Arabidopsis Chromatin Remodeler Brahma: Its Functional Interplay with Polycomb Proteins and The REF6 Histone Demethylase

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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ARABIDOPSIS CHROMATIN REMODELER BRAHMA: ITS FUNCTIONAL INTERPLAY WITH POLYCOMB PROTEINS AND THE REF6 HISTONE DEMETHYLASE
(Thesis format: Monograph)

by

Chenlong Li

Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
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Abstract

BRAHMA (BRM) is a SWI/SNF-type chromatin remodeling ATPase that plays an important role in regulation of gene expression. Tri-methylation of lysine 27 on histone H3 (H3K27me3) is a histone modification that is associated with transcriptionally repressed genes and catalyzed by Polycomb Group (PcG) proteins. BRM has been proposed to antagonize the function of PcG proteins but the underlying molecular mechanism is unclear. To understand how BRM regulates the function of PcG proteins during plant development, a genome-wide analysis of H3K27me3 in brm mutant was performed using chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq). Loss of BRM leads to increased H3K27me3 deposition at many Arabidopsis thaliana genes. It is shown that physical presence of BRM reduces the association of PcG proteins with genes. For example, without BRM, an elevated PcG occupancy at the flowering repressor gene SHORT VEGETATIVE PHASE (SVP) is accompanied by increased frequency of the H3K27me3 level and a concomitant reduction of the SVP transcription. Finally, genetic studies using gain- and loss-of-function mutants have established that BRM represses flowering transition by activating SVP transcription. This work highlights a crucial role of BRM in counter-balancing the activity of PcG proteins during plant development.

In Arabidopsis, H3K27me3 can be removed by H3K27 demethylase, called RELATIVE OF EARLY FLOWERING 6 (REF6). Even though both REF6 and BRM are thought to activate gene expression at the chromatin level, how their activities are coordinated remains unclear. It is shown here that BRM and REF6 share common DNA binding motifs, and co-localize on more than 1,000 Arabidopsis genes, many of which are involved in responses to various stimuli, including plant hormones. Furthermore, depletion of REF6 reduces the occupancy of BRM at hundreds of BRM-REF6 co-targets, indicating that REF6 facilitates the recruitment of BRM. Consistent with these observations, BRM and REF6 form a protein complex in vivo and co-activate the expression of a set of common genes. Together, these results demonstrate an unanticipated genome-wide coordination between an H3K27 demethylase and the BRM chromatin remodeling protein.
Keywords

SWItch/Sucrose NonFermentable, BRAHMA, Polycomb Group proteins, H3K27me3, histone demethylase, SHORT VEGETATIVE PHASE, RELATIVE OF EARLY FLOWERING 6, chromatin remodeling, Arabidopsis thaliana
Co-Authorship Statement

I performed all of the experiments described in this thesis except for the following:

The $XVE::aMIRBRM$ construct in section 2.5 was generated by Chen Chen in Dr. Cui’s laboratory. Using this construct, I transferred the vector into $Arabidopsis$ and performed all the downstream experiments.

Next generation sequencing in section 2.7 was performed at Mount Sinai Hospital in Toronto. ChIP-seq data analysis was conducted by me, Dr. Lei Gao and Dr. Lianfeng Gu in Dr. Xuemei Chen’s laboratory at the University of California, Riverside. I performed the ChIP assay.

RNA-seq in section 2.13 was performed at The Hospital for Sick Children in Toronto. I isolated the RNA. The data analysis was performed by Dr. Lei Gao in Dr. Xuemei Chen’s laboratory at the University of California, Riverside.

Co-IP in section 2.15 was performed by Dr. Chia-yang Chen in Dr. Keqiang Wu’s lab at the National Taiwan University. I prepared all vectors that were used for Co-IP assay.
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Foremost, I would like to thank my supervisor Dr. Yuhai Cui for giving me the opportunity to carry out my PhD in his group. His enthusiasm, guidance, continuous support and encouragement have been and will continue to be a great source of inspiration for my future. I feel extremely fortunate to have been given the freedom and support to pursue my own ideas.

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Last but not least, I would like to thank my family for always being there for me, for their constant love, patience, support, and encouragement
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<tbody>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ABI5</td>
<td>ABA INSENSITIVE 5</td>
</tr>
<tr>
<td>ABRC</td>
<td>Arabidopsis Biological Resource Center</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AG</td>
<td>AGAMOUS</td>
</tr>
<tr>
<td>aMIRBRM</td>
<td>An artificial microRNA that targets <em>BRM</em> message RNA</td>
</tr>
<tr>
<td>AN3</td>
<td>ANGUSTIFOLIA3</td>
</tr>
<tr>
<td>AP1</td>
<td>APETALA1</td>
</tr>
<tr>
<td>AP3</td>
<td>APETALA3</td>
</tr>
<tr>
<td>ARP4</td>
<td>ACTIN RELATED PROTEIN 4</td>
</tr>
<tr>
<td>ARP7</td>
<td>ACTIN RELATED PROTEIN 7</td>
</tr>
<tr>
<td>ARR6</td>
<td>ARABDOPSIS REPONSE REGULATOR 6</td>
</tr>
<tr>
<td>AS1</td>
<td>ASYMMETRIC LEAVES 1</td>
</tr>
<tr>
<td>AS2</td>
<td>ASYMMETRIC LEAVES 2</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>ATP monophosphatase</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bimolecular fluorescence complementation</td>
</tr>
<tr>
<td>BP</td>
<td>insertion of the att-B-containing DNA fragment into the att-P recombination sites in the Gateway donor vector</td>
</tr>
</tbody>
</table>
BR brassinosteroid
BRM BRAHMA
BSH BUSHY
C2H2 Cys2-His2
cDNA complementary DNA
ChIP Chromatin Immunoprecipitation
ChIP-qPCR ChIP assay followed by quantitative PCR
ChIP-seq ChIP assay followed by next generation sequencing
CHR12 CHROMATIN REMODELING 12
CHR23 CHROMATIN REMODELING 23
CLF CURLY LEAF
CO CONSTANS
COLDAIR COLD ASSISTED INTRONIC NONCODING RNA
Co-IP Co-Immunoprecipitation
$C_T$ cycle threshold
CTAB hexadecyltrimethylammonium bromide
DAG days after germination
DBD DNA-binding domain
DMSO Dimethyl sulfoxide
DNA deoxyribonucleic acid

xiv
dNTPs  dinucleotide triphosphates
DTT  Dithiothreitol
EDTA  ethylenediaminetetraacetic acid
EMF2  EMBRYONIC FLOWER2
ERF1  ETHYLENE-RESPONSE-FACTOR1ERF1
Esc  Extra sex combs
ET  ethylene
E(z)  Enhancer of zeste
FDR  false discovery rate
FIE  FERTILIZATION-INDEPENDENT ENDOSPERM
FIS2  FERTILIZATION INDEPENDENT SEED2
FLC  FLOWERING LOCUS C
FLM  FLOWERING LOCUS M
FOXO  forkhead box O
FT  FLOWERING LOCUS T
GA  gibberellin
GA3OX1  GIBBERELLIN 3 BETA-HYDROXYLASE 1
GFP  green fluorescence protein
GO  Gene Ontology
GUS  β-glucuronidase
H2AK119ub1  monoubiquitination of lysine 119 on histone
H3K27me3  trimethylation of lysine 27 on histone H3
HEC1  HECATE 1
IGB  Integrated Genome Browser
JA  jasmonic acid
JmjC  jumonji C
JMJD3  Jumonji D3
JmjN  jumonji N
kb  kilobase
KDM2B  lysine 4- and 36-specific demethylase 2B
KDM4  lysine demethylase 4
LEC2  LEAFY COTYLEDON 2
LHP1  LIKE HETEROCHROMATIN PROTEIN 1
LB  Left border primer of the T-DNA insertion
LP  primer specific to the 5’ flanking sequence of T-DNA insertion
LR  recombination between att-L and att-R sites of Gateway vectors
MDa  mega Dalton
MEA  MEDEA
miRNA  microRNA
mRNA  message RNA
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>Murashige and Skoog</td>
</tr>
<tr>
<td>MSI1–5</td>
<td>MULTICOPY SUPPRESSOR OF IRA1-5</td>
</tr>
<tr>
<td>Oligo (dT)</td>
<td>a short sequence of deoxy-thymine nucleotides</td>
</tr>
<tr>
<td>Pc</td>
<td>Polycomb</td>
</tr>
<tr>
<td>PcG</td>
<td>Polycomb Group</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl</td>
</tr>
<tr>
<td>PH</td>
<td>Polyhomeotic</td>
</tr>
<tr>
<td>PHB</td>
<td>PHABULOSA</td>
</tr>
<tr>
<td>PHO</td>
<td>Pleiohometric</td>
</tr>
<tr>
<td>PHV</td>
<td>PHAVOLUTA</td>
</tr>
<tr>
<td>PKL</td>
<td>PICKLE</td>
</tr>
<tr>
<td>PRC1</td>
<td>Polycomb Repressive Complexes 1</td>
</tr>
<tr>
<td>PRC2</td>
<td>Polycomb Repressive Complexes 2</td>
</tr>
<tr>
<td>PREs</td>
<td>Polycomb response elements</td>
</tr>
<tr>
<td>ProSVP:GUS</td>
<td>SVP promoter-GUS fusion reporter line</td>
</tr>
<tr>
<td>PSC</td>
<td>Posterior Sex Combs</td>
</tr>
<tr>
<td>Poly A</td>
<td>a long sequence of adenine nucleotides</td>
</tr>
<tr>
<td>pH</td>
<td>a measure of the acidity or basicity of an aqueous solution</td>
</tr>
<tr>
<td>qPCR</td>
<td>Real-time quantitative PCR</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcription-PCR</td>
</tr>
<tr>
<td>REF6</td>
<td>RELATIVE OF EARLY FLOWERING 6</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RP</td>
<td>primer specific to the 3’ flanking sequence of the T-DNA insertion</td>
</tr>
<tr>
<td>SA</td>
<td>salicylic acid</td>
</tr>
<tr>
<td>SCL1</td>
<td>SCARECROW-LIKE 1</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Su(z)12</td>
<td>Suppressor of zeste 12</td>
</tr>
<tr>
<td>SYD</td>
<td>SPLAYED</td>
</tr>
<tr>
<td>SOC1</td>
<td>SUPPRESSOR OF OVEREXPRESSION OF CO 1</td>
</tr>
<tr>
<td>SVP</td>
<td>SHORT VEGETATIVE PHASE</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>SWItch/Sucrose NonFermentable</td>
</tr>
<tr>
<td>SWN</td>
<td>SWINGER</td>
</tr>
<tr>
<td>SWP73</td>
<td>SWI/SNF ASSOCIATED PROTEINS 73</td>
</tr>
<tr>
<td>TAIR</td>
<td>The Arabidopsis Information Resource</td>
</tr>
<tr>
<td>T-DNA</td>
<td>transfer DNA</td>
</tr>
<tr>
<td>TFL2</td>
<td>TERMINAL FLOWER2</td>
</tr>
<tr>
<td>TrxG</td>
<td>Trithorax group</td>
</tr>
<tr>
<td>TSS</td>
<td>transcription start site</td>
</tr>
<tr>
<td>TTS</td>
<td>transcription termination site</td>
</tr>
</tbody>
</table>
ULT1  ULTRAPETALA1
UTX  tetratricopeptide repeat X
VRN2  VERNALIZATION2
WIG  Wiggle track format
WRKY23  WRKY DNA-BINDING PROTEIN 23
YFP  yellow fluorescence protein
ZnF  zinc finger
35S  cauliflower mosaic virus 35S RNA promoter
×g  times gravity
%  percent
β-ME  2-Mercaptoethanol

All numerical units included in this thesis are standard SI units. Arabidopsis gene names are written in italics and capital letters when referring to wild-type and in small letters if mutated. Protein names are written in capital letters.
Chapter 1 Introduction

1.1 Chromatin-Mediated Control of Gene Expression

In eukaryotic cells, DNA is packaged in a dynamic polymer called chromatin, whose basic repeating unit, the nucleosome, consists of around 146 nucleotides of DNA wrapped twice around an octamer composed of two copies of four canonical histones: H2A, H2B, H3 and H4 (Luger et al., 1997). Chromatin provides not only space to store large amounts of genetic material but also a means to package the same genetic material into different chromatin states. Histones are small basic proteins consisting of a global domain and an N-terminus (termed “histone tail”) that “sticks out” from the nucleosome. Specific amino acids in the histone tail are subject to at least eight different types of posttranslational modifications, including methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, and proline isomerization, which are catalyzed by a variety of chromatin-associated enzymes (Kouzarides, 2007). While genetic information is encoded in DNA, these chromatin modifications provide an extra layer of information. They affect the interaction between DNA and histones to alter chromatin structure, thereby leading to changes of gene transcription (Berger, 2002; Bannister and Kouzarides, 2011). Depending on the type of modifications and the amino acid being modified, these histone modifications have either a positive or a negative effect on gene expression. For example, histone acetylation is usually associated with transcriptionally highly active genes. Methylation on histone H3 lysine 9, or lysine 27 is linked to transcriptionally silenced genes, while, methylation on histone H3 lysine 4, or lysine 36 is normally found on transcriptionally active genes (Berger, 2007; Kouzarides, 2007). The fact that each histone tail can be simultaneously modified at multiple amino acid sites with different types of modifications led to the “histone code” hypothesis (Strahl and Allis, 2000; Jenuwein and Allis, 2001). This theory proposes that distinct histone modifications can work synergistically or antagonistically and that a specific combination of histone modifications defines a unique chromatin environment or state that provides a signal for the recruitment of transcriptional machinery, including specific transcription factors, consequently leading to changes in gene transcription.
Packaging of the eukaryotic genome into chromatin poses a barrier to many cellular processes that require access to DNA, such as DNA replication and gene transcription. Chromatin-mediated control of gene transcription is mainly achieved by chromatin remodelers and covalent histone-modifying enzymes (Narlikar et al., 2002; Goldberg et al., 2007; Li et al., 2007; Bannister and Kouzarides, 2011). Chromatin remodelers regulate gene transcription by controlling the accessibility of DNA to transcriptional machineries through alteration of nucleosome position, conformation and composition (Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011). Histone-modifying enzymes, on the other hand, add or remove chemical or protein modifications to histones, which serve as signals or tags for the recruitment of non-histone proteins (Kouzarides, 2007; Zentner and Henikoff, 2013). One of the major challenges in this field is to fully elucidate the functional crosstalk between chromatin remodeling machineries and histone modifiers.

