-7*, and *brm-3 abi5-7* double mutants after dehydration for 6 h. Values are mean ± SEM from two independent experiments (n > 53). Asterisks indicate statistical significance (***P < 1E-10). Inverted triangle indicates no statistical significance (P > 0.01).

(F) Representative pictures for data shown in (E).

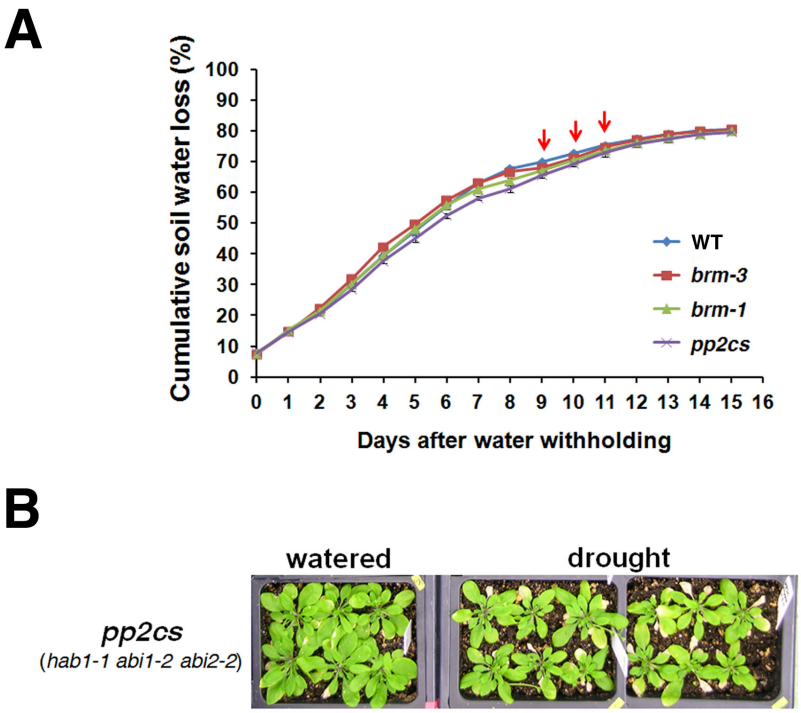


Figure 2-12. Measurement of soil water loss during drought treatment and drought resistance of positive control plants (*pp2c* triple mutants)

(A) Cumulative soil water loss during drought treatment of the plants shown in Figure 2-11. *pp2cs,* triple mutants, *hab1-1 abi1-2 abi2-2*, were included as a positive control for the drought treatment. Water was added three times to adjust water content in soil between the genotypes (red arrowheads). Values indicate mean ± SEM from two experiments. (B) Drought stress tolerance of *pp2cs* triple mutants. *pp2cs* triple mutants grown in soil without (left) or with (right) drought treatment. For drought treatment the picture was taken 5 days after rewatering.

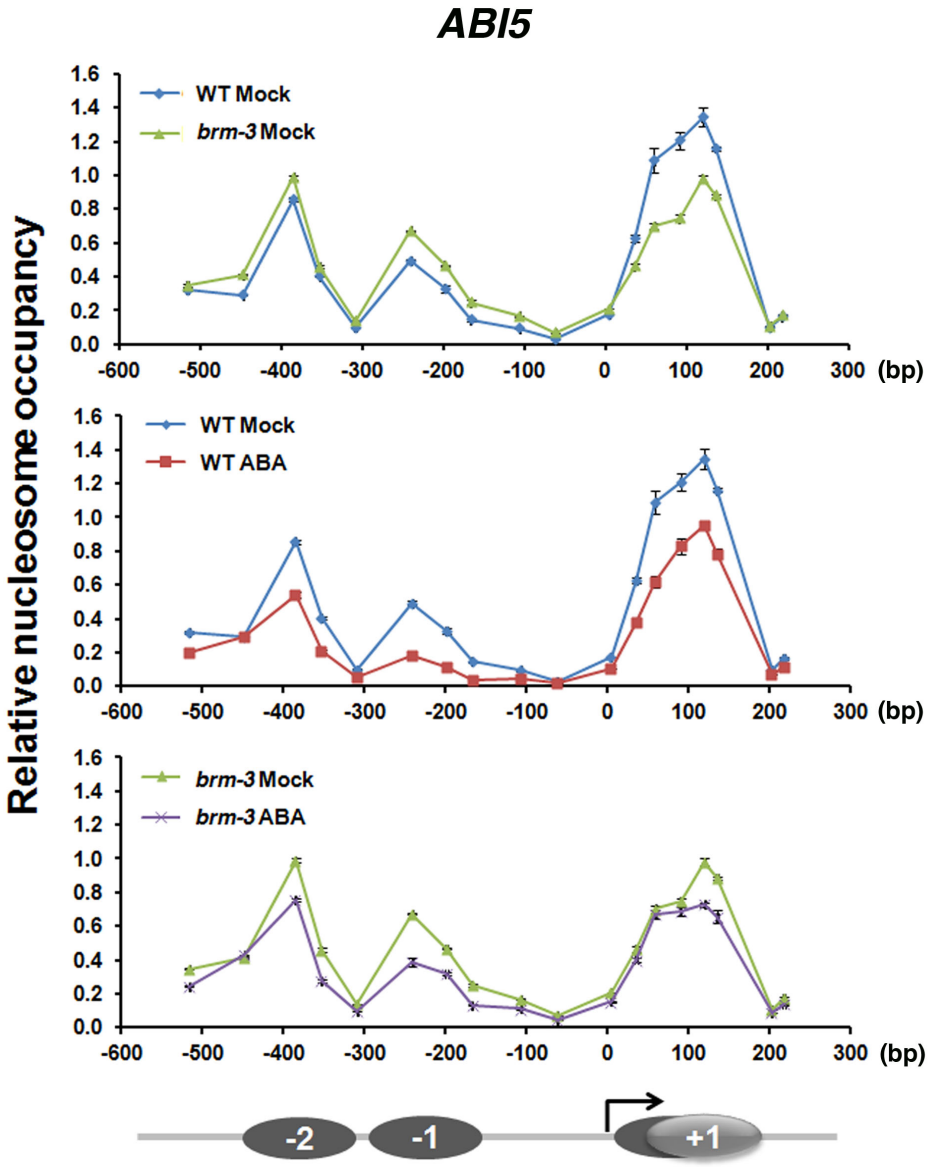

ABI5
Relative nucleosome occupancy
WT Mock
brm-3 Mock
WT Mock
WT ABA
brm-3 Mock
brm-3 ABA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-600
-500
-400
-300
-200
-100
0
100
200
300
(bp)
-2
-1
+1

Figure 2-13. BRM is required to maintain high occupancy of the +1 nucleosome at the *ABI5* locus.

MNase digestion followed by tiled primer qPCR to monitor nucleosome positioning and occupancy at the *ABI5* locus. MNase qPCR was performed after a 1-h mock or ABA treatment in 2-d-old wild-type (WT) and *brm-3* mutants. The fraction of undigested genomic DNA amplified for each amplicon was normalized to that of the 273 position of the negative control locus (gypsy-like retrotransposon; see Figure 2-14). Values are mean ± SEM of three technical replicates from one representative experiment. The number on the x-axis denotes distance (bp) from the TSS (0 bp). Below: Diagram of the positioned nucleosomes. Gray ovals, nucleosomes; black arrow, TSS; gray lines, genomic DNA; green arrowheads, ABREs.

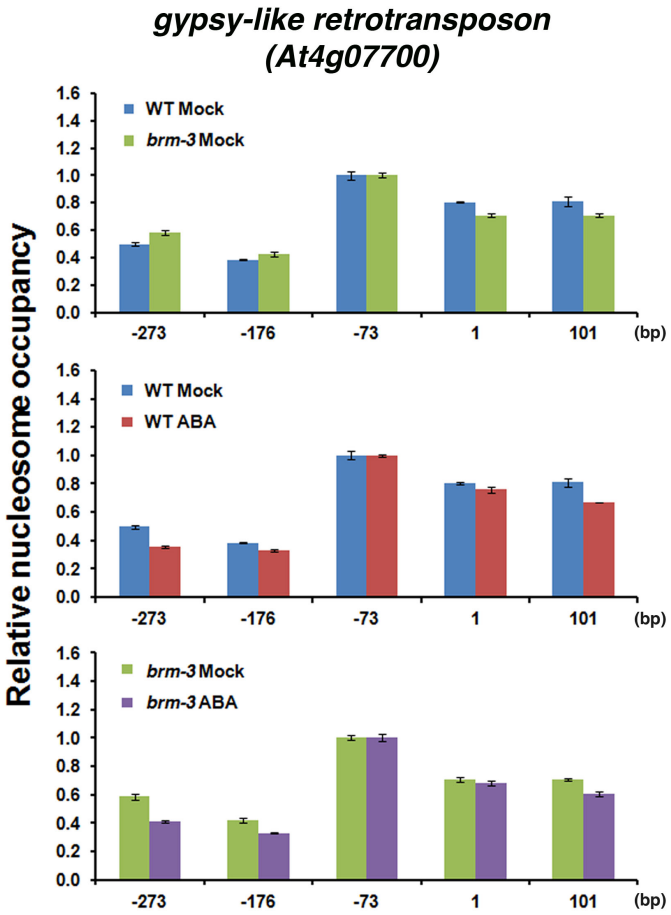


Figure 2-14. No change in nucleosome occupancy at the *gypsy-like retrotransposon* locus.

Nucleosome occupancy at a control locus (*gypsy-like retrotransposon, At4g07700*) using the same MNAse digested DNA as in Figure 2-13 and Figure 2.15

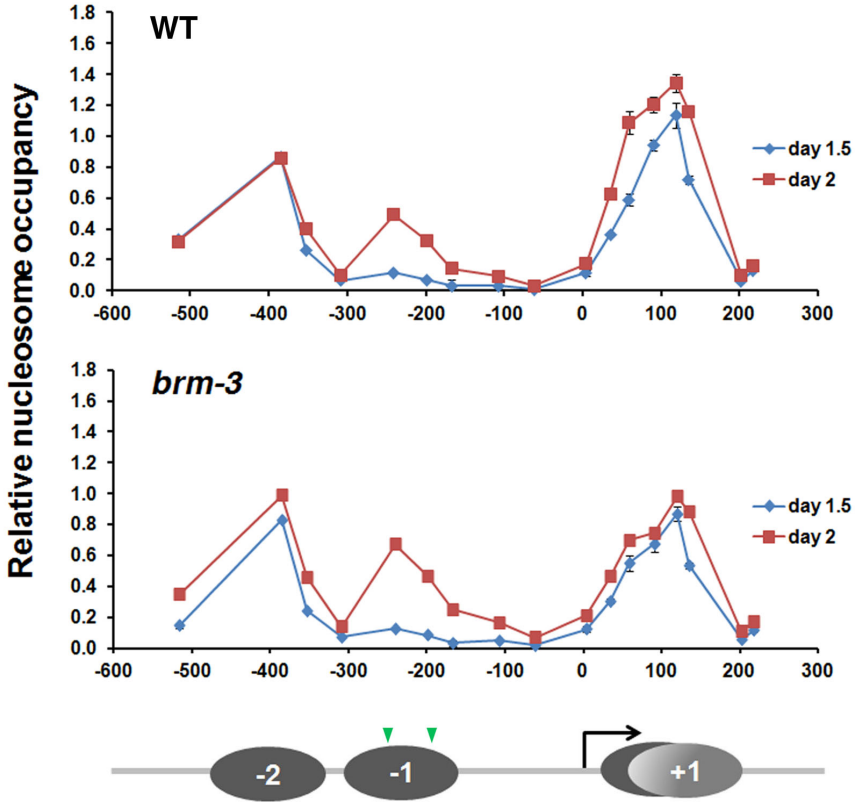
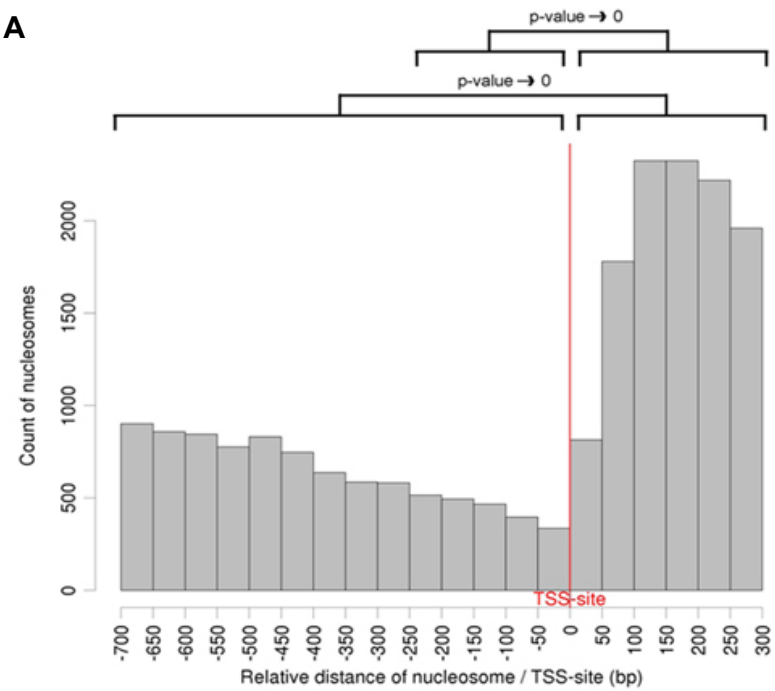