### 1.2 Polycomb Group Proteins

Polycomb Group (PcG) proteins are one of the histone modifiers that maintain the repressed state of genes in cells/tissues where the genes should be inactive (Schwartz and Pirrotta, 2007; Simon and Kingston, 2009; Margueron and Reinberg, 2011; Simon and Kingston, 2013). First discovered in *Drosophila melanogaster* as regulators of homeotic gene expression (a group of genes required for the proper body segmentation in *Drosophila*), PcG proteins are found in many eukaryotic organisms and represent a conserved system of long-term gene inactivation. It has been well accepted that PcG proteins repress gene transcription through a two-step mechanism involved in two multi-protein complexes known as Polycomb Repressive Complexes 1 (PRC1) and 2 (PRC2) (Figure 1A). The *Drosophila* PRC2 complex, which contains four core components, Enhancer of zeste [E(z)], Extra sex combs (Esc), Suppressor of zeste 12 [Su(z)12], and Nucleosome remodeling factor 55 (Nurf55), catalyzes the trimethylation at lysine 27 on histone H3 (H3K27me3) (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002). Subsequently, PRC1, which also consists of four core subunits,
Polycomb (Pc), Posterior Sex Combs (PSC), Polyhomeotic (PH), and dRING, recognizes and binds to histones that are marked by H3K27me3. Upon binding to H3K27me3 marked chromatin, PRC1 catalyzes monoubiquitination at lysine 119 on histone H2A (H2AK119ub1) and results in the compaction of chromatin and gene silencing (Shao et al., 1999; Fischle et al., 2003; Min et al., 2003; Eskeland et al., 2010) (Figure 1A). Although this classical, hierarchical model for the recruitment of PcG proteins to target genes has been widely accepted, three recently published studies are challenging this model (Blackledge et al., 2014; Cooper et al., 2014; Kalb et al., 2014). In their newly proposed model (Figure 1B), PRC1 complexes are classified into canonical and variant ones based on mutually exclusive subunits, as well as the ability to recognize the H3K27me3 mark and catalyze H2AK119ub1. The canonical PRC1, although poor in catalyzing H2AK119ub1, is good at recognizing H3K27me3 mark. The variant PRC1, on the other hand, can catalyze H2AK119ub1, but is unable to recognize H3K27me3. The variant PRC1 complex is recruited to target chromatin by lysine 4- and 36-specific demethylase 2B (KDM2B), a CXXC-type zinc finger (ZnF) containing protein that is able to directly bind to DNA. This leads to the deposition of H2AK119ub1 at target sites, which in turn facilitates the recruitment of PRC2. H2AK119ub1 stimulates the ability of PRC2 in catalyzing H3K27me3. Finally, H3K27me3, conferred by PRC2, recruits the canonical PRC1 (Figure 1B).

The composition and function of PRC2 are conserved between animals and plants (Hennig and Derkacheva, 2009; Bemer and Grossniklaus, 2012; Holec and Berger, 2012). In the model plant Arabidopsis thaliana, most of the PRC2 members are encoded by small genes families. Three E(z) orthologs [MEDEA (MEA), CURLY LEAF (CLF) and SWINGER (SWN)], three Su(z)12 homologues [(FERTILIZATION INDEPENDENT SEED2 (FIS2), VERNALIZATION2 (VRN2) and EMBRYONIC FLOWER2 (EMF2)] and five Nurf55 homologues [(MULTICOPY SUPPRESSOR OF IRA1-5 (MSI1–5)] of PRC2 complex have been identified in Arabidopsis (Pien and Grossniklaus, 2007; Hennig and Derkacheva, 2009). To date, three distinct PRC2 complexes have been described in Arabidopsis. The PRC2 complex containing MEA, FIS2, MSI1 and FERTILIZATION INDEPENDENT ENDOSPERM (FIE) (termed “MEA-FIS2-MSI1-FIE”) is thought to be required during seed development.
Figure 1: Two Models for Recruitment of Polycomb Group Complexes to Target Genes

(A) Classical hierarchical model for the recruitment of PcG complexes. Step 1: PRC2 is recruited to target chromatin through a recruiter, such as a DNA-binding protein or a non-coding RNA. PRC2 catalyzes the deposition of H3K27me3 (red star). Step 2: PRC1 recognizes and binds to H3K27me3, thus leading to the recruitment of this complex which then catalyzes H2AK119ub1 (blue hexagon).

(B) The new hierarchical model for the recruitment of PcG complexes. Step 1: Variant PRC1 is recruited to target chromatin through a recruiter, such as KDM2B or others. This leads to H2AK119ub1 (blue hexagon). Step 2: H2AK119ub1 directly or indirectly recruits PRC2 that catalyzes H3K27me3 (red star). Step 3: Canonical PRC1 recognizes and binds to H3K27me3, thus leading to the recruitment of this complex.

Figures were drawn based on the information from Comet and Helin (2014).
(Makarevich et al., 2006). The other two PRC2 complexes, CLF/SWN-EMF2-MSI1-FIE and CLF/SWN-VRN2-MSI-FIE, function in vegetative and floral development, respectively (Köhler et al., 2003; De Lucia et al., 2008). CLF and SWN are two H3K27 methyltransferases that are broadly expressed and play partially redundant roles in vegetative and reproductive development (Chanvivattana et al., 2004).

Although protein sequences comparison did not suggest orthologs of PRC1 subunits in plants, recent work has identified a functional counterpart of the Drosophila PRC1 complex in Arabidopsis (Zheng and Chen, 2011). Similar to the Pc subunit in the animal PRC1 complex, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), also called TERMINAL FLOWER2 (TFL2), binds to H3K27me3 and co-localizes with genes marked by H3K27me3 throughout the genome. Thus, it is thought that LHP1 fulfills the function of the Pc subunit of the animal PRC1 and exists in the putative plant PRC1 complex (Turck et al., 2007; Zhang et al., 2007a). In addition, RING homologs AtRING1A and AtRING1B and RING-finger homologs AtBMI1A and AtBMI1B have also been recently suggested as components of a plant PRC1 (Xu and Shen, 2008; Bratzel et al., 2010). Taken together, plant PRC1 complex probably consists of LHP1, AtRING1A/AtRING1B, and AtBMI1A/AtBMI1B.

Similar to animals, several thousands of Arabidopsis genes were reported to carry the H3K27me3 mark in young seedlings (Zhang et al., 2007b; Bouyer et al., 2011; Lafos et al., 2011; Lu et al., 2011). A fraction of PcG target genes was found to carry the H3K27me3 mark specifically in either the shoot apical meristem or leaf cells (Lafos et al., 2011), suggesting that PcG proteins can repress different genes in different cell types/tissues. What mechanisms guide the binding of PcG proteins to their target genes? Since none of the core components of PRC1 and PRC2 possess a DNA binding domain (Margueron and Reinberg, 2011), it is believed that targeting of PcG proteins must be specified elsewhere (Margueron and Reinberg, 2011; Bemer and Grossniklaus, 2012; Simon and Kingston, 2013). In Drosophila, the zinc finger protein Pleiohometic (PHO) was suggested to play a role in targeting PcG complexes to DNA (Mohd-Sarip et al., 2002). PHO is a sequence-specific DNA binding protein, which can also interact with both PRC1 and PRC2 (Wang et al., 2004). Analysis of the regulatory regions of several
PcG target genes allowed the identification of specific DNA elements called Polycomb response elements (PREs), which contain recognition sequences for recruiting PcG proteins in *Drosophila* (Simon and Kingston, 2009). However, no such PREs have been identified in plants (Zheng and Chen, 2011). Long non-coding RNAs (non-protein coding transcripts that are longer than 200 nucleotides) were also demonstrated as recruiters of PcG protein in animals, where these non-coding transcripts interact with PRC2 and are required for PcG-mediated silencing of target loci (Margueron and Reinberg, 2011). In *Arabidopsis*, COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR), a long non-coding RNA, physically associates with a component of PRC2 and recruits PRC2 to a floral repressor gene, *FLOWERING LOCUS C (FLC)* (Heo and Sung, 2011). In addition, it was reported recently that transcription factors also participate in recruitment of plant PcG proteins. In a study by Lodha et al. (2013), it was shown that the ASYMMETRIC LEAVES 1 (AS1) and 2 (AS2), two MYC-domain DNA binding transcription factors, physically interact with PRC2 and recruit it to target genes in *Arabidopsis* (Lodha et al., 2013).

### 1.3 RELATIVE OF EARLY FLOWERING 6, an Enzyme that Removes H3K27me3

As discussed above, H3K27me3 is associated with transcriptionally inactive chromatin, as established by the H3K27 methyltransferase in the PRC2 complex. The tri-methyl group of H3K27 can also be removed so that the repressed gene can be turned on. The enzyme involved in removing methyl groups from H3K27 is called H3K27 demethylase. In 2007, several groups identified ubiquitously transcribed tetratricopeptide repeat X (UTX) and Jumonji D3 (JMJD3) as two H3K27 demethylases in animals that catalyze the removal of tri-methyl groups from H3K27, thereby promoting expression of their target genes (Shi, 2007; Agger et al., 2008; Cloos et al., 2008; Simon and Kingston, 2013; Van der Meulen et al., 2014). The ability of removing tri-methyl groups is dependent on the catalytic Jumonji C (JmjC) domain, which contains conserved amino acids required for the binding of co-factors, such as iron and α-ketoglutarate. In order to activate gene
Figure 2: REF6 Mediates H3K27 Demethylation

Open and closed chromatin states can be facilitated by histone demethylase REF6 and histone methyltransferase PRC2 in Arabidopsis, respectively. REF6 erases methyl groups on H3K27, thus enabling gene transcription; while, PRC2 introduces methyl groups on H3K27, which leads to inhibition of gene transcription. Red stars: H3K27me3.

Figure was drawn based on information from Lu et al. (2011).
Open Chromatin

Closed Chromatin

PcG

REF6
expression, H3K27 demethylase UTX also interacts with a protein complex containing a H3K4 methyltransferse, which catalyzes the H3K4me3, a histone modification that is associated with transcriptionally active genes (Cho et al., 2007; Issaeva et al., 2007; Lee et al., 2007b). Furthermore, UTX was shown to be associated with several proteins that are involved in transcriptional elongation (Chen et al., 2012; Wang et al., 2012), thus fine-tuning gene expression. The H3K27 demethylases are evolutionarily conserved from roundworms (Caenorhabditis elegans) to humans (Homo sapiens) (Van der Meulen et al., 2014). However, a phylogenetic analysis did not suggest orthologs of UTX or JMJD3 in plants (Lu et al., 2008). Interestingly, RELATIVE OF EARLY FLOWERING 6 (REF6), initially considered as an ortholog of the metazoan lysine demethylase 4 (KDM4), a H3K9 demethylase, was depicted as a plant H3K27 demethylase (Lu et al., 2011) (Figure 2). It was shown that loss of REF6 caused increased H3K27me3 at hundreds of genomic loci, while plants overexpressing REF6 phenotypically resembled the PcG mutants (Lu et al., 2011). Similar to UTX and JMJD3 in animals, the REF6 contains a Jumonji N (JmjN) domain and a JmjC domain, which are responsible for the demethylase catalytic activity. Unlike animal H3K27 demethylases, which have no DNA-binding domain, the C-terminal part of Arabidopsis REF6 has four Cys2-His2 (C2H2) zinc finger domains (Lu et al., 2008). Zinc fingers are putative DNA binding domains; thus REF6 might have the ability to directly bind to a specific DNA sequence. These previous observations indicate the uniqueness of REF6 as a plant-specific histone H3K27 demethylase. Unlike the severe phenotypes of the UTX mutants of mice (Shpargel et al., 2012), C.elegans (Vandamme et al., 2012) and zebrafish (Lan et al., 2007), an impairment of REF6 causes relatively mild phenotypes (Noh et al., 2004; Yu et al., 2008), suggesting there might be a redundant H3K27 demethylase(s) in Arabidopsis.

1.4 The Chromatin Remodeling ATPase BRAHMA

As mentioned above, DNAs packaged in chromatin must be accessible for critical cellular processes such as transcription. ATP dependent chromatin-remodeling protein complexes are thought to utilize energy from ATP hydrolysis to mobilize, disrupt or
change nucleosomes in order to create an open chromatin structure for the access of transcription factors or other regulators (Saha et al., 2006; Clapier and Cairns, 2009). There are at least four families of ATP-dependent chromatin remodelers in eukaryotes: SWItch/Sucrose NonFermentable (SWI/SNF), Imitation Switch (ISWI), Nucleosome Remodeling Deacetylase (NURD), Inositol Requiring 80 (INO80) (Jerzmanowski, 2007; Clapier and Cairns, 2009). The ATPases in these remodelers have similar affinity to the nucleosome and display DNA- and nucleosome-dependent ATPase activity in vitro (Laurent et al., 1993). However, most chromatin remodelers contain multiple subunits, which can alter the activity of the core ATPase in vivo by (1) directly regulating the enzymatic activity of the complex, (2) facilitating the binding of the complex to transcription factors and other chromatin modifying enzymes, or (3) by guiding the complex to DNA (Hargreaves and Crabtree, 2011).

The SWI/SNF chromatin remodeling complex was originally identified in two independent genetic screens in *Saccharomyces cerevisiae* (Neigeborn and Carlson, 1984; Peterson and Herskowitz, 1992). This complex contains several subunits that are encoded by genes required for the mating-type switching and growth on sucrose, and therefore was named SWI (for “switch”) or SNF (for “sucrose nonterminating”) (Hargreaves and Crabtree, 2011). Yeast SWI/SNF is a 1.14 MDa complex of at least eight subunits, where SWI2/SNF2 subunit is the ATPase. SWI/SNF complex is evolutionarily conserved, and homologous proteins were identified in flies, mammals and plants (Jerzmanowski, 2007). The *Drosophila* SWI2/SNF2 ATPase homolog, BRM, was initially classified as a Trithorax group (TrxG) protein because it activates the transcription of homeotic genes and thus plays a role in antagonizing the function of PcG during fly development (Tamkun et al., 1992; Hargreaves and Crabtree, 2011). This is also the case in yeast where the SWI/SNF complex binds almost exclusively to promoters and activates the transcription of its direct target genes. However, recent studies in mammals and plants indicate that this complex is often found in intergenic regions where it both activates and represses gene transcription through increasing and decreasing accessibility of target DNA, respectively (Hargreaves and Crabtree, 2011; Ho et al., 2011; Han et al., 2012). Therefore, its role in regulation of gene transcription is not well understood and may
differ in different organisms. Moreover, the mechanism that governs the genomic targeting of the SWI/SNF complex remains a puzzle.

Although the biochemical activities of plant SWI/SNF complexes have not been examined, progress has been made to identify them through genetic and molecular analyses (Hurtado et al., 2006; Bezhani et al., 2007; Jerzmanowski, 2007; Archacki et al., 2013). Protein sequence comparison has predicted the existence of a (or possibly several) plant SWI/SNF complex. In Arabidopsis, four SWI2/SNF2 ATPase [BRM, SPLAYED (SYD), CHROMATIN REMODELING 12 (CHR12) and 23 (CHR23)]; four SWI3 proteins (SWI3A to SWI3D); two SWI/SNF ASSOCIATED PROTEINS 73 (SWP73A and SWP73B); two ACTIN RELATED PROTEINS (ARP4 and ARP7); and a single SNF5 subunit termed BUSHY (BSH) were predicted subunits of SWI/SNF complexes (Jerzmanowski, 2007). As documented in the in vitro protein-protein interaction experiments, these proteins can form SWN/SNF complexes, which was further supported by the purification of plant SWI/SNF complexes (Vercruyssen et al., 2014).