Figure 2-15. Developmental change in nucleosome occupancy at the *ABI5* locus
Relative nucleosome occupancy monitored in 1.5 day-old (blue) and 2 day-old (red) untreated wild-type (WT) and *brm-3* plants. The number on the x-axis denotes the distance (bp) from the transcription start site (TSS; 0 bp). Below: diagram of the nucleosome positions. Grey ovals: nucleosomes. Black arrow: transcription start site (TSS), Grey lines: genomic DNA.

A



B

GO-term	p-value
response to abscisic acid stimulus	3.8 x 10E-4
multicellular organismal development	3.8 x 10E-4
multicellular organismal process	5.7 x 10E-4
seed development	5.8 x 10E-4
response to hormone stimulus	7.6 x 10E-4
fruit development	8.0 x 10E-4
post-embryonic development	8.9 x 10E-4

Figure 2-16. Promoters are depleted of nucleosomes when compared to genic regions.

(A) The number of well-positioned nucleosomes was determined for all protein-coding genes and their position relative to the transcription start site (TSS; red vertical line) was plotted. The promoter region (-700 to 0bp) had reduced nucleosome occupancy relative to the genic region (0bp to +300 bp). Moreover, the density of well-positioned nucleosomes was significantly lower (p->0 for both comparison, χ^2-test) for promoter regions (-700 to 0 bp or -250 to 0 bp) compared to genic regions (0 to 300 bp).

(B) Gene Ontology term enrichment using AgriGo (Du *et al*., 2010) for genes with a well-positioned nucleosome near the transcription start site (TSS) based on our analysis of a published dataset (Chodavarapu *et al*., 2010). See text for details.

CHAPTER 3. Regulation of SWI/SNF complex activity by ABA sensing

3.1 Background

In my previous study, I showed that BRM binds to the *ABI5* promoter as strongly after ABA sensing as it does in the absence of ABA. However BRM only plays a role in *ABI5* transcription in the absence of ABA. It was therefore surprising that BRM occupancy at the *ABI5* locus were not reduced in response to ABA. These data suggest that BRM complex activity may be altered upon ABA sensing to allow up-regulation of *ABI5* by either altering the composition of the BRM complex or by post-translational modification of BRM in the presence of ABA. Saez *et al*., 2008 showed that one of the subunits of the SWI/SNF complex, SWI3B interact with the HAB1 phosphatase of the core ABA signal transduction pathway. Therefore I investigated a possible link between BRM and the ABA signal transduction pathway to test whether complex activity is regulated upon ABA sensing. I hypothesized that SnRK2 kinase can phosphorylate BRM upon ABA sensing and relieve BRM mediated repression of ABA responsive gene expression (Figure 3-1).

3.2 Results

3.2.1 BRM partially acts on ABA signal transduction pathway.

Overexpression of a negative regulator of ABA responses, the PP2C phosphatase HAB1 leads to ABA insensitivity (Saez *et al.*, 2004). When exogenous ABA is applied, the ABA insensitivity of 35S:HAB1 was partially suppressed in the *brm* mutant background suggesting that ABA signaling acts in part via BRM. This raised the possibility that ABA

signal transduction components may play a role in inactivation of BRM upon ABA sensing. To test this hypothesis I next examined the physical interaction between BRM and SnRK2 kinases as well as PP2C phosphatases.

3.2.2 BRM physically interacts with the components of ABA signal transduction pathway *in vitro* and *in vivo*.

Upon ABA sensing, ABA receptor PYR-PYL/RCAR family forms complex with type2 serine/threonine protein phosphatases (PP2Cs), which frees SnRK2 kinase activity to phosphorylate downstream targets (Figure 1-1). In addition to the well-known targets of phosphorylation by SnRK2 kinases, such as transcriptions factors that bind ABRE (ABA response elements) and ion channels involved in stomata closure, other SnRK2 substrates may be present in nucleus (Umezawa *et al*., 2010). To test for physical interaction between BRM and the components of ABA signal transduction pathway, I performed yeast two-hybrid assay (Figure 3-3A), Bimolecular Fluorescence Complementation (BiFC) (Figure 3-3B) and co-immunoprecipitation assays using *Arabidopsis* protoplasts (Figure 3-3C). In all assays tested, BRM interacted with both SnRK2 kinases (OST1) and PP2C phosphatases (HAB1). These data suggest that BRM may be a possible target of phosphorylation and de-phosphorylation mediated by SnRK2 kinases and PP2C phosphatases, respectively.

3.2.3 The physical interaction between BRM and HAB1 may be ABA signal dependent.

I found that the strong physical interaction between the possible BRM complex component, SWI3B, and HAB1 is abrogated in the presence of the ABA receptor and ABA (Figure 3-4), suggesting that the physical interaction between PP2Cs and SWI3B is regulated by ABA sensing. I hypothesized that BRM phosphorylation is also ABA signaling dependent. I will test if the interaction between BRM and HAB1 is also abrogated by ABA signal using the yeast three-hybrid system as well as *Arabidopsis* protoplasts (Figure 4-4).

3.2.4 BRM C-terminal region is phosphorylated in an ABA dependent manner.

The lab of our collaborator, Dr. Pedro Rodriguez (CSIC-UPV, Spain), found that the C-terminal domain of BRM is phosphorylated by a SnRK2 kinase in an ABA dependent manner (data not shown). They further identified serine 1760 as putative phosphorylation site (data not shown). In accordance with their data, published proteomic data (Wang *et al*., 2013) revealed that phosphorylation on serine 1760 and 1762 on BRM C-terminal fragment is induced by ABA treatment, in a manner dependent on three SnRK2 kinases, SnRK2.2, SnRK2.3 and SnRK2.6 (OST1) (Figure 3-5). These serine residues are well conserved in BRM orthologs in different plant species including monocots and eudicots (Figure 3-5). Interestingly, when I express small BRM C-terminal fragments in protoplasts, I observed high mobility shift in the PAGE compared to the wild type when BRM C-terminal fragments containing serine (S1760 and S1762) is subjected to altered ABA levels. Although this needs to be further confirmed, the data suggest that these residues may be phosphorylated upon ABA sensing.

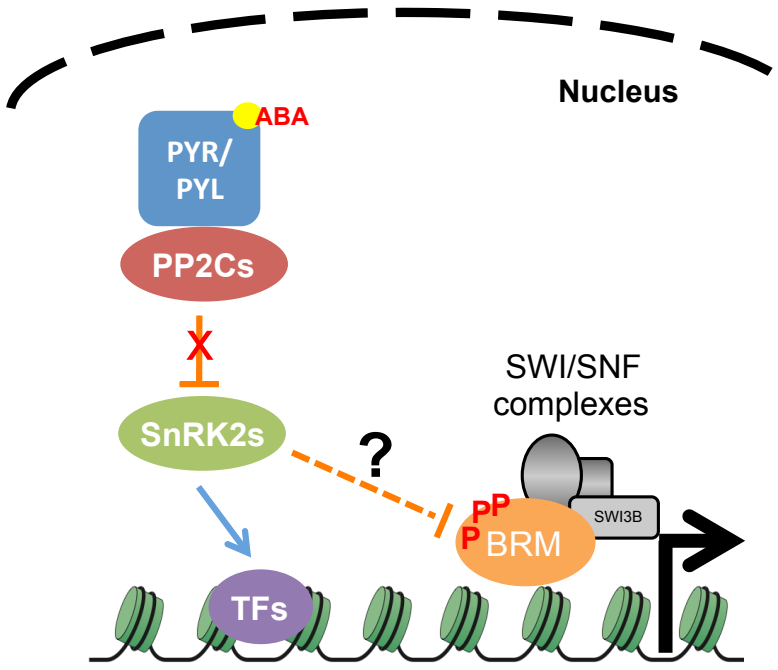


Figure 3-1. A hypothetical model for the regulation of BRM activity by the core ABA signaling pathway

Upon ABA sensing allows SnRK2 kinases to inactivate the BRM complex by phosphorylation, which relieves transcriptional repression of target genes by the BRM complex.

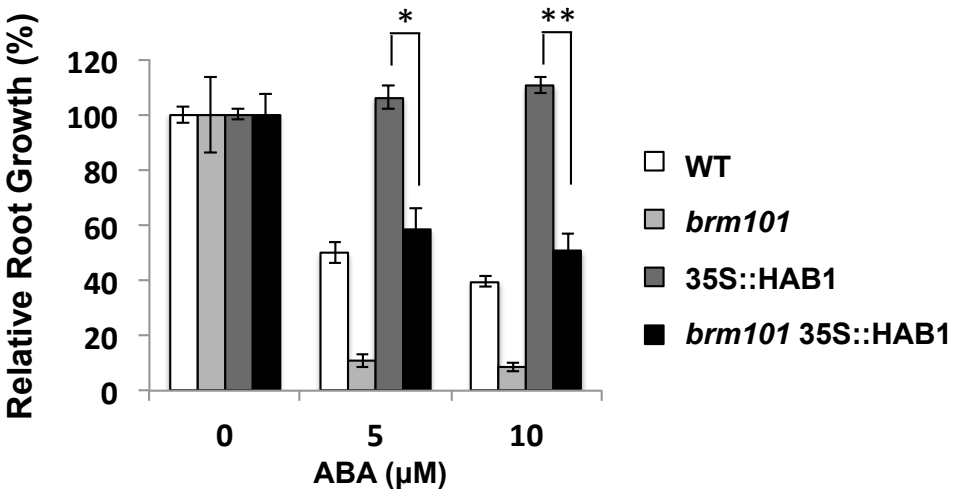
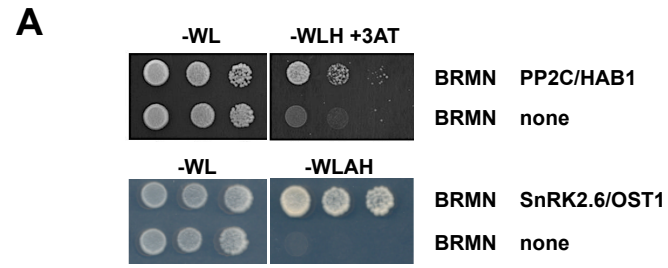
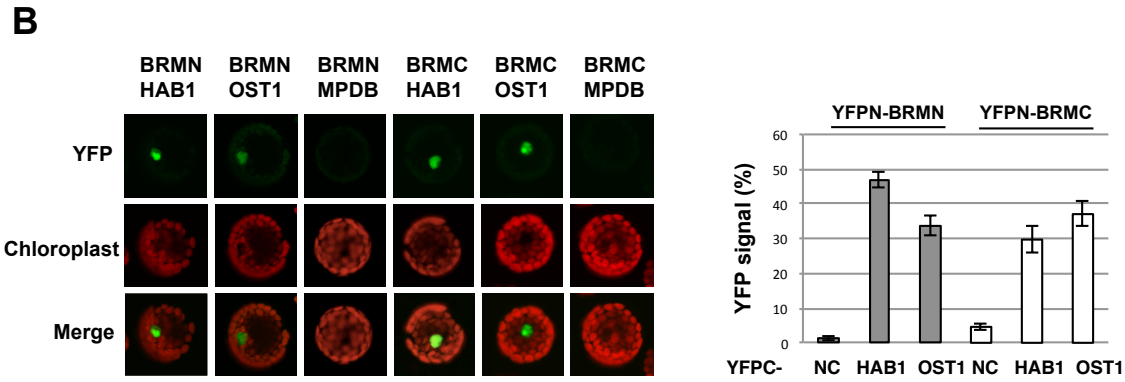


Figure 3-2. Genetic interaction between BRM and HAB1 PP2C phosphatase

Root growth of the *brm* mutant in an ABA-insensitive mutant background (35S:HAB1) in the presence and absence of ABA. The root length of the wild type (WT), 35S:HAB1, *brm-101*, and *brm-101* 35S:HAB1 double mutant was measured 10 d after stratification. 5 day-old seedlings were transferred to MS media with or without ABA and were grown 5 more days. Values are mean ± SEM from at least 15 plants. Asterisks indicate statistical significance between 35S:HAB1 and *brm-101* 35S:HAB1 double mutant based on one-tailed Student's t test ($n > 15$, * $P < 6.4E-07$, ** $P < 1.2E-E10$).


A
-WL
-WLH +3AT
BRMN PP2C/HAB1
BRMN none
-WL
-WLAH
BRMN SnRK2.6/OST1
BRMN none


B
BRMN HAB1
BRMN OST1
BRMN MPDB
BRMC HAB1
BRMC OST1
BRMC MPDB
YFP
Chloroplast
Merge
YFPN-BRMN
YFPN-BRMC
YFP signal (%)
60
50
40
30
20
10
0
YFPC-
NC
HAB1
OST1
NC
HAB1
OST1

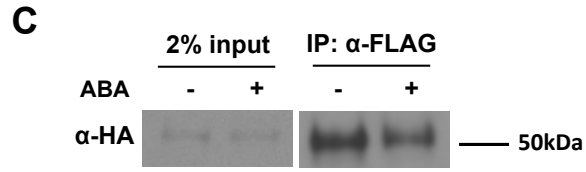

C
2% input
IP: α-FLAG
ABA
-
+
-
+
α-HA
50kDa

Figure 3-3. *in vitro* and *in vivo* interactions between BRM and the core components of ABA signal transduction pathway, SnRK2 kinases and PP2C phosphatases. BRM interacts with OST1 (SnRK2) and HAB1 (PP2C) in Y2H assays (A) or on the basis of bimolecular fluorescence complementation (BiFC) in protoplasts (B). (B) BRM N-terminus and C-terminus were used. MPDB was used as negative control for BRM interaction. Left: a representative fluorescent protoplast. Right: Percentage of YFP protoplasts. Transfection was done at the same time with equal amount of DNA. Values are mean ± SEM from 3 biological replicates. (C) Protoplast co-immunoprecipitation test using of FLAG-BRMN and HA-OST1. BRMN protein was precipitated by anti-FLAG and HA-OST1 was detected by immune blotting.

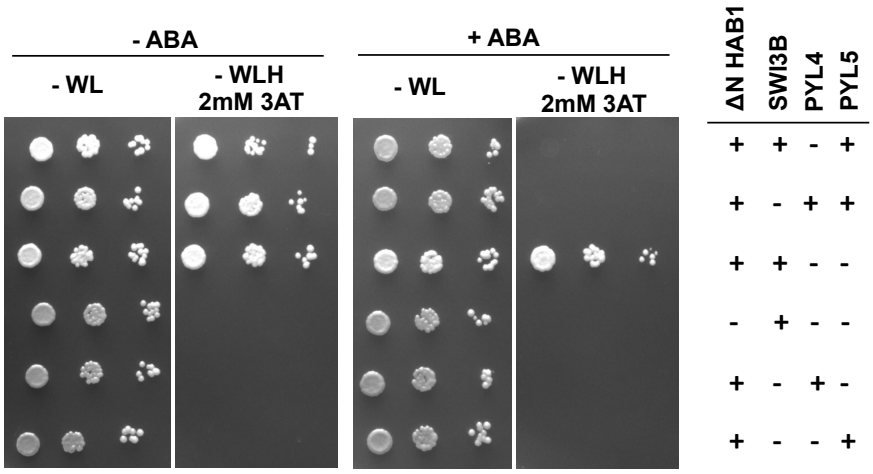


Figure 3-4. HAB1 binding to SWI3B is disrupted after ABA sensing.

Yeast-three hybrid assay reveals co-expression of ABA receptors PYLs abrogates the interaction of HAB1 with SWI3B in the presence of ABA. ΔN HAB1 was used to remove autoactivation of HAB1 in binding domain. ΔN HAB1 (BD) and ABA receptors, PYL4 and PYL5 (MCSII, Multiple Cloning Site II) were cloned into same vector, pBridge. 10 μM ABA was supplemented in the media.

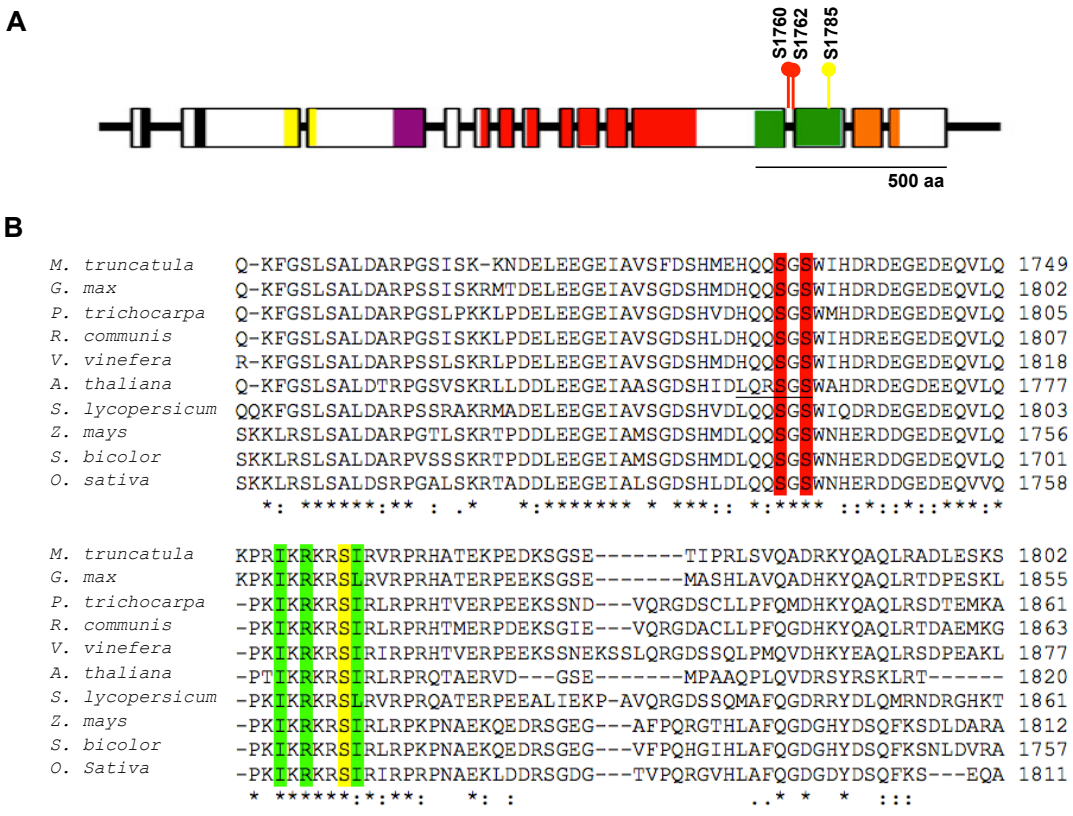


Figure 3-5. BRM C-terminal regions from different plant species contain putative BRM phosphorylation sites.

(A) Diagram of BRM coding region (2193 amino acids). Domains are marked in yellow, QLQ; purple, HSA; red, ATPase; green, AT-hook; orange, bromodomain. Diagram was modified from (Efroni *et al.*, 2013). Putative phosphorylation sites are marked in red (S1760, S1762) and yellow (S1865).

(B) The serine residues (S1760, S1762) are phosphorylated in an ABA and SnRK2s dependent manner (Wang *et al*., 2013). Consensus motif of SnRK2s target is shown in green and putative phosphorylation residue serine (S1865) is marked in yellow (Sirichandra *et al.*, 2010).

CHAPTER 4. Discussion and Future direction

4.1 Discussion

4.1.1 A role for BRM in repressing water stress responses during post-germination development in the absence of the stimulus

Newly germinated plant embryos are particularly vulnerable to drought stress, which triggers a growth arrest similar to that operating during seed development to induce desiccation tolerance (Lopez-Molina *et al*., 2001). Here, I implicate the SWI/SNF ATPase BRM in ensuring that the growth arrest pathway is triggered in germinated embryos only upon drought sensing/ increased endogenous ABA levels. *brm* mutants were hypersensitive to ABA, especially with respect to cotyledon greening and selectively derepressed expression of a subset of ABA response genes in the absence of the stimulus, among them *ABI3* and *ABI5*. Moreover, BRM bound to the regulatory regions of both genes. ABI3 acts upstream of *ABI5* during postgermination development (Lopez-Molina *et al*., 2002). In agreement with these combined observations, *ABI5* was epistatic to BRM with respect to inhibition of cotyledon greening, indicating that derepression of *ABI5* expression is the likely cause of the *brm* mutant's ABA hypersensitivity during this stage of development. Thus, BRM, ABI3, and ABI5 interact in a simple genetic pathway, which corresponds to a type 2 coherent feed-forward loop (Alon, 2007; Mangan and Alon, 2003), to regulate cotyledon greening (Figure 4-1). The type 2 coherent feed-forward loop displays an "off" delay (Alon, 2007; Mangan and Alon, 2003); upon ABA sensing, the upregulation of *ABI3* and *ABI5* would be delayed. This would ensure that growth arrest occurs only after a prolonged water stress or ABA

signal has been perceived. By contrast, the "on" switch in this type of feed-forward loop is rapid (Alon, 2007; Mangan and Alon, 2003). Thus, when ABA/ water stress levels fall below a certain threshold, BRM would rapidly repress *ABI3* and *ABI5* expression. The BRM/ABI3/ABI5 module is well suited to manage resource allocation to growth versus the stress responses. Not surprisingly, since *abi5* mutants show ABA-responsive root growth (Finkelstein *et al*., 2005; Miura *et al*., 2009), the ABA triggered inhibition of root elongation in *brm* mutants was only partially due to *ABI5* derepression. Thus, it is likely that BRM represses the expression of other transcription factors that act in parallel with ABI5 in root growth inhibition (Figure 4-1). Several additional transcription factors have been shown to have a role in the inhibition of root elongation in response to ABA, including WRKY transcription factors (Chen *et al.*, 2010a), Auxin Responsive Factor 2 (Wang *et al.*, 2011a), MYB transcription factors (Zheng *et al.*, 2012), and other bZIP transcription factors, such as ABF3 (Yoshida *et al.*, 2010), with the latter two reported to act as least in part in parallel with ABI5.