Arabidopsis SWI2/SNF2 ATPase BRM plays crucial roles in many aspects of plant development (Kwon et al., 2006; Jerzmanowski, 2007; Tang et al., 2008; Han et al., 2012; Wu et al., 2012; Zhu et al., 2012). brm single mutants show pleiotropic phenotypes, such as reduced plant architecture (Farrona et al., 2004; Hurtado et al., 2006), downward curling of leaves (Hurtado et al., 2006; Tang et al., 2008), mild floral homeotic defects (Hurtado et al., 2006; Wu et al., 2012), hypersensitivity to abscisic acid (ABA) (Han et al., 2012) and early flowering (Farrona et al., 2004; Tang et al., 2008; Farrona et al., 2011). The molecular mechanisms underlying these phenotypes of brm mutants are still far from clear. It was thought that BRM regulates plant growth and development mainly by directly regulating the expression of key genes involved in each specific pathway responsible for the observed phenotypes. The brm mutants show an early flowering phenotype as a result of an increase in the expression of the flowering integrators FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CO I (SOC1) (Farrona et al., 2011). These data suggest that BRM represses their expression. However, BRM does not directly bind to these loci, suggesting that BRM might indirectly repress their transcription by directly activating the expression of an upstream
repressor of \( FT \) and \( SOCI \). Alternatively, BRM might also directly repress the expression of an upstream activator of \( FT \) and \( SOCI \). No matter which mechanism, the precise roles of BRM in flowering time control remain unclear and therefore warrants further investigation.

1.5 SHORT VEGETATIVE PHASE, a Key Flowering Repressor

Plant development takes place in distinct phases, each of which is characterized by the activation of a particular set of genes and the repression of others. The precise control of gene expression in each phase is crucial for proper growth and development. Plants are sessile organisms that cannot move to optimal locations when the surrounding environment is not favorable. Therefore, they have developed strategies to adapt to the ever changing environment, enabling them to increase their chance to survive and reproduce. The transition from the vegetative to reproductive phase (termed “the floral transition”) is a key step for a successful life cycle of flowering plants. If the floral transition starts under unfavorable conditions, seed production will be heavily affected. Thus, the floral transition must be controlled precisely in response to environmental (e.g. temperature, day length, and light-quality) as well as endogenous signals (e.g. aging) (Mouradov et al., 2002; Boss et al., 2004; Andrés and Coupland, 2012; He, 2012).

In \textit{Arabidopsis}, a repressor complex that consists of two MADS-box transcription factors, FLC and SHORT VEGETATIVE PHASE (SVP), serves as a negative regulator of flowering time by directly binding to and repressing the expression of the floral pathway integrators \( FT \) and \( SOCI \) (Lee et al., 2007a; Li et al., 2008; He, 2012). Consistent with its role as a floral repressor, \( SVP \) is highly expressed during the vegetative phase (Hartmann et al., 2000; Li et al., 2008), but is down-regulated during the floral transition (Li et al., 2008), which results in the de-repression of \( FT \) and \( SOCI \) to promote flowering. In addition, \( SVP \) was also shown to repress floral transition by indirectly repressing the biosynthesis of plant growth hormone gibberellin (GA), a hormone that has been shown to induce flowering (Andrés et al., 2014). A recent study showed that the association of \( SVP \) with the \( FT \) locus is controlled by two alternative splice forms of \textit{FLOWERING}
Figure 3: Model Showing the Repression of SVP Floral Transition in Response to Temperature

SVP and FLC form a repressor complex that occupies the promoter of floral integrators $FT$ and $SOC1$. FLM$\beta$, a splice variant of FLM, which is produced mainly in lower temperatures, i.e. 16°C, interacts with SVP-FLC complex and enhances its binding ability to the promoter of $FT$ and $SOC1$. FLM$\beta$-SVP-FLC complex represses $FT$ and $SOC1$, thus prevents flowering transition. Upon higher temperatures, i.e. 22°C, the abundance of FLM$\beta$ decreases through an unknown mechanism, while more of the FLM$\delta$ splice form is produced. FLM$\delta$ competitively interacts with SVP-FLC. However, the FLM$\delta$-SVP-FLC complex is impaired in DNA binding ability, and thus unable to repress the promoter activity of $FT$ and $SOC1$. The increased expression of $FT$ and $SOC1$ leads to floral transition.

Figure was drawn based on the information in Posé et al. (2013)
**LOCUS M (FLM):** FLMβ and FLMδ (Figure 3, Posé et al., 2013). Lower ambient temperatures induce production of the FLMβ form, whereas more of FLMδ is produced at higher ambient temperatures (Balasubramanian et al., 2006). It was shown that the FLMβ and FLMδ competitively interact with SVP. Thus, at lower temperature, the FLMβ-SVP complex is dominant over the FLMδ-SVP complex. The FLMβ-SVP complex has the ability to bind to the promoter of *FT* and repress its transcription, resulting in the repression of the floral transition. At higher temperature, more of the FLMδ-SVP complex is formed. This complex is impaired in DNA binding ability and thus cannot bind to and repress *FT*, which in turn, leads to the floral transition (Posé et al., 2013). A model for the action of SVP is summarized in Figure 3. In addition, a study reported that the abundance of the SVP protein, but not the level of the SVP transcript, gradually decreases when the temperature is increased (Lee et al., 2013). These data suggest that SVP has been subjected to a posttranslational regulation mechanism in response to changes in temperature. Due to its key role in controlling flowering time, efforts have been made to obtain insights into how SVP regulates the floral transition; nevertheless, how the expression of *SVP* is regulated at the transcriptional level remains completely unknown. Particularly, no positive regulator(s) of *SVP* expression in the vegetative phase have been identified.

### 1.6 Thesis Objectives

Compared to rather extensive data on elucidating the mechanisms that govern the recruitment of PcG proteins, much less is known about mechanisms by which these proteins are prevented from binding to specific loci at a particular developmental stage. Although the roles of PcG proteins and BRM during plant development have been investigated, how their activities are coordinated is still poorly understood. Interestingly, a recent report in *Arabidopsis* showed that loss of the BRM activity led to the increased levels of H3K27me3 at two floral homeotic genes during floral development (Wu et al., 2012), suggesting an antagonistic relationship between BRM and PcG. However, the current model is solely based on the characterization of a few identified target genes of
BRM, thus it remains unknown to what extent BRM is required to antagonize the PcG function in plants. Other questions include, but are not limited to, the precise mechanism by which BRM antagonizes PcG activity during plant development and whether or not plant BRM might work synergistically with PcG proteins. On another front, both BRM and REF6 are thought to play roles in activating gene expression at the chromatin level. However, the genome-wide binding sites of BRM and REF6 have not been characterized, which hinder the functional studies of these important epigenetic regulators. Whether and how their activities are coordinated have not been addressed thus far.

To address these questions, the following research hypotheses guide my thesis work:

(1) In *Arabidopsis*, BRM activates gene expression by preventing the binding of PcG proteins.

(2) BRM and REF6 act interdependently to co-activate gene expression.

In order to test these hypotheses, the following objectives were set for my thesis work:

(1) To perform a genome-wide comparison of H3K27me3 profiles between wild-type *Arabidopsis* and the corresponding *brm* mutant using the ChIP-seq approach.

(2) To associate genes at which changes in H3K27me3 occur with the various *brm* phenotypes.

(3) To identify and compare genome-wide targets of BRM and REF6.

(4) To investigate whether associations of BRM and REF6 with chromatin are mutually dependent.

Addressing these and other related issues should help build a clearer picture with regard to the relationship between PcG, BRM and REF6 and how this affects the functioning of specific genes and pathways during growth and development of plants.
Chapter 2 Materials and Methods

2.1 Plant Material and Growth Conditions

*Arabidopsis* seeds were stratified at 4°C for 4 days in dark condition and then sown on soil. Alternatively, seeds were first grown on Murashige and Skoog (MS) plates (4.3 g/l Murashige and Skoog nutrient mix (Sigma-Aldrich), 1.5% sucrose, and 0.8% agar with pH 5.8) for 2 weeks and then transferred to soil. Before sowing to MS plate, seeds were surface-sterilized by gentle shaking for 10 min in 20% bleach 0.1% sodium dodecyl sulfate (SDS), followed by 5 washes with sterilized water. Plants were grown in growth rooms with 16h light/8h dark cycles (long-day) at 22°C or 16°C. T-DNA insertion mutants were obtained from the *Arabidopsis* Biological Resource Center (ABRC). The brm-1 (SALK_030046), brm-5, clf-29 (SALK_021003), tf12-1 (CS3796), svp-31 (SALK_026551) and ref6-1 (SALK_001018) mutants are all in the Columbia (Col) wild-type background and have been previously described (Larsson et al., 1998; Noh et al., 2004; Hurtado et al., 2006; Schönrock et al., 2006; Lee et al., 2007a; Tang et al., 2008). Homozygous T-DNA insertion mutants were identified by PCR-based genotyping in section 2.4. Transgenic seeds ProBRM:BRM-GFP (Smaczniaik et al., 2012) were kindly provided by Dr. Kerstein Kaufmann at Wageningen University, Pro35S:SVP by Dr. Jeong Hwan Lee at Korea University, ProSVP:GUS (Li et al., 2008) by Dr. Hao Yu at National University of Singapore, Pro35S:GFP-CLF and Pro35S:GFP (Masiero et al., 2004) by Dr. Xuemei Chen at the University of California, Riverside.

*Nicotiana benthamiana* plants were grown in soil using the same growth conditions as those for *Arabidopsis* plants.

2.2 Plant Genomic DNA Isolation

Leaf material (~30 mg) was ground in liquid nitrogen into fine powder and transferred into a 2 ml Eppendorf tube containing 300 µl of hexadecyltrimethylammonium bromide (CTAB) solution (0.1 M Tris-HCl pH 8, 1.4 M NaCl, 20 mM ethylenediaminetetraacetic
acid (EDTA) pH 8, 2% CTAB). The resulting leaf tissue mixture was mixed briefly by vortexing and 300 µl of chloroform were then added. The tube was vortex for 20 sec and centrifuged at 11,000 ×g for 10 min. The upper, aqueous phase (~250 µl) was transferred to a new tube and 250 µl of isopropanol was added. Gentle mixing was done by continually inverting the tube for 1 min, followed by centrifugation for 10 min at 14,000 ×g. A small white pellet containing DNA should be visible at the bottom of the tube. The aqueous phase was discarded and 300 µl of 70% ethanol was added to wash the white pellet. After centrifuging for 5 min at 14,000 ×g, the ethanol was discarded. The remaining ethanol was allowed to evaporate by incubating the opened tube for 10 min under vacuum at room temperature. The pellet was re-suspended in 40 µl of ddH2O.

2.3 General Polymerase Chain Reaction Setup

For polymerase chain reaction (PCR), the following components were added in 0.2 ml tubes: 0.5 µl of 10 µM each of forward and reverse primer, 2 µl of 10 x PCR buffer (GenScript Inc.), 2 µl of 25 mM dinucleotide triphosphates (dNTPs), 1 unit of Taq polymerase (GenScript Inc.), 2 µl of DNA template and H2O was added to a final volume of 20 µl. The mixture was then loaded onto a thermocycler, initialized for 5 min at 94°C, followed by 30-40 cycles of 94°C for 30 sec, 55-60°C for 30 sec and 72°C for 1 min, followed by a final extension phase of 72°C for 5 min.

2.4 Genotyping

To identify the T-DNA mutant line, a PCR-based genotyping method was used. There are three primers used in this method: LB (primer specific to the left border of the T-DNA), LP (primer specific to the 5’ flanking sequence of T-DNA insertion), and RP (primer specific to the 3’ flanking sequence of the T-DNA insertion). Two paired-PCR reactions were performed: LP+RP and LB+RP. For wild-type plants (no T-DNA insertion), a single PCR product should be produced in the LP+RP reaction, but not in the LB+RP
reaction. For Homozygous T-DNA insertion plants, a single PCR product should be produced in the LB+RP reaction, but not in the LP+RP reaction. For heterozygous T-DNA insertion plants, a PCR product should be produced in both LP+RP and LB+RP reaction. The primers used for genotyping T-DNA insertion line were designed from http://signal.salk.edu/tdnaprimers.2.html, and are listed in Appendix A.

2.5 Generating Stable Transgenic Plants

A REF6 genomic region including a 2 kb promoter without a stop codon was amplified by PCR reaction and subcloned into the pDONR221 vector (Invitrogen) by BP reaction according to the manufacturer’s instructions. The resulting transgene in the entry vector was sequenced to make sure that no sequence error was introduced during PCR. The transgene was then transferred into pMDC107 vector (Curtis and Grossniklaus, 2003) by LR reaction, according to the manufacturer’s instructions, to make the ProREF6:REF6-GFP. The construct was introduced into Agrobacterium tumefaciens (strain GV3101) which was then transformed into ref6-1 mutant plants using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected in MS media containing 50 µg/ml of hygromycin B and allowed to grow in soil to maturity to yield seeds. A line showing phenotypic complementation was chosen for further analysis.

The XVE::aMIRBRM construct was made by Chen Chen in Cui’s lab. I transferred the vector into Arabidopsis and performed all the downstream experiments. Briefly, the pRS300 vector (Schwab et al., 2006) was used as the backbone to first generate aMIRBRM. The primers used were designed according to Web MicroRNA Designer (WMD3) (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) and are listed in Appendix A. aMIRBRM was subcloned into the pDONR221 vector (Invitrogen), confirmed by sequencing, and then recombined into the pMDC7 vector (Curtis and Grossniklaus, 2003) where the aMIRBRM transgene is controlled by an estradiol-inducible promoter (Zuo et al., 2000). The construct was transformed into Col wild-type plants by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected for hygromycin B resistance and allowed to grow to maturity to yield seeds. Seven-day-old T2 transgenic
plants were treated either by 10 µM β-estradiol (to induce the activity of the promoter that controls aMIRBRM transgene) or dimethyl sulfoxide (DMSO) (as a mock control). Samples were collected at different time points after the treatment.

2.6 Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) was carried out as described (Gendrel et al., 2005; Li et al., 2009) with minor modifications. Briefly, two grams of 14-day-old seedlings grown on MS plates were harvested and cross-linked with 35 ml of 1% formaldehyde for 20 min under vacuum. Glycine was added to a final concentration of 0.125 M. The seedlings were then rinsed two times with water and ground in liquid nitrogen into a fine powder. 30 ml of extraction buffer 1 (0.4 M sucrose, 10 mM Tris-HCl pH 8, 10 mM MgCl$_2$, 5 mM β-ME (2-mercaptoethanol), 0.1 mM phenylmethylsulfonyl (PMSF), 2 tablets of complete protease inhibitor cocktail tablets (Roche) in 100 ml H$_2$O) were added to the samples which was then kept on ice for 5 min. The solution was filtered through a double layer of miracloth (Calbiochem) and then centrifuged for 20 min at 3,000 ×g at 4°C. The supernatant was discarded and the pellet was resuspended in 1 ml of extraction buffer 2 (0.25 M sucrose, 10 mM Tris-HCl pH 8, 10 mM MgCl$_2$, 1% Triton X-100, 5 mM β-ME, 0.1 mM PMSF, 1 tablet of complete mini protease inhibitor cocktail tablet (Roche) in 10 ml H$_2$O), centrifuged for 10 min at 12,000 ×g at 4°C. The supernatant was discarded and the pellet was gently resuspended in 300 µl of extraction buffer 3 (1.7 M sucrose, 10 mM Tris-HCl pH 8, 0.15% Triton X-100, 2 mM MgCl$_2$, 5 mM β-ME, 0.1 mM PMSF, 1 tablet of complete mini protease inhibitor cocktail tablet in 10 ml H$_2$O). Load resuspended pellet over another 300 µl of extraction buffer 3 and spun for 1 hr at 16,000 ×g at 4°C. The supernatant was discarded and the pellet was resuspended, by pipetting, in 300 µl of nuclei lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS, 0.1 mM PMSF, 1 tablet of complete mini protease inhibitor cocktail tablet in 10 ml H$_2$O). The solution was sonicated for three times, 15 sec each, with a 1 min incubation on ice between each sonication treatment. The solution was centrifuged for 10 min at 4°C to pellet the debris. About 300 µl of supernatant was transferred to a
new tube. Ten µl from each sample was taken for the input DNA control and stored at -20 °C. The sheared DNA fragments were 100 bp to 1,000 bp in length. The supernatant was made up to 3 ml with ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8, 167 mM NaCl). The sonicated chromatin was pre-cleared with 40 µl of protein A agarose beads for 1 hr at 4°C. Centrifuge for 1 min at 4°C, and transfer the supernatant into new tube and followed by incubation with 10 µl antibodies overnight at 4°C. The antibodies used in this study were anti-GFP (Ab290, Abcam), anti-H3K27me3 (07-449, Millipore), and anti-H3 (Ab1791, Abcam). After incubation, 50 µl of protein A agarose beads were added and incubated with rotating for another 2 hrs at 4°C. Beads were sequentially washed in the following 1 ml of buffers: (1) Low salt wash buffer; 150 mM NaCl, 0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) for 10 min. (2) High salt wash buffer; 500 mM NaCl, 0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) for 10 min. (3) LiCl wash buffer; 0.25 LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1) for 10 min. (4) TE buffer; 10 mM Tris-HCl (pH 8.1), 1 mM EDTA for 10 min. Immune complexes were eluted by adding 250 µl of elution buffer (1% SDS, 0.1 M NaHCO₃), followed by incubation for 15 min at 65°C with gentle agitation. This step was repeated and the supernatants were combined. Then, 20 µl of 5 M NaCl was added and incubated for 6 hrs at 65°C to reverse cross-link DNA to histones and other chromatin components. The input DNA control was made up to 500 µl with elution buffer and 20 µl of 5 M NaCl and incubated with the samples for 6 hrs at 65°C. Then, 10 µl of 0.5 M EDTA, 20 µl of 1 M Tris-HCl (pH 6.5) and 2 µl of 10 mg/ml proteinase K were added to the samples followed by 1 hr incubation at 45°C. To precipitate the DNA, 1 ml of ethanol, 50 µl of 0.3 M sodium acetate (pH 5.2) and 1 µl glycogen (20 mg/ml) were added. The sample was stored at -20 °C for 12 hrs and then centrifuged at 20,000 ×g for 20 min. The DNA was then recovered with the MiniElute PCR Purification Kit (Cat#28004, Qiagen) according to the manufacturer’s instructions.

The ChIP assay followed by quantitative PCR (ChIP-qPCR) was performed using the SsoFast™ EvaGreen® Supermix kit (Bio-Rad). All qPCR’s reactions were performed with the same cycling parameters: an initial phase of 98°C for 2 min, the cycling condition consisting of 40 cycles of 98°C for 5 sec and 60°C for 10 sec. The reactions
then entered melting curve analysis which began at 65°C and was increased incrementally by 0.5°C until a final temperature of 95°C was reached. Bio-Rad CFX96 real-time PCR detection system and CFX Manager™ Software (Bio-Rad) were used to detect and record fluorophore activity and determine the cycle threshold (C_T) value. Results were shown as percentage of input DNA using the formula $2^{-\Delta C_T} = 2^{-\left[C_{T\text{(ChIP)}}-C_{T\text{(input)}}\right]}$ according to the Champion ChIP-qPCR user manual (SABioscience). Three independent biological ChIP experiments were performed. Primer sequences used for ChIP-qPCR are listed in Appendix A.

2.7 ChIP-seq and Data Analysis

DNA from five ChIP assays was pooled to obtain 10 ng of DNA. Two biological replicates were prepared and sequenced. Next generation sequencing was performed in Mount Sinai Toronto. End repair, adapter ligation and amplification were carried out using the Illumina Genomic DNA Sample Prep Kit according to the manufacturer’s protocol. Illumina Genome Analyzer IIx or HiSeq 2500 was used for high-throughput sequencing of the ChIP-seq library.

Data analysis was finished with the help from Dr. Lei Gao and Dr. Lianfeng Gu in Dr. Xuemei Chen’s lab at the University of California, Riverside. The raw sequence data were processed using the Illumina sequence data analysis pipeline GA Pipeline 1.3.2. Then Bowtie (Langmead et al., 2009) was employed to map the reads to the *Arabidopsis* genome (version TAIR10) (Lamesch et al., 2012). Only perfectly and uniquely mapped reads were retained for further analysis. The data were then analyzed as described (Lu et al., 2011). Briefly, the alignments were first converted to Wiggle (WIG) files using program MACS (Zhang et al., 2008). Then WIG files were imported to Integrated Genome Browser (IGB) (Nicol et al., 2009) for visualization. Secondly, the program SICER (Zang et al., 2009) was used to identify ChIP-enriched domains (peaks) with the default parameter setup. Thirdly, quantitative comparisons between wild-type Col and mutants were performed using the ChIPDiff program (Xu et al., 2008). Regions with more than twofold changes were kept for further analysis. Finally, to assign the peaks to
proximal genes, the distance between each peak summit and the nearby transcription start site (TSS) of a gene was calculated. A peak summit that positioned within 2 kb upstream or 2 kb downstream of a TSS was assigned to that gene. If multiple genes were assigned to a peak, the closest TSS was selected. If no TSS was found in this window, the peak was left unassigned.

To generate heatmaps in Figure 21, the *Arabidopsis* genome was divided into bins with 100 bp in size. Then, the number of reads that fell within each bin was counted. The tag counts in each bin were divided by the total number of reads for both ChIPed sample (DNA obtained after Immunoprecipitation) and input sample following a previous method (Shen et al., 2012) to normalize the difference in the sequencing depths between different data sets. Normalized signal intensity values from the input sample were subtracted from those obtained from IP sample. Therefore, binding intensity for each 100 bp bin is computed as: normalized signal intensity = normalized signal intensity of ChIPed sample - that of input DNA sample. All of the bins overlapping each REF6 peak summit were identified, and binding intensity for each of those bins was calculated as described above. This process was repeated for the 10 bins upstream and 10 bins downstream of the peak summit (±1 kb). The binding intensity of BRM at each REF6 peak summit was also calculated using the same strategy. Then, the heatmaps were drawn using the heatmap.2 function in the gplots package in R (www.r-project.org) based on the normalized signal intensity which was transferred into a log2 scale.

To determine the distribution of the peak summits along the genes, all genes were scaled into 100 bins. Five kb upstream of TSS and 5 kb downstream of transcription termination site (TTS) were each divided into 50 bins of size 100 bp, respectively. Then the cumulative percentage of peak summits located in each bin was calculated.

### 2.8 Identification of DNA Motifs

The 300 bp sequences surrounding each peak summit (150 bp upstream and 150bp downstream) were extracted and searched for enriched DNA motifs using MEME-ChIP
(Machanick and Bailey, 2011). Searches were performed using default parameters. Motif comparisons were performed with a TOMTOM (Gupta et al., 2007) search against the JASPAR CORE PLANTS database at http://meme.nbcr.net/meme/cgi-bin/tomtom.cgi

2.9 Gene Ontology Term and Gene List Overlap Analysis

The BINGO 2.44 plugin for Cytoscape (http://www.psb.ugent.be/cbd/papers/BiNGO/Home.html) was used to determine which Gene Ontology (GO) categories are statistically enriched. To test if there is a statistical significance of the overlap between the two groups of genes, hypergeometric probability test was used at http://www.geneprof.org/GeneProf/tools/hypergeometric.jsp.

2.10 Histochemical GUS Staining

The standard GUS staining solution (0.5 mg/ml 5-bromo-4-chloro-3-indolyl-glucuronide, 20% methanol, 10 mM Tris-HCl, pH 7.0) was used. Seedlings immersed in 1 ml of GUS staining solution were placed under vacuum for 15 min, and then incubated at 37°C overnight. The staining solution was discarded and the samples were cleared by sequential incubation in 1 ml of 75% and 95% ethanol.

2.11 microRNA Northern Blot Analysis

RNA isolation and hybridization for microRNA (miRNA) detection was performed as described (Park et al., 2002; Tang et al., 2012). Digoxigenin-labeled miRNA probes were generated using the mirVana miRNA Probe Construction Kit (Ambion) according to manufacturer’s instructions. Oligonucleotide probes used are listed in Appendix A.
2.12 Gene Expression Analysis

Total RNA was isolated from ~50 mg of plant tissues using the Plant/Fungi Total RNA Purification Kit (Norgen). All RNA samples were treated with RNase-free DNase (Qiagen) to eliminate the genomic DNA contamination. One hundred ng of RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Random primers from the kit were used as primers.

Real-time quantitative PCR (qPCR) was conducted using the SsoFast™ EvaGreen® Supermix kit (Bio-Rad). All qPCR’s reactions were performed with the same cycling parameters: an initial phase of 95°C for 30 sec and a cycling condition consisting of 40 cycles of 95°C for 5 sec and 60°C for 10 sec. The reactions then entered melting curve analysis which began at 65°C and was increased incrementally by 0.5°C until a final temperature of 95°C was reached. Bio-Rad CFX96 real-time PCR detection system and CFX Manager™ Software (Bio-Rad) were used to detect and record fluorophore activity as well as determine the C_\text{T} value.

\[ \Delta \Delta C_T = x^{-} C_T \text{Target} - x^{-} C_T \text{GAPDH} \]

Standard deviation of the \( \Delta C_T \) value was calculated by:

\[ s \Delta C_T = [(s C_T \text{Target})^2 - (s C_T \text{GAPDH})^2]^{\frac{1}{2}}, \] where \( X^{\frac{1}{2}} \) is the square root of \( X \).

\( \Delta \Delta C_T \) values were calculated as the difference between each mutant genotypes \( \Delta C_T \) and wild-type (Col) \( \Delta C_T \) values:

\[ \Delta \Delta C_T = \Delta C_T \text{Mutant} - \Delta C_T \text{wild-type} \]
Standard deviation of $\Delta \Delta C_T$ was equal to that of $\Delta C_T$. Fold change in expression was calculated by: $2^{-\Delta \Delta C_T}$ with $+s \Delta \Delta C_T$ and $-s \Delta \Delta C_T$.

$\textit{GAPDH}$, a gene encoding glyceraldehyde-3-phosphate dehydrogenase, was used as the internal reference gene (Tang et al., 2008). PCR primers used in qPCR are listed in Appendix A.

2.13 RNA-sequencing Analysis

To measure gene expression in genome-wide, RNA-sequencing (RNA-seq) was performed. RNAs from 14-day-old seedlings of wild-type, $brm-1$, $clf-29$, $brm-1 clf-29$, $ref6-1$, and $brm-1 ref6-1$ were isolated as described in section 2.12. To minimize variations, the RNAs from three biological replicates were pooled and used for construction of sequencing libraries. PolyA+ mRNAs were enriched using oligo (dT) 25 beads and converted into sequencing libraries according to the Illumina TruSeq RNA library preparation protocol. The libraries were sequenced on an Illumina HiSeq 2500 using a paired end recipe (2×100 bp) with TruSeq v3 chemistry at The Hospital for Sick Children in Toronto. Data analysis was finished by Dr. Lei Gao in Dr. Xuemei Chen’s lab at the University of California, Riverside. Reads that passed the Illumina quality control steps were included in subsequent analysis, and reads with multiple copies were considered as a single read for the mapping procedure. The reads were mapped to the TAIR10 Arabidopsis genome using TopHat v2.0.4 with default settings (Kim et al., 2013). Reads that mapped to multiple regions were discarded. The number of reads mapped to each gene was counted using a Perl script. Genes showing at least 2 fold changes in mutants compared to wild-type were considered to be differentially expressed. An established method (Barrera-Figueroa et al., 2011) was applied to calculate the p-value for differential expression genes. The false discovery rate (FDR) was smaller than 0.05.
2.14 **Bimolecular Fluorescence Complementation Assay**

To test the protein-protein interaction in *planta*, Bimolecular Fluorescence Complementation (BiFC) assay was performed. The full length as well as the truncated *BRM* and *REF6* coding sequences were amplified by PCR and then separately transformed into the pDONR221 vector (Invitrogen) by BP reaction. The resulting entry vector was confirmed by sequencing to make sure that no sequence error was introduced by PCR. The transgene was then transferred into the modified pEarleyGate 201-nYFP (nYFP: N-terminal fragment of YFP consisting amino acids 1-174) or pEarleyGate 202-cYFP vectors (cYFP: C-terminal fragment of YFP consisting amino acids 175-239) (Lu et al., 2010) by LR reaction. The constructs were separately introduced into *Agrobacterium tumefaciens* GV3101, which were then used to infiltrate the lower epidermises of tobacco (*Nicotiana benthamiana*) leaves. After two days, the fluorescence signals were visualized using a confocal microscope (Leica Microsystems). The interaction between BRM and REF6 should result in a reconstituted YFP protein that can be visualized under confocal microscope. Primer sequences used are listed in Appendix A.

2.15 **Co-Immunoprecipitation Assay**

Entry vectors containing *BRM* or *REF6* coding sequences generated from section 2.14 were introduced into the pEarleyGate 104 or pEarleyGate 201 vectors (Earley et al., 2006) by LR reaction according to the manufacturer’s instructions (Invitrogen). Co-Immunoprecipitation (Co-IP) was performed as described (Yu et al., 2011) and was finished by Dr. Chia-yang Chen at National Taiwan University. I prepared all the vectors containing transgenes used in Co-IP assays. Briefly, transgene constructs, encoding the tagged proteins to be tested, were introduced into *Agrobacterium tumefaciens* and then co-infiltrated into tobacco leaves. Two days after infiltration, 1 g of tobacco leaves were harvested and ground in liquid nitrogen into a fine power. Proteins were extracted in 2 ml of extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), 20% glycerol, and 1% Igepal CA-630 [Sigma-Aldrich]) containing
protease inhibitor cocktail (Roche). Cell debris was pelleted by centrifugation at 20,000 ×g for 20 min. The supernatant was incubated with 5 µl of anti-GFP antibody (Ab290, Abcam) for 4 hrs at 4°C, then 50 µl of protein A agarose beads (Millipore) were added. After 2 hrs of incubation at 4°C, the beads were centrifuged at 3,800 ×g for 1 min and washed six times, each time with 1 ml of washing buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1mM DTT, 10% glycerol, and 1% Igepal CA-630). Proteins were eluted by heating at 100°C for 5 min in 40 µl of sample buffer (20% glycerol, 120 mM Tris-HCl pH 6.8, 4% SDS, 0.04% bromophenol blue, 5% β-ME) and analyzed by Western blotting using anti-GFP or anti-HA antibodies (Santa Cruz Biotechnologies).