4.1.2 Regulation of *ABI5* expression by BRM-dependent and BRM-independent alteration of nucleosome positioning and occupancy

brm mutants cause derepression of *ABI5* in the absence of the ABA as well as an ;40% reduction of the +1 nucleosome occupancy, with a preferential loss from the TSS proximal position. The +1 nucleosome is a frequent target of chromatin remodeling (Yen *et al.*, 2012). +1 nucleosomes positioned closer to the TSS are repressive and can interfere with the assembly or activity of the transcription initiation complex (Yen *et al.*, 2012). In addition, transcriptional activation of gene expression is associated with

positioning of the +1 nucleosome away from the TSS. Thus, I hypothesize that BRM represses *ABI5* transcription in the absence of water stress/ABA by promoting high occupancy of the +1 nucleosome and by directing this nucleosome from a more favorable predicted position to a position more proximal to the TSS. There is precedent for this model. Recently, derepression of HIV expression was observed upon loss of the human BAF SWI/SNF subfamily complex activity, which resulted in a reduction in the occupancy of the +1 nucleosome (Rafati *et al.*, 2011). Consistent with the idea that BRM causes increased occupancy and more TSS proximal positioning of the +1 nucleosome at the *ABI5* locus, BRM very strongly associated with the region of *ABI5* locus occupied by the +1 nucleosome.

I also observed stress hormone- and development dependent alterations of the nucleosome occupancy at this locus that may explain observed gene expression changes. *ABI5* expression is induced upon drought or ABA sensing (Lopez-Molina *et al*., 2001). Perception of the ABA stress hormone led to a destabilization (reduced occupancy) of all three nucleosomes at this locus. The most pronounced reduction in nucleosome occupancy was observed at the -1 position. This nucleosome is positioned over 2 *cis*-regulatory elements linked to ABA-responsive gene expression (Yamaguchi-Shinozaki and Shinozaki, 2005) and may hence modulate transcription factor access to their binding sites. The nucleosome destabilization by ABA was not BRM dependent; it was observed both in the *brm-3* mutants and in the wild type. Consistent with this, the fold increase in *ABI5* mRNA levels upon ABA treatment was similar in the wild type and in the *brm* mutant. It is possible that another chromatin regulator causes reduced nucleosome occupancy at the *ABI5* locus upon stress sensing; alternatively, the nucleosome

destabilization could be caused by increased transcriptional activity (Radman-Livaja and Rando, 2010).

Seed maturation is characterized by chromatin condensation, which is reversed during imbibition and germination (van Zanten *et al.*, 2011). Consistent with an open chromatin configuration in young germinated embryos, the -1 *ABI5* nucleosome was essentially absent at this stage. At the end of the postgermination phase, *ABI5* expression is developmentally repressed (Brocard *et al.*, 2002; Lopez-Molina *et al.*, 2001). In agreement with this, the occupancy of the -1 nucleosome strongly increased at this stage. It is likely that the observed chromatin changes underlie the developmental repression of *ABI5* expression at the end of postgermination development. Indeed, the *ABI5* locus continues to display high occupancy of the -1 nucleosome during later stages of vegetative development (Chodavarapu *et al*., 2010). The developmentally induced chromatin condensation at the *ABI5* locus was observed in both the wild type and in *brm* mutants. In agreement with this finding, *brm* mutants displayed normal developmental downregulation of *ABI5* expression (Figure 4-2). The chromatin condensation at the *ABI5* locus during seedling establishment may hence be triggered by other chromatin regulators. Mutations in several different chromatin regulators, histone deacetylases, Polycomb-repressive complexes, a putative histone methyltransferase, retinoblastoma proteins, and the CHD domain chromatin remodeling ATPase PICKLE (PKL), delay the switch from the embryo to the seedling stage (Aichinger *et al.*, 2009; Bouyer *et al.*, 2011; Gutzat *et al.*, 2011; Kim *et al.*, 2012b; Ogas *et al.*, 1999; Tanaka *et al.*, 2008; Tang *et al.*, 2008; Zhang *et al.*, 2012). Activity of these chromatin regulators likely contributes to the developmental downregulation of *ABI5* and *ABI3* (Figure 4-1B). Although BRM is not

required for developmental repression of *ABI5*, it does play a role in repression of a subset of seed storage proteins (Tang *et al*., 2008).

4.1.3 Drought Tolerance and Fitness Tradeoffs

I conclude that during postgermination development BRM ensures that costly stress responses are mounted only upon perception of water stress signals to enhance fitness of the organism. The role for BRM in restricting stress response gene expression would on one hand predict that *brm* mutants should display defects in growth that are due to constitutive activity of the water stress response pathway; on the other hand, *brm* mutants would be expected to display increased drought tolerance (Boyer, 1982; Grill and Ziegler, 1998). Both expectations were confirmed in our study, supporting the conclusion that BRM prevents stress responses in the absence of the stimulus. BRM is thus positioned at the nexus of the resource allocation decision between growth and drought tolerance.

In the coming years, we will likely encounter a global deficit in food supply due to increased drought (Battisti, 2009; Cominelli and Tonelli, 2010). To address this challenge, it is important to develop new crops that have improved water use efficiency. Efforts to engineer drought-resistant plants showed that a single gene change is often not sufficient to produce robust drought tolerance, especially in field conditions where water stress interacts with other stressors, such as heat and high light intensity (Mittler and Blumwald, 2010; Yang *et al.*, 2010). It was proposed that manipulating expression of a master transcriptional factor that targets multiple stress response genes would be a more promising approach (Cominelli and Tonelli, 2010). An even more global change in the plant's drought tolerance could be achieved via altered chromatin remodeling, as this

mechanism can modulate gene expression in many different pathways or of several master regulators simultaneously (Kwon and Wagner, 2007). Our studies show increased drought resistance of *brm* mutants at multiple developmental stages. While the molecular mechanism for this enhanced drought tolerance remains to be elucidated, a key challenge for the future is to generate conditional *brm* loss-of-function alleles that robustly enhance water stress survival without detrimental effects on growth or yield.

4.1.4 Possible mechanisms of regulation of BRM Activity

In response to ABA treatment, I observed a similar up-regulation of *ABI5* mRNA abundance relative to mock-treated plants and a similar *ABI5* promoter nucleosome destabilization in both wild-type and *brm* mutant germinated embryos. Thus, ABA induction of *ABI5* expression is apparently BRM independent. Since BRM was still bound to the *ABI5* locus upon ABA sensing, I hypothesize that BRM may be inactivated in the presence of ABA. A possible mechanism for BRM inactivation is alteration of BRM complex composition. Alternatively, BRM complex activity may be repressed via posttranslational modification(s). Both altered complex composition and posttranslational modifications can inactivate metazoan SWI/SNF subgroup complexes (Clapier and Cairns, 2009). Continued BRM presence at the *ABI5* locus may ensure that costly stress responses are mounted only upon perception of water stress signals to enhance fitness of the organism the growth arrest response is rapidly turned off once the desiccation stress/ABA signal has subsided. Given this model, why do *brm* mutants accumulate higher absolute levels of *ABI5* transcript upon ABA treatment than the wild type? The higher absolute *ABI5* accumulation in *brm* mutants upon ABA treatment is likely due to

the accumulation of transcripts for ABA-dependent transcription factors in *brm* mutant. ABA treatment both activates and stabilizes these transcription factors (Lopez-Molina *et al.*, 2003; Miura *et al.*, 2009; Nakashima *et al.*, 2009); this is expected to lead to a high absolute level of *ABI5* accumulation.

4.1.5 BRM activity is regulated by the core ABA signaling pathway

In our investigation on how BRM activity may be regulated upon ABA sensing, we found that BRM physically interacts with multiple core ABA signaling components, the SnRK2 kinase OST1 and the PP2C phosphatase HAB1, *in vitro* and *in vivo*. In addition BRM is phosphorylated by OST1 in an ABA dependent manner. A putative component of BRM complex SWI3B also interacts with OST1 and HAB1, this interaction is abrogated by ABA sensing *in vitro*. Therefore, activity of the BRM complex is likely regulated by ABA induced phosphorylation. However by which mechanism ABA induced signaling affects BRM nucleosome remodeling activity and which amino acids in BRM are the critical targets is currently unknown.

Phosphorylation is a well-described mechanism of signaling to chromatin-modifying factors (Badeaux and Shi, 2013). Activated kinase cascades can actively transmit signals to chromatin factors to interfere the chromatin landscape in a cue-dependent manner (Badeaux and Shi, 2013). Phorphorylation-mediated regulation of the chromatin remodeler complex components of the human SWI3 and BRG1 by ERK1 is important for chromatin compaction during mitosis. Phosphorylation inactivates the hSWI/SNF complex and subsequent dephosphorylation by hPP2A restores nucleosome-

remodeling activity (Sif *et al.*, 1998). It is not known whether which sites on hBRG1 and hSWI3 are phosphorylated and whether this mechanism is conserved in plants.

It is also possible that BRM phosphorylation changes protein-protein interaction between the complex components, which may affect chromatin complex activity. BAF60c, a SWI/SNF complex component, is associated with the tissue specific transcription factor Myo-D throughout muscle cell differentiation (Forcales *et al.*, 2012). However, BAF60c can recruit BRG1 to the Myo-D target genes only upon phosphorylation by differentiation signal activated p38 kinase (Forcales *et al.*, 2012) suggesting that the formation of SWI/SNF complex is regulated by a posttranslational modification.

4.1.6 Biochemical activity of chromatin remodelers and possible activity of BRM at the +1 nucleosome of *ABI5* Locus

ATP-dependent chromatin remodeling complexes can be broadly divided into four major subfamilies on the basis of their sequence, composition and activities: SWI/SNF, ISWI, CHD and the INO80 (Flaus *et al.*, 2006). All four chromatin remodelers share a SWI2/SNF2-family ATPase domain and utilize the energy derived from ATP hydrolysis to move, destabilize, eject, or restructure nucleosomes (Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011). Although the catalytic subunit is similar, the remodelers have specialized function. Each ATPase bears unique domains and forms a complex with unique subunits in different biological contexts (Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011).

Data from *in vitro* biochemical and yeast experiments shows that SWI/SNF subfamily proteins have many activities including the ability to slide and eject nucleosome, but they lack assembly function (Clapier and Cairns, 2009; Saha *et al.*, 2006). Therefore SWI/SNF chromatin remodelers are more disruptive and generally associated with transcriptional activation by disordering nucleosomes (Clapier and Cairns, 2009; Saha *et al.*, 2006). By contrast, ISWI complexes have central roles in chromatin assembly, through the organization and regular spacing of nucleosomes following DNA replication. ISWI hence primarily promotes transcriptional repression (Clapier and Cairns, 2009; Narlikar *et al.*, 2013; Saha *et al.*, 2006). However many mammalian studies indicate that SWI/SNF complexes can contribute to both repression and activation of gene expression in diverse developmental processes (Hargreaves and Crabtree, 2011). It is also known that certain ISWI containing complexes can assist transcriptional machinery leading to transcriptional activation (Morillon *et al.*, 2003). The CHD chromatin remodelers have characteristic two tandem repeats of chromodomains on the N-terminal region of the ATPase domain (Clapier and Cairns, 2009). Certain CHD complex also can slide or eject nucleosomes to promote transcription (Clapier and Cairns, 2009; Denslow and Wade, 2007), but others act as repressors by cooperating with histone deacetylase (HDAC) and methyl CpG-binding domain (MBD) proteins (Denslow and Wade, 2007). The CHD1 ATPase that belongs to this class also has a similar role to ISWI and has functional redundancy with ISWI for nucleosome spacing in the coding regions of transcribed genes (Gkikopoulos *et al.*, 2011). The INO80 complex has diverse functions including transcriptional activation and repression, DNA repair, DNA replication and chromatin-independent function (Conaway and Conaway, 2009). The

SWR1 ATPase is highly related to INO80, both remodelers can also exchange histones, while SWR1 remove H2A-H2B dimers and replace them with H2A.Z and H2B dimer insertion (Mizuguchi *et al.*, 2004), INO80 catalyzes the opposite reaction (Papamichos-Chronakis *et al.*, 2011).