2.16 Flowering Time Measurement

Wild-type and mutant plants were grown side by side in soil at 22°C or 16°C with 16h light/8h dark cycles. The number of rosette leaves was counted when the length of the inflorescence stem was 1 cm in length. For each genotype, at least 20 plants were analyzed at a time, and the analysis was independently repeated 3 times.

2.17 Primer Design

Unless indicated, all primers used in this thesis were designed using Primer3 at http://www.ncbi.nlm.nih.gov/tools/primer-blast/. The primer parameters are: melting temperature: 60°C; PCR product size: 80 bp-200 bp; GC content: 40%-60%. The primers listed in the Appendix A are shown in 5’ to 3’ orientation.
Chapter 3 Results

3.1 Arabidopsis Chromatin Remodeler BRAHMA Regulates Polycomb Function during Vegetative Development and Activates the Flowering Repressor Gene SVP

In flowering plants, the proper transition from vegetative phase to flowering stage is critical for their reproductive success and must be precisely controlled. Multiple genes have been shown to be involved in plant responses to environmental and endogenous signals that regulate flowering transition. In Arabidopsis, SVP is highly expressed during the vegetative phase and is crucial for repressing precocious flowering. However, the mechanism by which the high transcriptional level of SVP is maintained during vegetative phase is unknown.

In the following sections, I have performed a genome-wide study to look for the evidence of a functional relationship between the chromatin remodeler BRM and PcG proteins. Several hundred genes, including the SVP locus, showed increased H3K27me3 mark upon loss of BRM activity, demonstrating the critical role of BRM in preventing genes from H3K27me3-mediated repression in plant cells. BRM directly binds to and activates the expression of SVP by maintaining a low level of H3K27me3. Plants without BRM activity show decrease in the SVP transcription and early flowering. Together, these results demonstrate that BRM is essential for proper H3K27me3 deposition in plant genomes and this is essential during regulation of various developmental processes, including flowering.

3.1.1 Loss of BRM Activity Leads to Gain of H3K27me3 at Hundreds of Genes

To examine if BRM affects pattern of H3K27me3 deposition in a genome-wide scale, ChIP-seq experiments were performed in wild-type Col and brm-I, a null allele with a T-DNA insertion (Hurtado et al., 2006), using anti-H3K27me3 antibodies. Two independent biological ChIP DNA samples were generated and used for sequencing. The
reads were mapped to the *Arabidopsis* genome and H3K27me3-enriched regions were identified in both wild-type and *brm* mutants. Only H3K27me3-enriched regions identified in both biological replicates were chosen for further data analysis. In 14-day-old wild-type Col seedlings, 5,591 regions (corresponding to 7,230 genes) were marked by H3K27me3. The H3K27me3 marked genes identified in this thesis cover more than 95% of those reported in a previous ChIP-seq analysis (6,322 out of 6,634; Figure 4A). Furthermore, in both Col and *brm-1* mutant, the patterns of H3K27me3 at several well-characterized H3K27me3 target genes, such as *AGAMOUS* (*AG*), *APETALA3* (*AP3*), *FLC* and *FT*, are very similar to those reported by Lu et al. (2011) (Figure 4B). Furthermore, no H3K27me3 signals at two highly expressed genes, *ACTIN2/7* and *TUBLIN2*, were observed (Figure 4C), indicating the quality and reliability of the ChIP-seq data obtained.

Compared to wild-type, 276 genes showed more than a twofold change in H3K27me3 levels in the *brm-1* mutant. Out of these 276 genes, 258 (93.5%) genes showed more than a twofold increase in H3K27me3 in *brm-1*, while only 18 (6.5%) genes showed more than a twofold reduction in H3K27me3 in *brm-1* (Figure 4D). The genome-wide data indicate that BRM mainly acts to antagonize PRC2 activity during vegetative development. However, the decreased H3K27me3 at a smaller set of genes in *brm* mutant suggests that BRM could also promote PcG activity at certain loci.

To gain insight into the possible biological roles of the genes showing increased H3K27me3 deposition, their potential functional associations were examined by performing a GO analysis. In the classification of biological processes, these genes were highly enriched in “regulation of metabolic process” (P=9.69×10⁻⁴) and “regulation of gene expression” (P=3.8×10⁻⁴; Figure 4E), and in terms of molecular function, the most enriched category observed was “transcription regulator activity” (P=1.57×10⁻⁴). Thus, BRM is involved in a wide spectrum of cellular processes such as gene expression regulation and metabolism through preventing H3K27me3 deposition in *Arabidopsis*. To validate the ChIP-seq data, ChIP-qPCR was performed using an independent chromatin sample. The changes in H3K27me3 levels in *brm-1* at all 10 selected genes were confirmed (Figure 4F and 4G). The observed changes were specific to these genes,
Figure 4: Loss-of-function of BRM Resulting in Changes of H3K27me3 Distribution over Several Hundred Endogenous Genes

(A) A venn diagram showing the overlap between H3K27me3-targeted genes in Arabidopsis seedlings from ChIP-seq results in this thesis (left) and those from the ChIP-seq dataset reported by Lu et al. 2011 (right).

(B) ChIP-seq data for the well-known H3K27me3 target genes AG, AP3, FLC and FT from wild type Col (red; top) and brm-1 (orange; bottom). Gene structures are shown underneath each panel in blue.

(C) ChIP-seq data showing no H3K27me3 signal at two constitutively expressed genes ACTIN2/7 and TUB2 in wild-type Col (red; top) and brm-1 (orange; bottom).

(D) Numbers of genes showing at least a 2-fold change in H3K27me3 levels in brm-1 mutant compared to wild-type Col.

(E) GO analysis of genes showing increased H3K27me3 levels in brm-1. Numbers on the top are P values (hypergeometric test) for GO category enrichment generated by comparing the percentage of the corresponding categories in genes that showed increased H3K27me3 levels to those in the whole genome.

(F) ChIP-seq data showing changes in H3K27me3 levels at 10 selected genes in brm-1. Nine showed an increase and one showed a decrease in H3K27me3 levels. Data are shown in red at the top for the wild-type Col, and orange at the bottom for brm-1. Gene structures are shown underneath each panel in blue.

(G) ChIP-qPCR validation using an independent sample. Data are shown as percentage of input (% Input). ACTIN2/7 and AG, exhibiting no change in H3K27me3 deposition, were used as control loci. Error bars indicate standard deviations among three biological replicates.

(H) Expression analysis of selected genes by qRT-PCR. The expression of each gene was normalized to that of GAPDH, and the expression level in Col was set to 1. Error bars indicate standard deviations among three biological replicates.
as no marked changes were detected at PcG non-target *ACTIN2/7* and PcG target *AG* (Figure 4G).

The next question was whether the elevated H3K27me3 levels in the *brm-1* mutant is correlated with down-regulation of the corresponding genes. The expression levels of several selected genes that showed increased H3K27me3 levels in *brm-1* were measured by qRT-PCR. Decreased expression was observed for most but not all of the genes in *brm-1* (Figure 4H). These data indicate that, in general, there is a negative correlation between H3K27me3 levels and gene expression level in *brm-1*. However, the fact that the expression of some of the genes did not decrease suggests that increased H3K27me3 deposition alone in *brm-1* might not be sufficient for gene repression at some target loci. Interestingly, the increased expression was also found for *WRKY DNA-BINDING PROTEIN 23* (WRKY23) (Figure 4H), a gene with decreased H3K27me3 levels in *brm-1* (Figure 4F and 4G).

### 3.1.2 Removal of CLF Activity in *brm* Background Results in a Substantial Decrease of H3K27me3 Deposition at Some Genes

In *Arabidopsis*, CLF is a major H3K27 methyltransferase responsible for the deposition of H3K27me3 in tissues other than seeds (Goodrich et al., 1997; Schubert et al., 2006). LIKE HETEROCHROMATIN PROTEIN 1/TERMINAL FLOWER 2 (LHP1/TFL2) associates with regions containing H3K27me3 across the *Arabidopsis* genome and was proposed to be a key component of a plant PRC1 complex (Turck et al., 2007; Zhang et al., 2007a). Both *clf* and *tfl2* single mutants show upward curling of leaves (Figure 5A) (Goodrich et al., 1997; Larsson et al., 1998). Upward leaf curling in *clf* mutants was caused by ectopic expression of floral homeotic genes such as *AG*, *APETALA1* (*API*), and *AP3* in leaves (Goodrich et al., 1997; Chanvivattana et al., 2004). I reasoned that CLF might be required for the increased H3K27me3 levels at genes in the *brm-1* mutant. To test this hypothesis, the *CLF* gene was knocked out in the *brm* mutant background by generating a *brm clf* double mutant. In the *brm clf* double mutants, the upward curling of leaves was weaker than that in *clf* single mutants (Figure 5A), suggesting that mutation
Figure 5: Removal of CLF Activity in brm Background Resulting in a Substantial Decrease of H3K27me3 Deposition

(A) Loss of BRM activity partially rescues the upward leaf curling phenotypes of clf-29 and tfl2-1. Plants were grown in soil for three weeks. Scale bar: 1 cm.

(B) ChIP-seq data comparing H3K27me3 levels at 10 selected genes in Col, brm-1, clf-29 and brm-1 clf-29. Data for the wild-type Col are shown in red, brm-1 in orange, clf-29 in yellow and brm-1 clf-29 in green. Gene structures are shown underneath each panel in blue.

(C) ChIP-qPCR validation using independent samples. ChIP signals are shown as percentage of input. ACTIN2/7 and AG were used as negative and positive control loci, respectively. Error bars indicate standard deviations among three biological replicates.

(D) Analysis of CLF occupancy at selected genes as determined in brm-1 Pro35S:GFP-CLF plants using ChIP-qPCR. Pro35S:GFP-CLF plants were included as the negative control. ChIP signals are shown as percentage of input. ACTIN2/7 and AG were used as negative and positive control loci, respectively. Error bars indicate standard deviations among three biological replicates.
in BRM can partially suppress this phenotype in PcG mutant. A brm tfl2 double mutant was also generated. Leaves of brm tfl2 double mutant showed a downward curling phenotype that is almost identical to that of brm single mutants (Figure 5A), suggesting that brm suppresses the upward leaf curling phenotype of tfl2. These genetic data support a notion that BRM antagonizes PcG function in leave curling phenotype. Consistent with this notion, decreased expression of several floral homeotic genes, such as AG, AP1, and AP3, in brm clf double mutants compared to clf single mutants was observed (Figure 6).

To determine if CLF is required for the increased H3K27me3 levels at certain genes in the brm-1 mutant, the genome-wide distribution and level of H3K27me3 in brm clf double mutants were measured by ChIP-seq and compared with the brm single mutant. The removal of CLF activity led to a marked reduction of H3K27me3 levels at approximately half of the genes with increased H3K27me3 levels in brm-1 (133 out of 258; Figure 5B), indicating that the increased H3K27me3 levels at certain genes in brm mutants depends on the CLF activity. The reduction of H3K27me3 levels at nine selected genes in brm clf double mutants compared to brm mutants was validated by ChIP-qPCR experiments (Figure 5C). These observations are consistent with a scenario where BRM acts by sheltering certain loci from the CLF activity.

After confirming that the CLF activity is responsible for the elevated H3K27me3 in brm-1 mutants, I then asked whether the increase in H3K27me3 levels at these loci in brm mutants was due to increased CLF presence at these loci. To test this, the CLF occupancy level at these loci in the brm mutant relative to wild-type was measured by ChIP-qPCR. For this purpose, a GFP-tagged CLF transgenic line in the clf mutant background (35S:GFP-CLF) (Schubert et al., 2006) was obtained. The transgene fully rescued the clf mutant phenotype, suggesting it is functional in vivo (Schubert et al., 2006). The line was then crossed with a brm-1 mutant to generate brm-1 35S:GFP-CLF clf plant. ChIP assays were performed using brm-1 35S:GFP-CLF clf and 35S:GFP-CLF clf. As shown in Figure 5D, CLF occupancy was increased for half of the selected genes, suggesting that, in the absence of BRM, CLF is allowed to access genomic regions, resulting in increased H3K27me3 levels. However, the fact that increased CLF presence was not observed for other selected genes suggests that there might be other strategies employed by BRM to
Figure 6: Partial Restoration of Ectopic Expression of Floral Homeotic Genes in the *clf* Mutant by Removing BRM Activity

Expression data of floral homeotic genes in different genetic backgrounds were determined by qRT-PCR with three biological replicates. Bars indicate standard deviation among three biological replicates.
antagonize PcG, such as keeping PcG proteins in an inactive state rather than keeping them physically away from the target sites. Alternatively, it is also possible that, instead of CLF, SWN is recruited to those genes in brm mutants. At the WRKY23 locus, CLF occupancy was reduced in brm mutant (Figure 5D), consistent with the decreased H3K27me3 levels observed at this locus (Figure 4G and 4F).

### 3.1.3 BRM Directly Binds to Genes Preventing H3K27me3 Deposition

Next, I asked how BRM antagonizes PcG function during vegetative growth, i.e., whether it does so directly or indirectly. One possibility that could explain increased H3K27me3 deposition and PcG occupancy in brm is the elevated expression level of genes encoding PcG subunits. To address this issue, the transcription levels of genes encoding PRC2 components in brm mutants, including CLF, SWN, EMF2, VRN2, FIE and FIS2, were examined. The expression of these genes was not increased in brm-1 compared to that of wild-type (Figure 7), indicating that BRM does not antagonize PcG through repressing the transcription of PcG-encoding genes. Another possibility could be that loss of BRM causes increased H3 occupancy at these genes, which in turn, indirectly results in the increase level of H3K27me3. To address this issue, histone H3 levels were measured at selected loci. Although a slight increase in H3 levels was found in brm-1 (Figure 8), the increase in H3 levels was very small and thus could not fully account for the increase in H3K27me3 levels.

I then asked whether BRM directly acts on the affected genes by physically binding to them. ChIP-qPCR experiments were performed to examine BRM occupancy at the affected genes. For the ChIP assay, a transgenic Arabidopsis line expressing a GFP-tagged BRM transgene under the control of the BRM native promoter (ProBRM:BRM-GFP) was used (Smaczniak et al., 2012). The transgene could fully rescue the morphological defects of the brm-1 null mutant (Figure 9A), suggesting that it is functional in vivo. ChIP was performed with anti-GFP antibodies, and Pro35S:GFP plants were used as the negative control. DNA obtained from ChIP assay was analyzed by qPCR to examine the enrichment of BRM at selected genes. The data indicate that all
Figure 7: Expression Analysis of PcG-encoding Genes in brm-1 and Col Seedlings as Determined by qRT-PCR

The expression levels of each gene were normalized to that of GAPDH, and the expression level in Col was set to 1. Error bars indicate standard deviation among three biological replicates.
Figure 8: ChIP-qPCR Analysis of Histone H3 Levels at Selected Genes in *brm-1* and *Col* Seedlings

ChIP signals are shown as fold changes relative to that in wild-type plants. Error bars indicate standard deviation from three biological replicates.
selected loci were enriched by BRM-GFP protein (Figure 9B). The physical association of BRM with these selected genes, in combination with increased H3K27me3 deposition and decreased expression of the genes in brm mutants, strongly suggests that BRM acts directly at these target loci to keep the PRC2 activity off and thus promotes gene expression. Loss of the BRM activity allows access of PRC2 to these loci, which turns off or decreases gene expression.

3.1.4 BRM Positively Activates the Expression of Flowering Repressor SVP

In the sections below, I present evidence demonstrating that SVP is the main target of BRM during regulation of floral transition. SVP is a key negative regulator of flowering in Arabidopsis and loss of SVP results in precocious transition from vegetative to reproductive phase (Hartmann et al., 2000; Li et al., 2008). Consistent with its role in maintaining the vegetative phase, SVP is highly expressed in young seedlings but barely detectable in inflorescence tissues (Hartmann et al., 2000). Interestingly, as shown in Figure 4F and 4G, H3K27me3 levels as well as CLF occupancy were drastically increased at the SVP locus in brm-1 compared to wild-type. The increase in H3K27me3 levels at the SVP locus in brm raises the possibility that BRM may act to keep expression of SVP on by antagonizing PcG activity during the vegetative growth. To test this hypothesis, I first extended the single time point expression analysis of SVP in brm-1 as presented in Figure 4H by examining the expression of SVP in the brm-1 mutant spanning more time points. Indeed, the expression of SVP in the brm-1 mutant was consistently lower than that in wild-type plants over a time course spanning 8 to 14 days after germination (DAG, Figure 10A), suggesting that BRM activity is required for high levels of SVP expression in seedlings. The decreased expression of SVP was unlikely due to the accelerated floral transition of brm-1 plants, since the expression of AP1, a marker gene for the vegetative-to-floral developmental transition (Mandel et al., 1992; Benlloch et al., 2007), remained low throughout the time course (Figure 11).

To confirm that BRM activates the transcription of SVP, an XVE::aMIRBRM transgenic line that harbors an inducible artificial microRNA (aMIRBRM), that targets BRM mRNA,
Figure 9: Physical Occupancy of BRM at Selected Genes

(A) ProBRM:BRM-GFP complements the brm-1 phenotype. GFP signals were detected by confocal microscopy in roots and leaves of 14-day-old brm-1 ProBRM:BRM-GFP. Scale bar: 50 µm.

(B) BRM occupancy at selected genes as determined by ChIP using anti-GFP antibody in brm-1 ProBRM:BRM-GFP plants with Pro35S:GFP plants as control. ChIP signals are shown as percentage of input. TA3, a transposable element gene that is not targeted by BRM (Wu et al., 2012), was used as a negative control. Error bars indicate standard deviations from three biological replicates.
was generated (Figure 10B). As shown in Figure 10C, BRM transcript levels were gradually decreased by approximately 50% during a 24h time course in 7-day-old XVE::aMIRBRM seedlings treated with β-estradiol. SVP transcript levels showed similar reduction kinetics during the same time period (Figure 10D). This result further supports that the proper BRM activity is required for SVP expression.

To further confirm that BRM activates SVP expression at the transcriptional level, I obtained a previously developed SVP promoter-GUS fusion reporter line (ProSVP:GUS, Li et al., 2008), and introduced it into the brm-1 background by genetic crosses (brm-1 ProSVP:GUS). As shown in Figure 10E, GUS activity in brm-1 ProSVP:GUS was almost invisible compared to that in ProSVP:GUS at all three time points (blue color indicates GUS activity) (Figure 10E), suggesting that the promoter of SVP has no detectable activity when BRM is absent. As negative controls, Col wild-type and brm-1 mutants were also stained but no GUS staining was observed (Figure 10E). Documented Arabidopsis gene expression data (Schmid et al., 2005) indicate a temporal and spatial overlap of SVP and BRM expression patterns in leaves (Figure 12), which is consistent with BRM’s role as a positive regulator of SVP in developing seedlings. All together, these data demonstrate a positive spatial and temporal correlation between BRM and SVP expression, and when combined with BRM-GFP ChIP data (Figure 9B), which showed a direct BRM binding to the SVP locus; this suggests that BRM directly promotes SVP expression during the plant vegetative development.

3.1.5 BRM Represses Flowering Mainly via Activating SVP Transcription

Having shown a positive role for BRM in regulating SVP expression, I next sought to investigate whether the BRM-SVP module could explain the early flowering phenotype of the brm mutant. Both brm and svp single mutants show early flowering phenotypes under long-day growth conditions (Hartmann et al., 2000; Farrona et al., 2004; Lee et al., 2007a; Tang et al., 2008; Farrona et al., 2011). However, it is not known whether there is a common molecular mechanism underlying their early flowering phenotype. As the careful comparison of flowering time for brm and svp mutants has never been performed,
Figure 10: A Tight Control of SVP Expression by BRM

(A) The transcription of SVP is drastically decreased in developing brm-1 seedlings compared to that in Col (grown at 22°C) as determined by qRT-PCR.

(B) Schematic diagram of the region between the right and left T-DNA borders of the XVE::aMIRBRM construct. XVE is a DNA sequence encoding a chimeric transcription factor containing the DNA-binding domain (DBD) of the bacterial repressor LexA (X; residues 1-87); the acidic transactivation domain of VP16 (V; residues 403-479); and the carboxyl region of the human estrogen receptor (E; residues 282-595) (Zuo et al., 2000). The binding of β-estradiol to E carboxyl region leads to translocation of XVE from cytoplasm to nucleus. The precursor of aMIRBRM was inserted behind a LexA operator sequence fused to the-45 35S minimal promoter (O_{LexA-45}). Other components of the vector were described previously (Curtis and Grossniklaus 2003).

(C) BRM expression in 7-day-old XVE::aMIRBRM transgenic seedlings mock treated or treated with 10µm β-estradiol for 0, 8, 12, and 24h.

(D) SVP expression in 7-day-old XVE::aMIRBRM transgenic seedlings mock treated or treated with 10µm β-estradiol for 0, 8, 12, and 24h. The expression of each gene in A, C, and D was normalized to that of GAPDH. Error bars indicate standard deviations from three biological replicates.

(E) GUS activity patterns of ProSVP:GUS in Col and brm-1 backgrounds in 7, 11, and 14-DAG seedlings. Col and brm-1 were included as negative controls. Scale bar: 0.5 mm.
Figure 11: Expression Analysis of *AP1* in *brm-1* and Col Seedlings as Determined by qRT-PCR

The transcription level of *AP1* gene was normalized to that of *GAPDH*. Error bars indicate standard deviation from three biological replicates.
Figure 12: Expression Patterns of \textit{BRM} and \textit{SVP}

Data were taken from Schmid et al. (2005) and displayed using the AtGenExpress Visualization Tool (http://jsp.weigelworld.org/expviz/expviz.jsp)
both mutants were grown side by side and flowering time was measured by counting the number of rosette leaves at bolting time. As shown in Figure 13A and 13B (top panel), brm-1 and svp-31, a null T-DNA insertion mutant (Lee et al., 2007a), flowered at the same time. svp-31 heterozygous plants flowered significantly later than their homozygous siblings but earlier than wild-type Col plants, indicating that SVP controls flowering in a dosage-dependent manner, which is consistent with previous observations (Hartmann et al., 2000). Taking advantage of the dosage-dependent flowering control by SVP, SVP transcript levels were quantified by qRT-PCR in mutant plants to estimate the contribution of SVP to flowering control by BRM (Figure 13B, middle panel). The qRT-PCR data confirmed that svp-31 is a null allele and heterozygous plants accumulated approximately half the amount of SVP transcripts found in wild-type plants (Figure 13B, middle panel). SVP expression in brm-1 was drastically reduced to less than half that of the svp-31 heterozygous plants. In brm-1 ProBRM:BRM-GFP plants, both the flowering time and SVP expression were restored to the wild-type level (Figure 13A and 13B), further confirming that BRM activity is responsible for the normal expression level of SVP. The quantification of flowering time and SVP transcript levels in brm-1, when compared quantitatively to those from svp-31 mutants, suggests that 1) BRM is a major activator of SVP expression; and 2) The early flowering phenotype of the brm-1 mutant can be largely accounted for by the down regulation of SVP transcription in the mutant, although other BRM targets might also have minor contributions.

In order to provide additional evidence to further support this conclusion, I examined whether restoration of SVP in the brm mutant background could overcome its early flowering phenotype by driving the SVP expression from a constitutive promoter that is not controlled by BRM (Pro35S:SVP) (Mizukami and Ma, 1992) into brm-5, a chemically induced mutant that has a single nucleotide change in the region coding the ATPase domain (Tang et al., 2008). Indeed, introduction of Pro35S:SVP into brm-5 did rescue the early flowering phenotype of brm-5 mutant (Figure 13C). To test the genetic interaction between BRM and SVP in flowering time control, a brm-1 svp-31 double mutant was also generated. This double mutant was found to flowering only slightly earlier than either single mutant (Figure 13B), suggesting that BRM and SVP largely act in a common pathway in determining flowering time, with a possible minor contributions
Figure 13: Repression of Flowering by BRM through Activating SVP Transcription

(A) Comparison of flowering phenotypes of plants with various genetic backgrounds shortly after bolting. For direct comparison, pictures of wild-type and \textit{brm-1} ProBRM:BRM-GFP, heterozygous of \textit{svp-31 [svp-31(HE)]} and homozygous of \textit{svp-31 [svp-31(HM)]}, \textit{brm-1} and \textit{brm-1 svp-31} were taken at the same age. All plants were grown at 22°C under long-day conditions. Scale bar: 2 cm.

(B) Decreased steady-state level of the \textit{SVP} mRNA is associated with early flowering of \textit{brm-1} at 22°C. Top panel: rosette leaf number at bolting of plants in different genetic backgrounds. Error bars indicate standard deviations from at least 20 plants. Lowercase letters indicate significant differences among genetic backgrounds determined by Post-hoc Tukey’s HSD test. Middle panel: transcription analysis of \textit{SVP}. Bottom panel: transcription analysis of \textit{FT}. Transcription level of \textit{SVP} and \textit{FT} was calculated relative to that of \textit{GAPDH}. Error bars indicate standard deviations transcription from three biological replicates.

(C) Overexpression of \textit{SVP} rescues the early flowering phenotype of \textit{brm} mutant. Top panel: flowering phenotype of \textit{brm-5}, \textit{Pro35S:SVP} and \textit{brm-5 Pro35S:SVP} plants grown for five weeks at 22°C under long-day conditions. Scale bar: 2 cm. Bottom panel: rosette leaf number of \textit{brm-5}, \textit{Pro35S:SVP} and \textit{brm-5 Pro35S:SVP} plants at bolting.
from other BRM targets. It is worth mentioning that three other flowering time genes also displayed increased H3K27me3 levels in the brm mutant (Figure 14A). When the transcription levels of these genes were examined at the mRNA level, a strong decrease in steady-state level of the AGAMOUS-LIKE24 (AGL24) mRNA, but not the other two genes, in brm-1 was observed (Figure 14B-D). AGL24 is a MADS-box protein involved in flowering time control (Gregis et al., 2006). agl24 mutants show delayed flowering while agl24 svp double mutants are early flowering similar to svp single mutants (Gregis et al., 2006). Thus, these data suggest that the early flowering phenotype of brm mutants is unlikely caused by these flowering time genes. In addition, I also examined the expression of FT, a target gene that is negatively regulated by SVP in various genetic backgrounds (Figure 13B, bottom panel). As expected, FT transcript levels were negatively correlated with those of SVP and positively with flowering time in corresponding genetic backgrounds. In summary, those observations strongly suggest that BRM represses flowering mainly through activating the expression of SVP.

3.1.6 Both BRM and SVP Act in the Ambient Temperature Pathway

It is well-known that flowering time of Arabidopsis is temperature sensitive, i.e., Arabidopsis plants flower late when grown at lower temperature, such as 16°C, than at higher temperature, such as 23°C (Samach and Wigge, 2005; Lee et al., 2008). SVP apparently plays a key role in the so-called ambient temperature pathway because the svp mutant is insensitive to changes in ambient temperature and flowers early even at low temperature (16°C, Lee et al., 2007a). To test whether BRM also participates in ambient temperature in flowering time control, brm mutants were grown at low temperature (16°C) and flowering time was measured. For comparison, svp mutants were also grown at the same time. The brm mutant displayed early flowering at 16 °C which is similar to that observed in svp mutant (Figure 15A and 15B). Consistent with the early flowering phenotype of brm mutant under low temperature, the expression of SVP was drastically decreased in the brm mutant at 16°C (Figure 15B), and the expression of FT was elevated correspondingly (Figure 15B). This observation suggests that BRM also participates in
Figure 14: Analysis of H3K27me3 and Expression Levels in brm-1 at Several Genes Involved in Flowering Time Control

(A) ChIP-seq data showing an increase in H3K27me3 levels at several genes in brm-1. Data from wild-type Col and brm-1 are shown in red at the top and orange at the bottom, respectively.

(B) ChIP-qPCR validation using independent samples. Data are shown as percentage of input. Error bars indicate standard deviations from three biological replicates.

(C) Expression analysis of AGL24 and SMZ by qRT-PCR. Expression of each gene was normalized to that of GAPDH, and the expression level in Col was set to 1. Error bars indicate standard deviations from three biological replicates.

(D) Small RNA Northern blot analysis of miR156 in brm-1 compared to Col. Two different time points were used (10 and 14 days after germination). Levels of small RNAs in brm-1 were compared to those in Col, which was set to 1. Numbers below the gel images indicate relative abundance. U6, a small nuclear RNA, was used as the loading control.
the ambient temperature pathway in the control of flowering, thus providing further evidence in support of the genetic link between BRM and SVP.
Figure 15: Repression of Flowering by BRM in Ambient Temperature Pathway

(A) Flowering phenotype of Col, brm-1 and svp-31 plants grown at 16°C under long-day conditions. Scale bar: 2 cm.

(B) Top panel: rosette leaf number at bolting of plants in different genetic backgrounds. Error bar indicates the standard deviation from at least 20 plants. Middle and Bottom panel: transcription analysis of SVP and FT, respectively. The transcription levels of SVP and FT were calculated relative to that of GAPDH. Error bars indicate standard deviations from three biological replicates.
A  

Col  

brm-1  

svp-31  

16°C

B

Rosette leaf number

Col  

brm-1  

svp-31

16°C

SVP/GAPDH

Col  

brm-1  

svp-31

FT/GAPDH

Col  

brm-1  

svp-31
3.2 Concerted Actions of the *Arabidopsis* Histone H3 Lysine-27 Demethylase REF6 and BRM in Activating Gene Expression

In the following sections, I describe how chromatin-remodeling ATPase BRM functionally interplays with Histone H3 lysine 27 demethylase REF6 in activating gene expression. I first mapped the global binding sites of BRM and REF6 in *Arabidopsis*. I then showed that *Arabidopsis* BRM and REF6 co-localize over thousands of genomic regions, many of which are involved in response to various endogenous and environmental stimuli. I further revealed that REF6 physically associates with BRM and facilitates the recruitment of BRM to hundreds of genomic regions. Together, this work demonstrates a concerted action between chromatin remodeler BRM and H3K27 demethylase REF6 during plant development.