One of the main finding from my studies is that the Arabidopsis SWI/SNF subgroup ATPase BRM contributes to the placement and occupancy of the transcription start proximal nucleosome at the *ABI5* locus. The +1 nucleosome is just downstream of the TSS. The nuclease protected region, ~200 bp of DNA, is broader than DNA protected by mononuclosome (~150 bp). This suggests that +1 nucleosome may be present in two alternative positions. A nucleosome position prediction program identified nucleosome start sites around 45bp proximal region from the TSS, while MNase mapping showed that start of +1 nucleosome is more close to the TSS, suggesting that a subset of the +1 nucleosome is positioned more TSS proximal than predicted. Mutations in *brm* cause ~40% reduction of the +1 nucleosome occupancy with preferential loss from the TSS proximal position, as well as derepression of *ABI5* in the absence of the ABA. BRM strongly associated with the region of *ABI5* locus occupied by the +1 nucleosome. Therefore I hypothesize that BRM represses *ABI5* transcription in the absence of ABA by promoting high occupancy of the +1 nucleosome and by directing this nucleosome from a more favorable predicted position to a position more proximal to the TSS. How does BRM activity cause increased occupancy and more TSS proximal positioning of the +1 nucleosome at *ABI5* locus? One mechanism is that BRM deposits the nucleosome to maintain high occupancy at the +1 nucleosome position. However it is well known that most SWI/SNF remodelers lack assembly functions, by contrast most ISWI remodelers

conduct nucleosome assembly and spacing in nucleosomal arrays. For example, the human ISWI-containing factor RSF (remodeling and spacing factor) and ACF (ATP-dependent chromatin-assembly factor)/ CHRAC (Chromatin assembly complex) complex were found to mediate nucleosome deposition onto DNA and form regularly spaced nucleosome arrays in the presence of ATP (Fyodorov *et al.*, 2004; Loyola *et al.*, 2003). The CHD subgroup member of chromatin remodelers, CHD1 plays a role in histone deposition during chromatin assembly in *Drosophila* in an ATP-dependent manner (Konev *et al.*, 2007). Although these ISWI and CHD1 remodelers are involved in nucleosome positioning and deposition, genome-wide nucleosome position studies from yeast revealed that deletion of ISW1 and CHD largely did not affect +1 nucleosome position (Gkikopoulos *et al.*, 2011). This raises the possibilities that another chromatin remodeler is needed to direct +1 nucleosome positioning and occupancy. The ISWI2 in yeast is known to affect the positioning of the +1 nucleosome (Whitehouse *et al.*, 2007) and is localized to the +1 nucleosome (Yen *et al.*, 2012), although its activity is more likely in nucleosome sliding. In *Arabidopsis,* two ISWI ATPases have been identified and one of them is necessary for normal cell expansion during late embryogenesis and has an essential role during female gametogenesis (Huanca-Mamani *et al.*, 2005). What types of chromatin changes these roles of ISWI ATPase in plants are due to chromatin changes remains to be determined. *Arabidopsis* SWI/SNF ATPases were identified based on the sequence similarity with metazoan SWI/SNF and interaction with *Arabidopsis* SWI3 proteins (SWI/SNF core complex components)(Flaus *et al.*, 2006; Knizewski *et al.*, 2008; Kwon and Wagner, 2007), however the biochemical activity of SWI/SNF ATPase has not been studied in any plant system. Whether the *Arabidopsis* BRM complex

functions in chromatin assembly as seen in ISWI complexes in other species needs to be confirmed through the testing of biochemical activity, or alternatively, through an analysis of genome-wide nucleosome positioning and occupancy in *brm* mutant. Intriguingly, it has been shown that the SWI/SNF complex is involved in rapid nucleosome assembly at the *PHO5* promoter during transcriptional repression in *S. cerevesiae* (Schermer *et al.*, 2005). In addition, earlier biochemical experiments showed that human SWI/SNF proteins have the ability to transfer histone octamers from donor nucleosomes to acceptor DNA (Phelan *et al.*, 2000).

Another possible mechanism for increasing occupancy and positioning at +1 nucleosome is nucleosome sliding by chromatin remodelers, which is a common activity of SWI/SNF family remodelers. If it is mediated by the sliding activity of BRM, a nucleosome shift towards nucleosome free region (NFR) or the nearby nucleosomes is expected to see the in *brm* mutants. There is precedent for a nucleosome-sliding model of SWI/SNF proteins to form repressive nucleosome. Recently, derepression of HIV expression in human memory T- cells was observed upon loss of human SWI/SNF family complex activity, which resulted in a reduction in occupancy of the +1 nucleosome (Rafati *et al.*, 2011). In their nucleosome mapping study, the position shift of nucleosome to a favored nucleosome-positioning site in the promoter was observed as a consequence of loss of SWI/SNF (Rafati *et al.*, 2011). This suggests that human SWI/SNF is essential for positioning the repressive nucleosome site to repress HIV gene expression, and that in this case this is mediated by sliding activity of SWI/SNF. However in my nucleosome mapping data at *ABI5* locus, a subtle increase or no increase was observed in nucleosome occupancy at nucleosome free region (NFR) or at -1 nucleosome in several independent

biological replicates. The 3' end of +1 nucleosome in *brm* mutant even shifts towards to TSS. I cannot rule out the possibility that this subtle change is due to the tissue complexity that I used for the MNase assay, which might dilute changes in specific cell types. Statistic analyses need to be performed to see if subtle increases in nucleosome occupancy around the NFR and -1 nucleosome are significant in multiple biological replicates. We could also isolate the BRM-expressing cells from germinated embryos by FACS sorting to avoid the tissue complexity issue.

In summary, I favor the hypothesis that BRM contributes to the high occupancy of the +1 nucleosome and position to more proximal to the TSS at *ABI5* locus by a nucleosome assembly mechanism, in part because we do not see a clear sliding effect in the nucleosome profile in *brm* mutant. However there thus far is little evidence that SWI/SNF remodeler functions in nucleosome assembly.

4.1.7 Repressive role of BRM in *ABI5* transcription

Although studies have established the role of the SWI/SNF complex in transcriptional activation in yeast (Hirschhorn *et al.*, 1992; Martens and Winston, 2003; Petesch and Lis, 2012; Sudarsanam *et al.*, 2000), studies in mammals provide evidence that SWI/SNF family proteins can function in both transcriptional activation and repression and even switch between the two modes of action at the same gene (Hargreaves and Crabtree, 2011; Ho *et al.*, 2009; Rafati *et al.*, 2011; Trotter and Archer, 2008; Zhang *et al.*, 2007). Studies indicate the remodeling proteins can play critical roles in gene silencing through interactions with a variety of transcriptional co-repressors such as HDAC and REST

(Trotter and Archer, 2008). Moreover, genome-wide binding studies of SWI/SNF complex components in ES cells showed that they are required for keeping developmental genes repressed and in optimizing the levels of ES cell-specific genes to maintain the core circuitry (Ho *et al.*, 2009). Genome-wide binding study in Hela cells also showed that SWI/SNF components associate with many target genes that are transcriptionally repressed (Euskirchen *et al.*, 2011). Therefore it is not surprising that BRM can directly repress *ABI5* transcription. I hypothesized that BRM represses the basal level of *ABI5* transcription increasing +1 nucleosome at the TSS proximal region. Consistent with my idea, the +1 nucleosome is a frequent target of chromatin remodelers (Yen *et al.*, 2012) and +1 nucleosomes positioned near the TSS are repressive and can interfere with the transcriptional activation (Yen *et al.*, 2012). *Arabidopsis* SWI/SNF complexes also can function in activation and repression of transcription. In *Arabidopsis thaliana*, there are only a few genes known as direct targets of SWI/SNF complexes because genome-wide binding data is not as yet available for these chromatin remodelers. BRM and a putative component of SWI/SNF complex are known to have a role in transcriptional repression of several target genes including; *ABI5* (Han *et al.*, 2012), seed storage proteins (Tang *et al.*, 2008), *FLC* (Farrona *et al.*, 2011; Jegu *et al.*, 2014) and transposable elements (Zhu *et al.*, 2013). However the repressive mechanisms mediated by SWI/SNF complex at these target genes are different in each case; increase of +1 nucleosome occupancy (Han *et al.*, 2012), increase of open chromatin by loss of BRM (Tang *et al*., 2008), formation of a DNA loop (Jegu *et al.*, 2014) and long non-coding RNA association (Zhu *et al.*, 2013), respectively. Because of limited information on the role of SWI/SNF complexes and their target genes in plants, the common or distinct

mechanism of different chromatin remodelers in transcriptional regulation remains largely unknown.

4.1.8 Chromatin changes at *ABI5* locus upon loss of BRM

In *brm* mutant plants, I observed derepression of *ABI5* expression in the absence of ABA treatment. Consistent with this observation, I found a moderate (~40%) reduction in nucleosome occupancy at the + 1 position of the *ABI5* locus coupled with a shift away from the TSS in the absence of ABA treatment in *brm* mutants relative to wild-type germinated embryos. I observed changes by loss of BRM only at the +1 nucleosome, not at the -1 and -2 nucleosomes that I monitored. The reduction in nucleosome occupancy and shifting at the +1 nucleosome are also found in response to ABA treatment in wild type plants. The observed ABA-dependent change in wild type was very similar to germinated *brm* mutants, suggesting that this effect in wild type was likely due to a loss of BRM in the presence of ABA. However, unexpectedly we observed strong binding of BRM at the *ABI5* locus in the presence of ABA where the +1 nucleosome resides. Therefore I hypothesized that BRM complex activity may be inactivated upon ABA sensing, which resembles *brm* mutant. To test whether BRM ATPase activity is required for +1 nucleosome occupancy and position, we could utilize an ATPase dead mutant of BRM. It is expected that these mutants may display *brm* like phenotype including *ABI5* derepression, reduced nucleosome occupancy and enhanced ABA responses. However it needs to be confirmed that ATPase mutation does not affect BRM complex assembly and recruitment to *ABI5* locus.

Other models are also possible other than a direct role of BRM in +1 nucleosome placement and occupancy at the *ABI5* locus. Repressor complex such as HDAC (Histone deacetylase complex) could associate with BRM complex to repress *ABI5* transcription in the absence of ABA or water stress. Human BRG1 containing SWI/SNF complex has been shown to interact with a variety of transcriptional co-repressors (Trotter and Archer, 2008). Derepression of *ABI5* upon loss of BRM may be a consequence from enhanced transcriptional activity due to a failure of a repressive-complex recruitment. Upon ABA sensing, repressor complex can leave the *ABI5* locus while BRM is retained at that position, which allows *ABI5* transcription.

4.1.9 Chromatin changes at *ABI5* locus during development

I observed development-dependent changes in the -1 nucleosome occupancy at the *ABI5* promoter just prior to seedling establishment. The occupancy of the -1 nucleosome was very low at day 1.5 but increased at day 2 in both wild type and *brm* mutants (Figure 4-3, D1.5 and D2). In addition, the occupancy and position at this nucleosome was not changed in *brm* mutants compared to wild type at both developmental stages, suggesting that this change is BRM independent. Hence another chromatin remodeler may be involved in this -1 nucleosome assembly. Increased nucleosome occupancy at this position may trigger chromatin condensation at *ABI5* locus during developmental transition. In agreement with this finding, *ABI5* expression and its ABA inducibility was normally down regulated at the end of postgermination in both wild type and *brm* mutants. Upon seedling establishment (Figure 4-3, D3), *ABI5* expression is no longer

derepressed in *brm* mutants and reverts to the wild type level in ABA response. This suggests that BRM does not play a role in development-dependent repression of *ABI5* and that other chromatin regulators are involved in this process.