3.2.1 Genome-Wide Identification of BRM- and REF6-Associated Sites

To begin the studies on how BRM and REF6 may functionally interplay *in vivo*, their genome-wide locations in *Arabidopsis* were mapped by ChIP-seq. For profiling BRM global binding sites, ChIP-seq analysis were carried out using transgenic plants expressing a BRM and green fluorescent protein (GFP) fusion protein under the control of the native *BRM* promoter in *brm-1* mutant background (*ProBRM:BRM-GFP brm-1*; Smaczniak et al., 2012). ChIP assays were performed using an anti-GFP antibody and two independent biological ChIP DNA samples were sequenced. Only BRM-enriched regions identified in both biological replicates were chosen for further data analysis. Analysis of ChIP-seq data identified 5,272 genes occupied by BRM across the genome. These BRM target genes identified by ChIP-seq included many previously reported BRM targets, such as HECATE 1 (HEC1), a transcription activator (Vercruyssen et al., 2014); ARABDOPSIS REPONSE REGULATOR 6 (ARR6), a type-A cytokinin response regulator (Efroni et al., 2013); ABA INSENSITIVE 5 (ABI5), a member of the basic leucine zipper transcription factor family, involved in ABA signaling (Han et al., 2012); GIBBERELLIN 3 BETA-HYDROXYLASE 1 (GA3OX1), an enzyme involved in GA biosynthetic gene; and SCARECROW-LIKE 1(SCL1), a transcription factor implicated...
Figure 16: ChIP-seq Genome-browser Views of BRM Occupancy at Previously Identified BRM Targets

Gene structures are shown underneath each panel in blue.
BRM ChIP-seq

ABI5  SCL1  AR6  HEC1  GA3OX1
in positive regulation of the GA pathway (Archacki et al., 2013) (for ChIP-seq data at these genes see Figure 16).

To map the genomic distributions of REF6, a transgenic Arabidopsis line expressing a GFP-tagged REF6 driven by its native promoter (ProREF6:REF6-GFP) was generated. It was observed that the transgene could fully rescue the morphological defects (such as delayed flowering) of the ref6-1 null mutant (Figure 17), indicating that it is functional in vivo. ChIP was then performed using an anti-GFP antibody and again, two biological replicates were included. Analysis of the ChIP-seq data identified 3,164 REF6 target genes across the Arabidopsis genome. Twelve previously identified REF6 direct targets (Lu et al., 2011) were also found in the list of REF6-associated genes (Figure 18).

Next, BRM- and REF6-association patterns were compared with published genome-wide histone modification data (Luo et al., 2013). For this comparison, histone modifications that are associated with active genes such as H3K4me2, H3K4me3, H3K36me3, H3K9Ac, and H3K18Ac, as well as H3K27me1, H3K27me3, and H3K9me2, which tend to mark repressed genes, were chosen. For a direct comparison, the histone modification data from a ChIP-seq study, that also used 14-day-old seedlings grown on MS medium, were used (Luo et al., 2013). Interestingly, both BRM and REF6 were found to co-localize with the active histone marks, but not with the repressive ones (Table 1).

GO term enrichment analysis revealed that both BRM- and REF6-associated loci contain many common GO categories. Particularly, genes involved in responses to different types of stimuli were highly enriched for both BRM- and REF6-associated genes (Figure 19). For BRM target genes, 18 out of the top 20 GO categories were genes associated with responses to stimulus/stress, including abiotic stress, light, radiation, chitin, osmotic stress, salt, temperature, and plant hormones. For GO molecular functions, both REF6 and BRM target genes were significantly overrepresented for genes with functions related to kinase, transferase, and catalytic activity (Figure 19). GO category enrichment analysis suggests a non-random distribution of both BRM and REF6 targets identified by the ChIP-seq, implying that REF6 and BRM may localize to genes with similar molecular and biological functions such as those involved in stress tolerance.
Figure 17: Rescuing of ref6-1 Phenotype by ProREF6:REF6-GFP

Plants were allowed to grow in soil for 45 days in long-day condition before the picture was taken.
Figure 18: ChIP-seq Genome-browser Views of REF6 Occupancy at Twelve Previously Identified REF6 Targets

Gene structures are shown underneath each panel in blue.
Table 1: Overlapping Analysis of BRM- and REF6-Binding Genes with Different Epigenetic Marks

<table>
<thead>
<tr>
<th>Active marks (Covered genes)</th>
<th>Overlapped BRM targets (Percentage of overlap)</th>
<th>BRM random overlap (Percentage of overlap)</th>
<th>P value</th>
<th>Overlapped REF6 targets (Percentage of overlap)</th>
<th>REF6 random overlap (Percentage of overlap)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3K4me2(19,406)</td>
<td>3221 (9%)</td>
<td>1898 (58%)</td>
<td>1.5e-22</td>
<td>1551 (50%)</td>
<td>1126 (38%)</td>
<td>4.4e-04</td>
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<tr>
<td>H3K4me3(14,233)</td>
<td>3615 (69%)</td>
<td>2219 (42%)</td>
<td>1.8e-05</td>
<td>1551 (50%)</td>
<td>1278 (41%)</td>
<td>9.7e-04</td>
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<td>H3K27ac(10,073)</td>
<td>2844 (28%)</td>
<td>1704 (33%)</td>
<td>0</td>
<td>1522 (42%)</td>
<td>1844 (52%)</td>
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</tr>
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<td>H3K18ac(3,283)</td>
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<td>511 (8.7%)</td>
<td>4.8e-73</td>
<td>383 (10%)</td>
<td>294 (9%)</td>
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</table>

<table>
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<tr>
<th>Repressive marks (Covered genes)</th>
<th>Overlapped BRM targets (Percentage of overlap)</th>
<th>BRM random overlap (Percentage of overlap)</th>
<th>P value</th>
<th>Overlapped REF6 targets (Percentage of overlap)</th>
<th>REF6 random overlap (Percentage of overlap)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3K27me2(1,408)</td>
<td>89 (1.3%)</td>
<td>227 (9.1%)</td>
<td>0.39</td>
<td>37 (1.1%)</td>
<td>142 (4.8%)</td>
<td>0.99</td>
</tr>
<tr>
<td>H3K27me3(7,230)</td>
<td>882 (16%)</td>
<td>1050 (20%)</td>
<td>0.39</td>
<td>421 (12%)</td>
<td>621 (19%)</td>
<td>0.99</td>
</tr>
<tr>
<td>H3K9me2(1,148)</td>
<td>48 (5.9%)</td>
<td>173 (3.4%)</td>
<td>0.39</td>
<td>20 (0.9%)</td>
<td>110 (3.5%)</td>
<td>0.99</td>
</tr>
</tbody>
</table>

The gene lists for all histone modifications were described in Luo et al., 2013.
Figure 19: Gene Ontology Analysis of the BRM- and REF6-Target Genes

The BINGO 2.44 program was used to determine which GO categories are statistically enriched. The terms were ranked by P-value.
Occupancy profiles of individual genomic regions showed that both BRM and REF6 proteins occupy defined locations within the genome. Two types of BRM sites composed of locally distributed regions characterized by single, defined narrow peaks, as well as regions more broadly distributed were found (Figure 20A). The average length of all BRM binding sites was 2,155 bp (Figure 20B). In contrast, only one major type for REF6, characterized by single, defined narrow peaks, was found (Figure 20A), with an average length of 1,355 bp (Figure 20B). Examination of the distribution of these sites relative to annotated genes revealed similar localization patterns for both proteins, which ~80% of these sites were located in genes, and ~20% were in intergenic regions (Figure 20C). The distribution of the peak’s summit position along the target genes was further analyzed. As shown in Figure 20D, both BRM and REF6 tend to enrich around the transcription start site (TSS). This enrichment preference at TSS is consistent with their role as transcriptional regulators. Similar genomic distribution patterns have been recently reported for BRM homologs and H3K27 demethylases in animal model systems (De Santa et al., 2009; Ho et al., 2009; Tolstorukov et al., 2013; Morris et al., 2014).

3.2.2 BRM and REF6 Co-Localize Genome-Wide

Given their similar genomic distribution patterns as described above, I wondered whether BRM and REF6 would co-localize on chromatin. Indeed, a total of 1,276 genes were found to be co-occupied by BRM and REF6 (Figure 21A), a fraction much larger than that expected by chance alone (hypergeometric test, $P < 7 \times 10^{-162}$), indicating that BRM and REF6 can bind to the same genomic regions. Furthermore, BRM were strongly enriched right at the summits of REF6-bound sites (Figure 21B). Figure 21C showed the co-localization of BRM and REF6 at a set of randomly selected loci. To validate the co-occupancy of BRM and REF6 on the chromatin, ChIP-qPCR experiments were performed for a set of randomly selected genes (Figure 21C) using independent plant materials. The results clearly showed that BRM was targeted to REF6-binding genes (Figure 21D). The gene functions of these co-target genes of BRM and REF6 were
Figure 20: Genome-Wide Localization of BRM and REF6

(A) ChIP-seq genome-browser views of occupancy of BRM (top) and REF6 (bottom) at the same genomic coordinates on chromosome 5. Red boxes highlight the single, defined BRM peaks, while blue boxes highlight the broad BRM peaks. White arrows point to the REF6 peaks. Gene structures are shown underneath the panel.

(B) Average peak width of BRM and REF6 sites with x-axis showing log2 of the width.

(C) Pie figures showing the distributions of BRM and REF6 peaks at annotated genic regions in the genome.

(D) Distributions of BRM and REF6 occupancy (peak summits) relative to gene structure including 5-kb upstream and 5-kb downstream of TSS and TTS, respectively.
also examined by GO term analysis. Many co-target genes were reportedly and/or predicted to be involved in response to various stimuli (Figure 21E). In particular, many BRM-REF6 co-targets were genes involved in response to different plant hormones such as ABA, auxin, ethylene (ET), salicylic acid (SA), and brassinosteroid (BR) (Figure 21E). In addition, the BRM-REF6 co-targets also tend to co-localize with genes marked by H3K4me2, H3K4me3, H3K36me3, H3K9Ac, and H3K18Ac but not those with H3K27me1, H3K27me3, and H3K9me2 (Table 2), as do targets of BRM or REF6 individually (Table 1).

### 3.2.3 Shared Genomic DNA Motifs for BRM and REF6 Targeting

I next attempted to define consensus sequence(s) targeted by these two proteins, particularly REF6. I was inspired by the observation that REF6 localizes to single, well-defined genomic sites (shown as narrow peaks in sequence alignment profiles; Figure 20A) and the fact that REF6 contains four C2H2 zinc finger domains at its carboxyl terminal part, which can potentially directly bind to DNA (Lu et al., 2008). BRM, on the other hand, does not appear to have the intrinsic ability to specifically bind DNA (Farrona et al., 2007), thus, it might depend on other factors for genomic targeting. A motif discovery analysis was conducted using MEME-ChIP program (Machanick and Bailey, 2011) and significant enrichments of several motifs for both BRM- and REF6-associated sites were revealed (Figure 22A). Interestingly, two out of the five most enriched motifs for REF6, CTCTGTTT and GAAGAAGA, were also found in BRM targets (Figure 22A). In fact, 44% of BRM- and 81% of REF6-associated sites contain the CTCTGTTT motif, while 24% of BRM- and 34% of REF6-associated sites contain the GAAGAAGA motif. The data suggest that these two proteins could target nearby or the same cis element in the genome.

Whether the BRM-specific binding motifs (motifs not shared by REF6) are shared by other transcription factors was also examined. Interestingly, one BRM-specific motif, G/AAG/AGAGA, was found to be a motif for ANGUSTIFOLIA3 (AN3) (Figure 22B), which has been recently identified as a BRM interacting protein during leaf development.
Figure 21: Co-Occupancy of BRM and REF6 over a Large Number of Genomic Regions

(A) A Venn diagram displaying a statistically significant overlap between genes occupied by BRM and those by REF6 (1277;* P< 7×10^{-162}, according to the hypergeometric test).

(B) Heatmap representation of the co-occupancy of BRM and REF6 in the genome. The heatmap at the left shows the binding intensity of each REF6-bound region. At the same time, the binding intensity of BRM at each REF6-bound region was also calculated to make the heatmap at the right. The horizontal lines of the heatmap represent each of REF6-bound regions. Columns indicate the nucleotide position around each peak summit. Binding intensity is indicated by shades of red. The middle red regions in the both heatmaps support BRM binding occurred at many of REF6-binding sites. At the same time, the red region in BRM sample was wider than that of REF6 sample, which suggested that REF6 had sharper peak width than BRM. The detailed description of generating heatmap can be found in section 2.7.

(C) ChIP-seq genome-browser views of BRM and REF6 co-occupancy at selected genes. Gene structures are shown underneath each panel.

(D) ChIP-qPCR validation of BRM and REF6 occupancy at common targets using independent ChIP samples. Data are shown as percentage of input. Pro35S:GFP plants and TA3 locus were used as negative control sample and locus, respectively. Error bars indicate standard deviations from three biological replicates.

(E) Gene Ontology analysis of the BRM-REF6 co-target genes showing that BRM and REF6 co-regulate a large number of genes involved in responses to stress. The inset figure shows genes involved in plant responses to hormones are enriched among BRM-REF6 co-target genes.
Table 2: Overlapping Analysis of BRM-REF6 Co-Target Genes as well as REF6 Dependent BRM Target Genes with Different Epigenetic Marks

<table>
<thead>
<tr>
<th>Active material (Covered genes)</th>
<th>Overlapped BRM-REF6 targets (Percentage of overlap)</th>
<th>BRM-REF6 random overlap (Percentage of overlap)</th>
<th>P value</th>
<th>Overlapped REF6-dependent BRM targets (Percentage of overlap)</th>
<th>REF6 random overlap (Percentage of overlap)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMm5ac201 (2,460)</td>
<td>686 (70%)</td>
<td>225 (41%)</td>
<td>0.04-102</td>
<td>137 (20%)</td>
<td>1138 (35%)</td>
<td>3.6e-15</td>
</tr>
<tr>
<td>HMm5ac14 (2,202)</td>
<td>623 (65%)</td>
<td>335 (57%)</td>
<td>4.2e-07</td>
<td>70 (40%)</td>
<td>1278 (41%)</td>
<td>0.001</td>
</tr>
<tr>
<td>HMm5ac15 (2,073)</td>
<td>790 (65%)</td>
<td>445 (55%)</td>
<td>1.3e-07</td>
<td>84 (42%)</td>
<td>65 (25%)</td>
<td>0.055</td>
</tr>
<tr>
<td>HMm5ac18 (2,383)</td>
<td>251 (18%)</td>
<td>102 (6%)</td>
<td>3.2e-08</td>
<td>74 (12%)</td>
<td>18 (96%)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Repressive number (Covered genes)</th>
<th>Overlapped BRM-REF6 targets (Percentage of overlap)</th>
<th>BRM-REF6 random overlap (Percentage of overlap)</th>
<th>P value</th>
<th>Overlapped REF6-dependent BRM targets (Percentage of overlap)</th>
<th>REF6 random overlap (Percentage of overlap)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMm5ac2 (1,430)</td>
<td>62 (18%)</td>
<td>91 (40%)</td>
<td>0.06</td>
<td>21 (1.1%)</td>
<td>0 (4.8%)</td>
<td>0.06</td>
</tr>
<tr>
<td>HMm5ac2 (7,533)</td>
<td>74 (9%)</td>
<td>365 (59%)</td>
<td>0.03</td>
<td>98 (12%)</td>
<td>40 (25%)</td>
<td>0.08</td>
</tr>
<tr>
<td>HMm5ac2 (7,140)</td>
<td>12 (1.4%)</td>
<td>48 (3.5%)</td>
<td>0.08</td>
<td>2 (1.5%)</td>
<td>7 (3.5%)</td>
<td>0.32</td>
</tr>
</tbody>
</table>

The gene lists for all histone modifications were described in Luo et al., 2013.
Figure 22: DNA Motifs Associated with BRM and REF6.