Developmentally dependent repression of *ABI5* has been previously described (Perruc *et al.*, 2007). In this study, it is shown that a CHD subgroup chromatin remodeler in *Arabidopsis,* PKL, mediates chromatin changes that are required for eventual shut down of *ABI5* expression at the end of the postgermination period. Increased expression of *ABI5* in *pkl* mutants relative to the wild type in the presence of ABA treatment is correlated with a reduced level of two repressive histone modifications, H3K9me2 and H3K27me2, at the promoter of *ABI5* and a failure of developmental repression of *ABI5*. Although it is not known that *ABI5* expression is directly regulated by PKL, repressive histone modifications triggered by PKL may be an important role for the formation of a condensed chromatin structure at the *ABI5* locus upon seedling establishment (Figure 4-3, D3). Besides PKL, it is also known that mutations in several chromatin regulators such as histone deacetylase, Polycomb repressive complexes and retinoblastoma proteins (Bouyer *et al.*, 2011; Gutzat *et al.*, 2011; Kim *et al.*, 2012b; Tanaka *et al.*, 2008) delay the developmental switch from embryo to seedling growth. It is possible that these chromatin regulators cooperate with PKL to generate the repressive state of the *ABI5* locus.

Interestingly, the transcriptional activity of *ABI5* is regained during later vegetative development (Brocard *et al.*, 2002; Mizoguchi *et al.*, 2010). I observed that *ABI5* expression is derepressed at 21 days in *brm* mutant plants and that BRM binds to promoter region of *ABI5* locus at this time in development (Figure 4-3, D21). In addition,

nucleosome occupancy at the -1 nucleosome was decreased in *brm* mutants and ABA treated wild type (data not shown in my thesis), which was similar to the effect seen at the +1 nucleosome during the postgermination stage of development. Because I did not tested BRM binding and nucleosome change in *brm* mutants at +1 nucleosome region at this stage, I do not know whether BRM has an effect on +1 nucleosome at this developmental stage. In summary, BRM plays a role in *ABI5* repression during postgermination and later vegetative stages. This conclusion is based upon *ABI5* derepression, BRM binding to the locus and reduction in nucleosome occupancy at *ABI5* locus in *brm* at both developmental stages. However the location where the BRM acts to promote occupancy and direct the positioning of nucleosome appears to be different between two stages. In addition, I observed that *ABI5* expression was synergistically increased in *brm* mutants treated with ABA compared to wild type treated with ABA during vegetative development (Figure 4-3, D21), indicating that BRM activity at this stage is not fully inactivated by ABA.

4.1.10 Chromatin changes at *ABI5* locus by ABA treatment during postgermination development

I detected reduced occupancy of all three nucleosomes (-2, -1 and +1) in response to ABA treatment. As I described above, a reduction in occupancy at +1 nucleosome in the presence of ABA seems to be due to BRM inactivation upon ABA sensing. The ABA-dependent changes in the upstream nucleosomes (-2 and -1) were similar in both *brm* and wild type, suggesting that this reduction is BRM independent. Reduced occupancy in

these nucleosomes is positively correlated with *ABI5* induction in the presence of ABA. The most pronounced reduction is observed at the -1 nucleosome. This nucleosome is positioned over 2 *cis*-regulatory elements called ABA-responsive elements (ABRE). These elements are bound by ABA-activated transcription factors and linked to ABA-responsive gene expression. Transcription factor binding to these sites may modulate the accessibility of DNA in the context of chromatin (Radman-Livaja and Rando, 2010). Therefore the transcriptional phenotype of *ABI5* in ABA treatment or drought may result from two combined effects; increased transcription factor binding to ABREs causing a destabilized -1 nucleosome, and increased activity of the transcription initiation complex due to a reduction in the +1 nucleosome occupancy by BRM inactivation upon ABA sensing.

4.1.11 Link between drought response and BRM

I observed a drought tolerant phenotype of *brm* mutant during vegetative development. My hypothesis was that drought tolerance of *brm* mutants is due to increased *ABI5* expression. The ectopic expression of *ABI5* can induce stress-responsive gene expression so that the plant better tolerates water loss during vegetative development (Lopez-Molina *et al.*, 2001). To further test this hypothesis, I examined drought responses in *brm abi5* double mutant during the vegetative stage. However *brm abi5* plants were as tolerant as *brm* alone to drought. Thus, these results suggest that either increased *ABI5* expression in *brm* mutant does not contribute to drought tolerance or that ABI5 acts redundantly with other ABA-responsive transcription factors such as ABF3 in drought response, ABF3

expression was also derepressed in *brm* mutants during vegetative development (Figure 2-10B). Because the more prominent role of BRM on *ABI5* expression is during the postgermination stage, testing drought tolerance of *brm abi5* double mutant at this stage may help to understand whether increased *ABI5* expression contribute to drought tolerance of *brm* mutant during this postgermination period. For further insight into the role of BRM in drought response during vegetative stage, we need to perform genome-wide analyses to identify the direct target genes of BRM at this stage and as well as genes differentially expressed in *brm* mutant upon water stress.

4.2 Summary and Future direction

My study showed that the SWI/SNF chromatin remodeling ATPase BRM plays a role in the regulation of drought-responsive gene expression and that BRM promotes positioning and occupancy of a nucleosome close to TSS to prevent upregulation of a master drought response regulator, *ABI5*, in the absence of ABA. This mechanism ensures that costly stress responses are mounted only upon perception of water stress signals to enhance fitness of the organism. This regulatory role of nucleosome positioning and of occupancy by BRM is not limited to the genes that I analyzed in my thesis study. Therefore, for the future study it would be interesting to perform genome-wide approach as such as MNase-seq to monitor nucleosome position or occupancy change in wild type and *brm* mutant upon ABA treatment. It would furthermore be of interest to conduct BRM ChIP-seq to monitor genome-wide binding of BRM. In addition, RNA-seq using wild-type and *brm* mutant treated ABA or non-treated could be performed to identify ABA-regulated genes

whose expression is altered in *brm* mutants. The data from such genome-wide analyses would provide us with the subset of drought responsive genes that are regulated by nucleosome positioning by the BRM chromatin remodeling ATPase.

To gain insight into the detailed mechanism how ABA sensing regulators of SWI/SNF chromatin remodeling complex activity, we could test two possibilities. First, does BRM complex composition change upon drought stress? We could isolate the BRM complex from *Arabidopsis* nuclei using well-established epitope tagged BRM transgenic lines to see if BRM complex composition changes under water stress conditions. Second, are core BRM complex components differentially post-translationally modified in stress versus non-stress conditions to regulate nucleosome-remodeling activity of BRM? Biochemical activity can be tested using the full-length BRM cDNA expressed in insect cells with a baculovirus-based expression system, followed by treatment with active or kinase dead SnRK2 kinase.

In summary, I have explored the link between chromatin regulators known to alter genome accessibility, the SWI/SNF chromatin remodeling ATPases, and drought stress response in the plant model system *Arabidopsis thaliana*. My thesis work and future studies will provide mechanistic insight how environmental stress dependent genome accessibility is regulated by the SWI/SNF chromatin remodelers. It may contribute to our ability to modulate the drought stress response-pathway, which is crucial for enhancing plant tolerance to water stress.

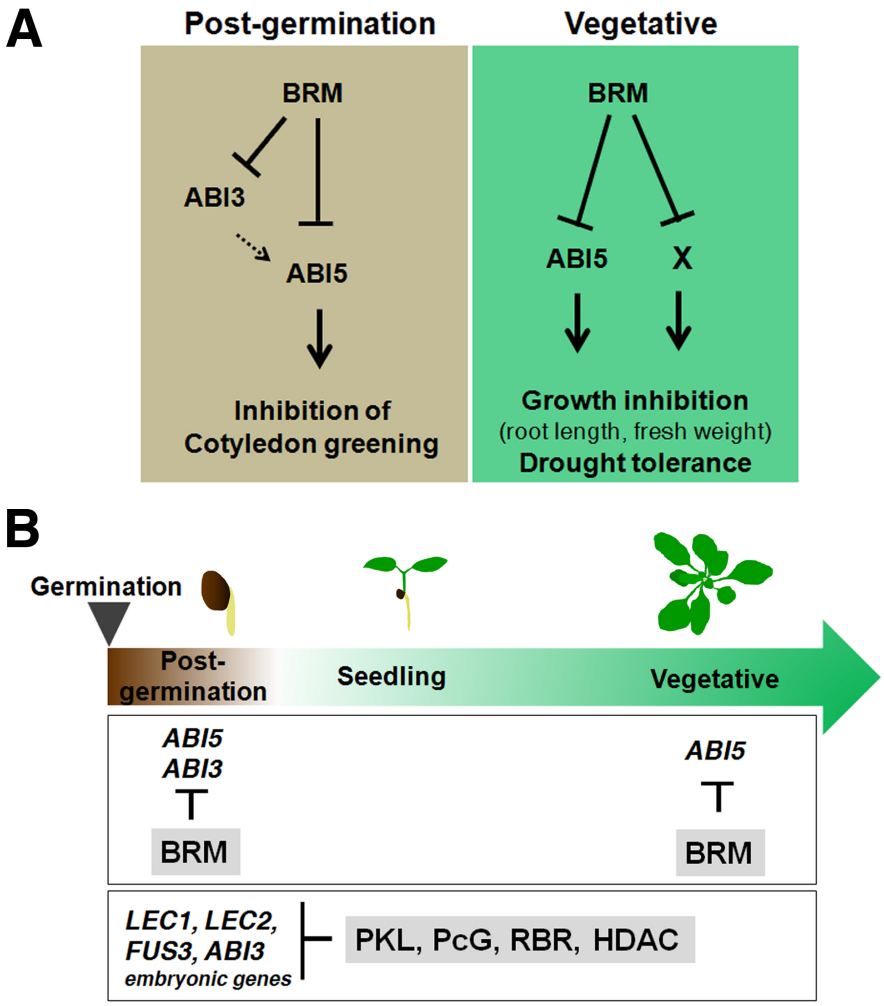

A
Post-germination
Vegetative
BRM
ABI3
ABI5
Inhibition of
Cotyledon greening
BRM
ABI5
X
Growth inhibition
(root length, fresh weight)
Drought tolerance
B
Germination
Post-
germination
Seedling
Vegetative
ABI5
ABI3
BRM
ABI5
BRM
LEC1, LEC2,
FUS3, ABI3
embryonic genes
PKL, PcG, RBR, HDAC

Figure 4-1. Model for role of BRM in ABA Responses

(A) Role of BRM in ABA response at different developmental stages. Left: Inhibition of cotyledon greening during postgermination development. BRM negatively regulates the expression of two key ABA-related transcription factors *ABI5* and *ABI3*. *ABI3* acts upstream of *ABI5* (Lopez-Molina *et al*., 2002). Solid arrows, direct regulation; dashed arrows, direct or indirect regulation. Right: Inhibition of growth during vegetative development. Additional direct BRM targets remain unidentified that act in parallel with ABI5. ABI5 has been implicated in drought tolerance (Lopez-Molina *et al*., 2001), although the increase of *ABI5* expression alone was not responsible for the *brm* mutant drought tolerance.

(B) Role of chromatin regulators in expression of ABA-responsive transcription factors during postgermination and vegetative development. BRM represses *ABI5* expression during postgermination and vegetative development and *ABI3* during postgermination development. Several chromatin regulators influence the developmental transition from postgermination development to seedling establishment. HDAC, histone deacetylase; PcG, Polycomb; RBR, Retinoblastoma-related protein.

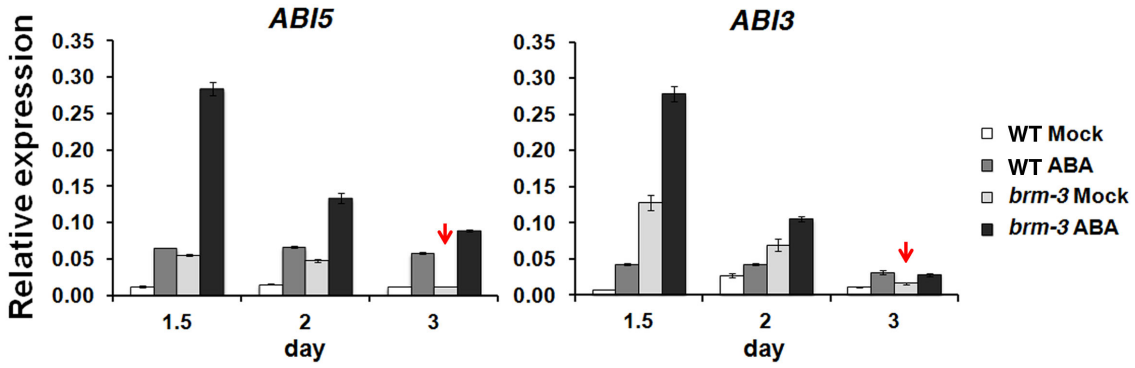


Figure 4-2. Developmental regulation of *ABI5* and *ABI3* expression in the wild type and in *brm* mutants

qRT-PCR expression analysis of 1.5, 2 and 3-day-old wild type (WT) and *brm-3* mutants one hour after mock or ABA (50 μM) treatment. Arrow points to transcript level in *brm-3*, which is indistinguishable from that in the WT on day 3.

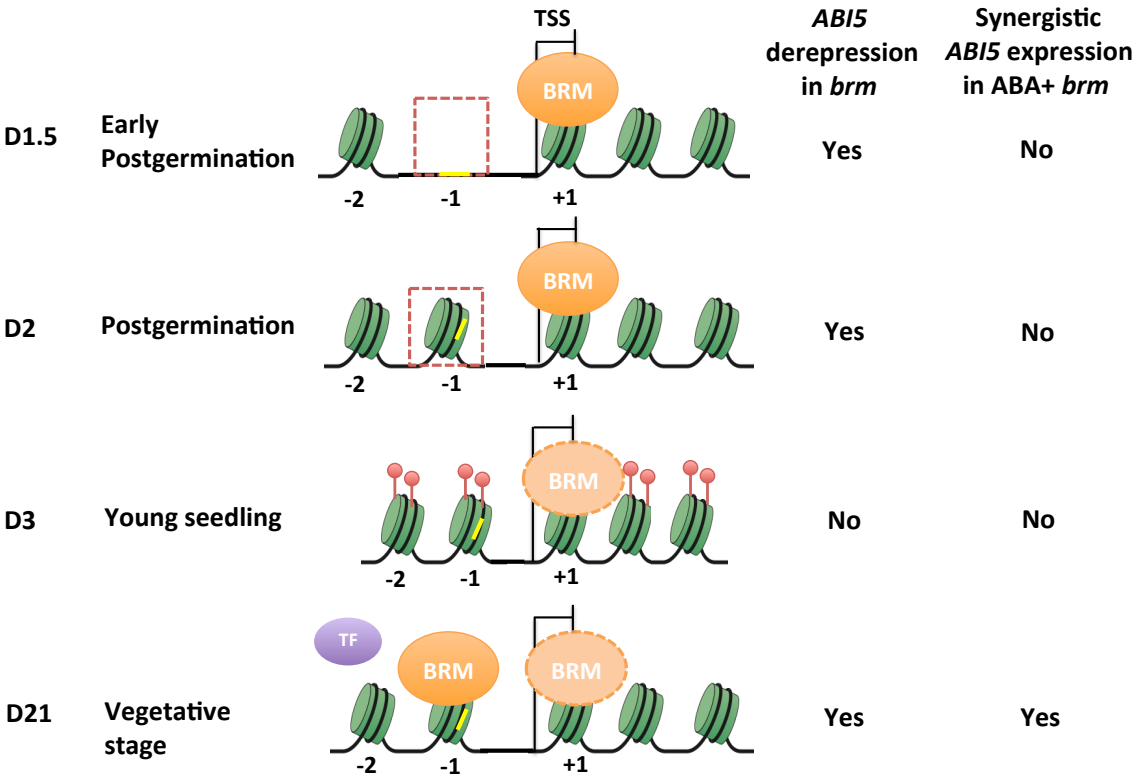


Figure 4-3. A model for the role of BRM at *ABI5* locus during development

D1.5 (1.5 day after stratification): BRM binds to the +1 nucleosome. The -1 nucleosome is absent at this stage (marked by the red box). *ABI5* derepression was observed in *brm* mutant. Fold change (ABA treated/ Mock treated) in *ABI5* expression was the same in wild type and *brm* mutant (*ABI5* expression was not synergistically regulated by BRM and ABA).

D 2: BRM binds to the +1 nucleosome. The -1 nucleosome (in red dashed box) positioned over 2 *cis*-regulatory elements (ABRES, marked in yellow) in the *ABI5* promoter. *ABI5* derepression in *brm* mutants is still observed, although ABA inducibility of *ABI5* is slightly decreased.

D3: BRM binding at +1 nucleosome has not been tested (dashed orange oval). Derepression of *ABI5* at this stage is not observed. Overall *ABI5* expression becomes very low and ABA inducibility is decreased. PKL triggered repressive histone modifications (H3K9me2 or H3K27me2) are generated (small red circles), which may contribute to formation of condensed chromatin and developmental shut down of *ABI5* expression (Perruc *et al.*, 2007)

D21: BRM binds to -1 nucleosome and promote -1 nucleosome occupancy. BRM binding to +1 nucleosome was not tested (dashed orange oval). *ABI5* derepression is regained. Fold change (ABA treated/ Mock treated) in *ABI5* expression was higher in *brm* mutant than wild type (*ABI5* was synergistically increased by loss of BRM and ABA treatment). Transcription factors such as ABF3 (purple oval) induced by ABA or water stress also affect destabilization of -1 nucleosome.

CHAPTER 5. Materials and Methods

5.1 Plant Growth

The Arabidopsis thaliana genetic resources used in this study were mostly in the Columbia ecotype and have been previously described: *swi3c-2* (Sarnowski *et al.*, 2005), *brm-3* (Farrona *et al*., 2007), *brm-1* (Hurtado *et al*., 2006), *syd-5* (Bezhani *et al*., 2007), *bsh-1* (Tang *et al*., 2008), *abi5-7* (Yamagishi *et al*., 2009), and 35S:HAB1 (Saez *et al*., 2004). *brm-101* (Kwon *et al*., 2006) was in the Landsberg *erecta* ecotype and partly introgressed into Columbia. The strong loss-of function *minu1-2* (CS413977) and *minu2-1* (SALK_057856) mutants (Sang *et al*., 2012) and the weak *syd-6* (SALK_116266) mutant were obtained from the ABRC stock center. The pBRM:BRM-GFP construct was previously described (Wu *et al.*, 2012). Plants on plates and in soil were stratified at 4°C for 3 d. Plant growth was in inductive photoperiod (16-h-light/8-hdark cycles) or constant light at 22°C under white fluorescent light (fluence rate: 110 μmol/m2 s for soil-grown plants; 90 μmol/m2 s for media-grown plants). Plant growth on plates was in the presence of 1% Sucrose unless indicated otherwise.

5.2 ABA and Drought Treatments

For germination assays, wild-type, *brm-3*, and *brm-1*/+ seeds were placed on Murashige and Skoog (MS) plates (no Sucrose) and supplemented with the ABA concentration indicated. Radicle emergence was scored 3d after stratification (Müller *et al.*, 2006). Well-ripened seeds from plants grown under the same growth condition were used. For seedling growth (green cotyledon) assays, seeds were placed on MS media supplemented

with various concentration of ABA (0, 0.5, 0.8, 1.0, and 1.5 μM). Plants that had formed green cotyledons were counted 7 d after stratification unless indicated otherwise. For root growth assays, seeds were germinated on MS plates and seedlings were grown vertically for 2 or 5 d, followed by transfer to fresh media lacking or containing ABA (1, 5, or 10 μM). Plates were incubated vertically for an additional 5 d before measuring root length. For ABA treatment for gene expression, ChIP, and MNase studies, seeds were stratified for 3 d followed by growth in constant light for the time indicated. Liquid MS media with or without 50 μM ABA (Sigma-Aldrich; A1049) was added to the plates for 1 h. For studies on 3-week-oldplants, 9-d-old seedlings grown on MS plates were transplanted to soil and grown for 12 more days before treatment. 100 μMABA in 0.5mM Tris-HCl, pH 8.0, or 0.5 mM Tris-HCl, pH 8.0, alone was applied to 3-week-old plants by spraying the leaves with an atomizer.

5.3 Dehydration and drought treatment

Dehydration and drought treatment were performed as previously described (Li *et al.*, 2008). For drought tolerance test, 9-day-old seedlings grown on MS were transferred to soil and grown for an additional 12 days. Each pot was filled with the same amount of soil. The pots were evenly spaced in the tray and their position was changed daily to minimize edge effects. After placement in the growth chamber, pots were weighed daily to ensure equal soil water content until the plants were three weeks old. Next I withheld water for two weeks under 24°C ± 1°C and 45% ± 5 % relative humidity. All pots dried at a similar rate based on daily assessment of the pot weight (Figure 2-12). After two weeks, watering was resumed. Phenotypes were recorded 5 days after watering. The

experiment was repeated four times with at least 12 plants per genotype per experiment. One representative experiment is shown.

2-week-old plants grown on MS plates were dehydrated by placement on filter paper (Yamaguchi-Shinozaki and Shinozaki, 1994; Li *et al*., 2008) with airflow for 3 hours (25°C ± 1°C, 25% ± 2 % relative humidity) then rehydrated. Survival rate was scored and pictures were taken 5 days after rehydration. Plants that had more than four green leaves were scored as surviving. The experiment was repeated three times and >20 plants per genotype per experiment were used. One representative experiment is shown.

Stainless mesh (S3895, Sigma) was placed on plates containing MS media and seeds were sown on top. After 2 weeks of growth, plants were pulled out carefully from the media and placed in empty petri plates after removal of excess moisture and media. The plants were dehydrated by placement of the plates on a lab bench (24°C ± 1°C, 27% ± 3 % relative humidity) for 6 hours. Pictures were taken at the time indicated. 6 hours after dehydration, water was added and plants were moved back to growth chamber.

5.4 Gene Expression Analyses

RNA purification, reverse transcription, and quantitative PCR (qPCR) were performed as described previously (Pastore *et al.*, 2011) except amplification was monitored by EvaGreen fluorescent dye (Biotium). The sequences of primers used are listed in the Table 6.1. Confocal imaging was performed as previously described (Winter *et al.*, 2011).

5.5 Chromatin imunoprecipitation

For the GFP-tagged BRM ChIP, 1.5-d-old *brm-1* ProBRM:BRM-GFP germinated seeds (0.2 g) were used. The ChIP procedure was as previously described (Kwon *et al.*, 2005). Five microliters of anti-GFP rabbit polyclonal antibody (Invitrogen; A6455) was employed per 0.2 g of tissue. To quantify BRM enrichment on the genomic DNA, qPCR was performed using a StepOnePlus Real-Time PCR system (Applied Biosystems) with EvaGreen fluorescent dye (Biotium). The percentage of input was calculated by determining $2^{-\Delta Ct}$ (= $2^{-[Ct(ChIP)-Ct(Input)]}$) as per the ChampionChIP qPCR user manual (SABioscience). To facilitate comparison of different genotypes and treatments, the calculated percent input of the wild type (control) at the regions tested was set to 1. The relative enrichment represents the fold change to the wild type. The exon region of retrotransposon *TA3* (Johnson *et al.*, 2002) was used as negative control. Primer sequences are listed in the Table 6.2.

5.6 MNase Assay

A total of 0.2 g of 1.5- or 2-d-old plants was harvested in liquid nitrogen. Nuclei and chromatin were isolated as previously described (Chodavarapu *et al*., 2010) with the following changes. The isolated nuclei were washed twice with HBB buffer (25 mM Tris-Cl pH 7.6, 0.44 M Suc, 10 mM MgCl2, 0.1% Triton-X, 10 mM betamercaptoethanol), and the isolated chromatin was digested with 0.1 units/ µL - 0.2 units/µL (final concentration) of Micrococcal Nuclease (Takara) for 10 min in digestion buffer at 37°C. Subsequent steps were performed as previously described (Chodavarapu *et al*., 2010). Mononucleosomes were excised from 1.5% agarose gels and purified using

a gel purification kit (Qiagen). The purified DNA was quantified using a NanoDrop ND-1000 spectrophotometer. Two nanograms of purified DNA were used for qPCR to monitor nucleosome occupancy. The fraction of input was calculated as $2^{-\Delta Ct}$ ($2^{-[Ct\ (mono)-Ct\ (gDNA)]}$) using undigested genomic DNA (Gévry *et al.*, 2009) followed by normalization over that of gypsy-like retrotransposon (At4g07700) 273 loci for each sample. The tiled primer sets for *ABI5* locus used for realtime PCR are listed in Table 6.3. Oligonucleotide sequences for gypsy like gene (At4g07700) nucleosome mapping are as previously used in (Kumar and Wigge, 2010).

5.7 Statistical Analysis

For root length and fresh weight measurement, P values were calculated with the one-tailed Student's t test. For green cotyledons, germination, and survival rate assays, x2 analysis was performed. Two random variables (ex. the wild type and *brm-3*) with two types of data (survival or death) were entered in a 2 x 2 contingency table and x2 statistic and P values were calculated using a java-based script (http://www.physics.csbsju.edu/stats/contingency_NROW_NCOLUMN_form.html). For *brm-1/+* populations, I assumed Mendelian inheritance of a recessive trait. For statistical significance cutoff, I employed a P value lower than 0.01.

5.8 Yeast two hybrid assay

To test the interaction between BRM and the components of core ABA signaling components, BRM N-terminal domain (At2g46020, 1-976 aa) was cloned into either

pBridge or pGADT7, and OST1 (At4g33950, 1-362 aa) and HAB1 (At1g72770, 1-511 aa) were cloned into pGBKT7 or pGADT7, respectively. To test the abrogation of interaction between BRM and HAB1 in the presence of ABA signaling, PYL4 (At2g38310, 1-207 aa) and PYL5 (At5g05440, 1-203 aa) were inserted at MCSII (Multiple Cloning Site II) of the pBridge-BRM (BD) construct. To test the abrogation of HAB1-SWI3B interaction upon ABA signaling, ΔNHAB1 (179-511aa) and PYLs cloned into pBridge and used pACT1-SWI3B (Saez *et al*., 2008). Each bait and prey plasmids were co-transformed into AH109 yeast strain according manufacturer's manual (MATCHMAKER GAL4 Two-Hybrid User Manual, Clontech Laboratories, INC). Transformed cells were plated on -Trp -Leu SD media. The resulting colonies were grown in -Trp -Leu SD liquid media overnight, adjusted for equal cell density, serially diluted (1~ 10^{-4}) and spotted on selection media (-Trp -Leu –His /SD media with 0.1mM, 0.5mM, 1mM, 2mM 3-AT). To test an abrogation of interaction, 10 μM ABA was supplemented into the selection media. The growth of interactors on the different media was scored. The interaction test was performed at least 2 times for each bait/prey combination.

5.9 Bimolecular fluorescence complementation (BiFC)

BiFC was performed by transforming plasmids harboring BRM-N (1-976 aa) or BRM-C (1541-2193 aa) fused with N-terminal YFP in pSPYNE(R)173 and HAB1 or OST1 fused with C-terminal YFP in pSPYCE(MR) (Waadt *et al.*, 2008) into *Arabidopsis* leaf protoplasts. Protoplast isolation and transformation was performed as previously

published methods (Yoo *et al.*, 2007). Confocal microscopy imaging for protoplast BiFC was done as preciously described (Yamaguchi *et al.*, 2013).

5.10 Co-immunoprecipitation

For co-immunoprecipitation assays using protoplast, FLAG-BRMN (Wu *et al*., 2014 submitted) in pUC19 and PYL4 in pSPYNE(R)173 were co-transformed into protoplast isolated from 35S::HA-HAB1 transgenic lines. FLAG-BRMN in pUC19 and pSPYNE(R)173-HAB1 were co-transformed into the protoplast isolated from 35S::HA-OST1 transgenic plants. pSPYCE(MR)-MPDB was used as a negative control for BRM interaction. The nuclear fraction of protoplasts was prepared as previously described (Ryu *et al.*, 2007). Anti-FLAG (2368S, Cell Signaling) and anti-HA-peroxidase high affinity (3F10, Roche) were used for co-IP or Western blot, respectively.

5.11 Accession Numbers

Sequence data for the genes in this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: BRM (AT2G46020), SWI3C (AT1G21700), SYD (AT2G28290), MINU1 (AT3G06010), MINU2 (AT5G19310), BSH (AT3G17590), ABI5 (AT2G36270), ABI3 (AT3G24650), ABF3 (AT4G34000), HY5 (AT5G11260), ABF2/AREB1 (AT1G45249), EIF4A1 (AT3G13920), TA3 (AT1G37110), gypsy-like retrotransposon (AT4G07700), HAB1 (AT1G72770), OST1 (AT4G33950), PYL4 (AT2G38310), PYL5 (AT5G05440). Mutants investigated in this study are listed in Methods.

5.12 Computational identification of well-positioned nucleosomes

(This analysis is performed by Dr. Qi Zheng from the lab of Dr. Brian Gragory at Penn) To get a genome-wide overview of the nucleosome positioning in *Arabidopsis*, we utilized the published MNase-seq dataset (Chodavarapu *et al*., 2010) and re-implemented the mapping and position-calling procedure as previously described with slight modifications, as the published results were based on an earlier genome assembly (TAIR 7); also the exact coordinates of the called nucleosome positions are not publically available. Therefore, we downloaded the raw Illumina sequencing reads for *Arabidopsis* MNase-Seq (qseq) sample (GSM543296) from NCBI SRA and dumped them into FASTQ files. The reads were mapped to the TAIR9 genome assembly using bowtie, with non-default options set as "-n 2 -e 160 -l 34 -y -k 1 -m 1 -B 1 –nomaqround" and a seed-match search of up-to 2 mismatches (~6%) of the 34-bp seeds and up-to 4 total mismatches (~8%) of the entire 55 nt reads for uniquely-mapped reads. An additional parsing step was performed after the alignment to guarantee the 2 and 4 mismatches in the seed and full-length reads. The mismatch cut-off levels as well as the seed-lengths were chosen after examining the overall base-wide sequencing quality of the whole library (data not shown) to facilitate both mapping sensitivity and specificity.

The nucleosome positions on *Arabidopsis* genome were predicted as "nucleosome enriched regions" as previously described (Kaplan *et al.*, 2008) as in the published article of the data source (Chodavarapu *et al.*, 2010). All aligned reads were first extended to 147 bp as the average nucleosome size, and the read coverage of every genome base was calculated; then genomic regions with aberrantly high read coverage (defined as > 10

times the median genomic coverage value) were "trimmed" by filling these regions with values 10 times the median coverage value, and normalized against the genomic average values to get the base-wise nucleosome occupancy values, as $occ_i = \log 2\left(C_i / \bar{C}\right)$, where the occ_i is the normalized nucleosome occupancy for the i^{th} base-pair, C_i is the (trimmed) read coverage for the i^{th} base-pair and the $\bar{C}$ is the genome average read coverage. Last, nucleosome positions on the *Arabidopsis* genome were called as consecutive genomic regions >= 50 bp with a minimum occupancy value >= T*e*, where T*e* was set to 0.75 to optimize the nucleosome calling accuracy as previously described (Kaplan *et al*., 2008). As a result, we called 96,078 well-positioned nucleosomes on *Arabidopsis* genome with median size of 152 bp which is close to the average nucleosome size.

Table 5.1 Oligonucleotide sequences for expression analyses

Gene	Forward (5'->3')	Reverse (5'->3')
EIF4A1	aaactcaatgaagtacttgagggaca	tctcaaaaccataagcataaataccc
ABI5	acctaatccaaacccgaacc	taccctcctcctcctgtcct
ABI3	atgtatctcctcgagaacac	ccctcgtatcaaatatttgcc
ABF3	ccttacatgtttgggcgagt	tttgagttgcgcaatttctg
HY5	atgaggagatacggcgagtg	ttcagccgcttgttctcttt
ABF2/AREB1	ttacaacgaaagcaggcaag	aaggtcccgactctgtcctc

Table 5.2 Oligonucleotide sequences for BRM-GFP/HA ChIP

Loci	Forward (5'->3')	Reverse (5'->3')
ABI5-p1	aacatttgtgtagccgaagtca	aggcgtgaaggtcaacatct
ABI5-e1	aattctccggcggctttt	ccggtggctttgtgttcc
ABI5-e2	acctaatccaaacccgaacc	taccctcctcctcctgtcct
ABI3-p1	tgtcgcatagccacgtagag	acgatgacatatggccgaac
ABI3-p2	aagtgatttacggcccacac	cgtaatgctcctcctcgaaa
ABI3-e1	attgaatcagcggcaagaag	aagagaggttggtggtggtg
NC1 (TA3)	ctgcgtggaagtctgtcaaa	ctatgccacagggcagttt
NC2 (BRM)	tacaccaaccccaagaggag	cccccaagctttgtttcttt

Table 5.3 Oligonucleotide sequences used for *ABI5* nucleosome mapping.

COA*	Forward (5'->3')	Reverse (5'->3')
-516	tctaacaagtctactttcaccagcta	aggttagattcaagatgttatgaaaga
-448	tctttcataacatcttgaatctaacct	ttgttttgtgaaattgacggatta
-385	gatcaatcaaattaatccgtcaa	gaacgtgaaatttggattagaag
-309	cgctcttctaatccaaatttca	cacgtgtgacttcggctaca
-241	aacatttgtgtagccgaagtca	gtgtcctgcacgtgtctctc
-199	cacgtgtcgagcctgtga	tccaggcccatgatcaga
-167	gagagacacgtgcaggaca	tttcgaccaatggaatgc
-107	tctgatcatgggcctgga	cgcgtggggtctaagaag
-62	gcattccattggtcgaaa	aatgggtaggaggcggtaa
4	tcacggtgagaacataaatatcaatc	cgccggagaattttgactg
35	tttaccgcctcctacccatt	ggaggttctcctccttcacatag
59	tctctctttctcaaaacctttcagtc	ctgagagaatccgcttcttgtt
90	aattctccggcggctttt	ccggtggctttgtgttcc
119	ggaggagaacctccataacaaga	cctgaaaatgaaatctgtgtgtctaa
135	aacaagaagcggattctctca	acaagaaaagtgttaacctgaaaatg
202	ccggtttttagacacacagatt	ccagaaaacgaagacctaaactt
217	tcattttcaggttaacacttttctt	aaggaccagaaaacgaagacc

* Center Of Amplicon: distance (base pairs) form Transcription Start Site

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