(A) Targeting sites of BRM, REF6, and BRM-REF6 co-targets were used for de novo motif discovery using MEME-ChIP (see section 2.8 for details). Five most significantly enriched motifs are shown. Boxes highlight the common motifs shared by BRM and REF6. Percentage of peaks containing a given motif is shown under each motif. P values were determined by MEME-ChIP.

(B) Motifs shared between BRM or REF6 and known transcription factors.
**A**

<table>
<thead>
<tr>
<th>Motif</th>
<th>Bound by</th>
<th>Transcription Factor</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCTGT</td>
<td>MYC2, MYC3, MYC4</td>
<td>Fernández-Calvo et al., 2011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BZR1, BES1</td>
<td>Oh et al., 2012</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PIF3, PIF4, PIF5</td>
<td>Hornitschek et al., 2012</td>
<td></td>
</tr>
<tr>
<td>GAAGA</td>
<td>HY5</td>
<td>Zhang et al., 2011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FHY3</td>
<td>Ouyang et al., 2011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PIL5</td>
<td>Oh et al., 2006</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABF1</td>
<td>Choi et al., 2000</td>
<td></td>
</tr>
<tr>
<td>CACGG</td>
<td>BRM</td>
<td>AN3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Verzruysen et al., 2014</td>
<td></td>
</tr>
<tr>
<td>GAAGA</td>
<td>BRM</td>
<td>ERF1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lorenzo et al., 2003</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheng et al., 2013</td>
<td></td>
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</tbody>
</table>

**B**

Motifs Shared Between BRM or REF6 With the Known Transcription Factors
Yet another BRM-specific motif, the G-box motif (CACGT/CG) (Figure 22B), was also shared by a number of important transcriptional factors involved in light and hormone responses, including MYC2, MYC3, and MYC4 (Fernández-Calvo et al., 2011; Kazan and Manners, 2013), BZR1 and BZR2 (Oh et al., 2012), PIF3, PIF4 and PIF5 (Hornitschek et al., 2012), HY5 (Zhang et al., 2011), FHY3 (Ouyang et al., 2011), PIL5 (Oh et al., 2009) and ABF1 (Choi et al., 2000). REF6-specific motifs were also compared to the public database using the TOMTOM program (see section 2.8). It was observed that one REF6-specific motif, CC(A/G)CC(A/G), was shared by the Arabidopsis ETHYLENE-RESPONSE-FACTOR1ERF1 (ERF1) (Figure 22B), an important upstream component of jasmonic acid (JA) and ET signaling, which are involved in pathogen resistance and stress response (Lorenzo et al., 2003; Cheng et al., 2013).

Motifs for BRM-REF6 co-target genes were also derived and found that the two shared motifs by BRM and REF6 were also highly overrepresented in BRM-REF6 co-target genes (Figure 22A). The observation that BRM and REF6 share a common set of targeting consensus sequences indicates that they localize to the same or neighboring sites in the genome and this is consistent with their extensive co-occupancy in the Arabidopsis genome.

### 3.2.4 Physical Association between BRM and REF6 in *Planta*

Given the fact that BRM and REF6 co-localize on many genes, I further tested the physical association between these two proteins in plant cells. To this end, a BiFC assay was performed using full length REF6 and BRM. REF6 was fused with the N-terminal portion of the yellow fluorescent protein (YFP) (*REF6-nYFP*), and BRM was fused to the C-terminal portion of YFP (*BRM-cYFP*). When *REF6-nYFP* and *BRM-cYFP* were cotransformed into *Nicotiana benthamiana* leaves, a clear yellow fluorescence signal was observed (Figure 23A and 23B). As the negative controls, no interaction was observed, when *REF6-nYFP* and *cYFP*, or *BRM-cYFP* and *nYFP* were cotransformed, suggesting the specificity of the interaction between BRM and REF6. To map out the domains
Figure 23: Physical Association of BRM with REF6

(A) Diagrams of the conserved domains of BRM (top) and REF6 (bottom). The putative function of each domain in BRM can be found in Farrona et al. (2004). The domains of REF6 have been described in section 1.3.

(B) BiFC assay showing interaction between BRM and REF6 in tobacco leaf cells. Images are overlays of fluorescence and bright field views. The yellow spots indicate YFP signal. nYFP and cYFP are N- and C-terminal portions of YFP, respectively. Scale Bar: 10 µm.

(C) Co-immunoprecipitation analysis showing interaction between BRM and REF6. Proteins extracted from tobacco leaves expressing BRM-AT-hook-YFP and REF6-4ZnF-HA, BRM-AT-hook-YFP alone, or REF6-4ZnF-HA alone were used for immunoprecipitation with anti-GFP antibody. Immunoblot was probed with anti-HA or anti-GFP antibodies. The asterisks indicate nonspecific bands.
A

B

C

<table>
<thead>
<tr>
<th>BRM-AT-hook-YFP</th>
<th>Input</th>
<th>IP: GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Anti-GFP

<table>
<thead>
<tr>
<th>Anti-HA</th>
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</table>

*
responsible for their physical association, several truncated versions of BRM and REF6 were generated, and BiFC assays were performed. The results revealed that the C-terminal part of REF6, which contains the four zinc finger domains (REF6-4ZnF), interacts with full length BRM, and that both the AT-hook and bromodomain of BRM interact with REF6 (Figure 23A and 23B). These observations indicate a specific interaction between BRM and REF6 in vivo.

To confirm the physical association between BRM and REF6 in planta, a Co-IP assay was carried out. The BRM-AT-hook-YFP and a HA-tagged version of REF6-4ZnF (REF6-4ZnF-HA) were co-expressed in tobacco leaves, and immunoprecipitation with anti-GFP antibody was performed. As shown in Figure 23C, REF6-4ZnF-HA protein was detected in the immunoprecipitated protein complex containing BRM-AT-hook-YFP by Western blotting. These results, when combined with the observations that BRM and REF6 were co-targeted to many common genes, strongly suggest that these two proteins could form a complex at common target genes.

### 3.2.5 REF6 Is Required for Genomic Occupancy of BRM at a Subset of Target Loci

Given the genome-wide co-localization of REF6 and BRM, and their physical association, I next determined whether their binding to genes is mutually dependent. In order to test whether the association of REF6 to chromatin is dependent on BRM, BRM activity was inactivated in ProREF6:REF6-GFP transgenic plants by crossing brm-1 mutant with ProREF6:REF6-GFP plants to obtain brm-1 ProREF6:REF6-GFP. Notably, loss of BRM activity had no effect on REF6 transcription (Figure 24A), as well as the nuclear localization of the REF6 protein (Figure 24B). Then ChIP-seq analysis was performed to compare the genome-wide occupancy profiles of REF6 in brm-1 mutant background to that in wild-type. It was shown that no loci in the brm-1 mutant were found with significant loss in REF6 occupancy. ChIP-seq data for a set of selected genes are shown in Figure 25A; these were further validated by ChIP-qPCR (Figure 25B). These data suggest that BRM is likely not required for the binding of REF6 to chromatin.
Figure 24: Imperceptible Change in REF6 Transcription in brm Mutant

(A) qRT-PCR analysis of REF6 transcript levels in brm mutant compared to wild-type. Error bars indicate standard deviations from three biological replicates.

(B) Confocal images of root tips showing nuclear localization of GFP-tagged REF6 in brm-1 and wild-type plants. Scale bar: 50µm
**A**

REF6 expression

Fold change

WT  brm-1

**B**

ProREF6:REF6-GFP  brm-1 ProREF6:REF6-GFP
Figure 25: REF6-Dependent Recruitment of BRM to Genomic Loci

(A) ChIP-seq genome-browser views of REF6 occupancy at selected loci in wild-type and brm-1 mutant plants. Gene structures are shown underneath each panel.

(B) REF6 occupancy at selected genes as determined by ChIP using anti-GFP antibody in brm-1 ProREF6:REF6-GFP and ProREF6:REF6-GFP plants. ChIP signals are shown as percentage of input. TA3 was used as a negative control locus. Error bars indicate standard deviations from three biological replicates.

(C) ChIP-seq genome-browser views of BRM occupancy in wild-type and ref6-1 mutant plants. These are the same genes as those in Figure 2B. Gene structures are shown underneath each panel.

(D) Decreased BRM occupancy at selected genes as determined by ChIP-qPCR using anti-GFP antibody in ref6-1 ProBRM:BRM-GFP and ProBRM:BRM-GFP plants. ChIP signals are shown as percentage of input. TA3 was used as a negative control locus. AT2G17550, a gene showing no change of BRM occupancy in brm compared to wild-type based on the ChIP-seq data, was also included as a control. Error bars indicate standard deviations from three biological replicates.

(E) Motifs derived from REF6-dependent BRM targets. Percentage of peaks with a given motif is shown under each motif. P values were determined by MEME-ChIP program.
Figure 26: Undetectable Change in BRM Transcription in *ref6* Mutant

(A) BRM transcription in *ref6* mutant compared to wild-type. Error bars indicate standard deviations from three biological replicates.

(B) Confocal images of root tips showing nuclear localization of GFP-tagged BRM in *ref6-1* and wild-type plants. Scale bar: 50µm
Next, I investigated if the occupancy of BRM on chromatin would be dependent on REF6. Again, ChIP-seq analyses were carried out by comparing the BRM occupancy profiles in ref6 mutant to that in wild-type (ref6-1 ProBRM:BRM-GFP vs ProBRM:BRM-GFP). A substantial loss of BRM occupancy (more than a two-fold decrease) in a total of 199 genes in the absence of REF6 was found (Figure 25C), indicating that the association of BRM with a subset of its target genes requires REF6. Notably, the loss of REF6 did not affect the transcription level of BRM nor its nuclear localization (Figure 26A and 26B), excluding the possibility of BRM occupancy decrease as a result of decreased BRM abundance in the nuclei. Importantly, all 199 genes were direct REF6 targets, suggesting that BRM recruitment at REF6 targets is indeed controlled by the physical presence of REF6. The ChIP-seq data, showing REF6-dependent BRM targeting, were also validated by ChIP-qPCR experiments using independent plant materials (Figure 25D). Interestingly, among the 199 REF6-dependent BRM target genes, only half showed increased H3K27me3 levels in ref6 mutant (Lu et al., 2011), indicating that the decreased BRM occupancy upon loss of REF6 protein is not necessarily accompanied by an increase in H3K27me3. Together, these results demonstrate that REF6 mediates the recruitment of SWI/SNF complex to a subset of its target genes, and that its own genomic targeting is independent of SWI/SNF.

It was also interesting to determine if there are any specific targeting motifs that could be derived from these 199 genes. As shown in Figure 25E, the MEME-ChIP search results showed that CTCTGT/CTT and GAAGAAGA were the two enriched cis elements for the REF6-dependent BRM targets. These two motifs have been found to be the common targeting consensus sequences shared amongst BRM and REF6 targets and the BRM-REF6 co-targets as described above in Figure 22A.

3.2.6 BRM and REF6 Activate a Common Set of Target Genes

It was reasoned that if REF6 cooperates functionally with BRM, the two proteins should regulate the expression of a common set of target genes. To test this hypothesis in an unbiased manner, RNA-seq analysis of the global transcriptome landscapes in brm-1,
ref6-1, and brm-1 ref6-1 backgrounds was performed and compared to that in wild-type plants. In total, 203 genes showed at least a two-fold decrease of expression in the brm mutant, while 49 genes showed up-regulation. In the ref6 mutant, there were 69 and 8 genes that were down- and up- regulated, respectively. In the brm-1 ref6-1 double mutant, 399 genes and 63 genes exhibited decrease and increase in regulation, respectively. When the cut-off was lowered to 1.5-fold, these numbers were roughly doubled. When comparing the differentially expressed genes in brm-1 and ref6-1 mutants, it was found that there was a significant overlap between down-regulated (Figure 27A) but not between the up-regulated genes (Figure 28A) in the two mutants. In addition, there was no significant overlap between down-regulated genes in brm-1 and up-regulated genes in ref6-1, or vice versa (Figure 28A). These data suggest that BRM and REF6 preferentially activate a common set of genes.

To unambiguously define commonly regulated genes by the BRM and REF6, I further compared the ChIP-seq data with the RNA-seq data. There was a significant overlap between BRM-REF6 co-associated genes and the down-regulated genes in brm-1, ref6-1, or brm-1 ref6-1 double mutants (Figure 27B and 28B). In contrast, the overlap between BRM-REF6 co-associated genes and the up-regulated genes in brm-1, ref6-1, or brm-1 ref6-1 mutants was not significant (Figure 28C), indicating that BRM and REF6 preferentially co-bind expressed genes. Furthermore, there was also a significant overlap between 199 REF6-dependent BRM targets and the down-regulated genes (Figure 27C), but not those up-regulated (Figure 28D) in brm-1 ref6-1 double mutants. The RNA-seq data were further validated by qRT-PCR on a set of selected genes (Figure 27D). Double mutant analysis further indicated that there was no additive effect on expression at most of the examined genes (i.e., AT1G76590, SNRK2.8, NPF6.4, CYP707A1, NAC079, and AT5G51670) (Figure 27D). These results suggest that BRM function in the same pathway with REF6 to activate expression of some genes. In summary, a significant overlap between the down-regulated genes in mutants and BRM-REF6 co-associated genes was observed, suggesting that BRM and REF6 directly co-activate a set of common target genes. This conclusion is consistent with the findings noted above, where both BRM and REF6 tend to co-localize with histone modification marks associated with active genes (Table 2).
Figure 27: Expression of BRM-REF6 Co-Target Genes in \textit{brm}, \textit{ref6}, and \textit{brm ref6} Backgrounds

(A-C) Venn diagrams showing statistically significant overlaps between genes down-regulated in \textit{brm} and those in \textit{ref6} (A), between BRM-REF6 co-bound genes and genes with reduced expression in \textit{brm ref6} (B), and between REF6-dependent BRM target genes and genes with reduced expression in \textit{brm ref6} (C).

(D) qRT-PCR analysis showing decreased expression of selected genes in \textit{brm-1}, \textit{ref6-1}, and \textit{brm-1 ref6-1} plants compared to wild-type. The expression level of each gene was normalized to that of \textit{GAPDH}. The expression level in Col was set as 1. Error bars indicate standard deviations from three biological replicates.
Figure 28: BRM and REF6 Directly Co-Activate the Expression of a Set of Common Genes

(A) Venn diagrams showing a statistically significant overlap between genes up-regulated in brm and ref6.

(B) Venn diagrams showing a statistically significant overlap between BRM-REF6 co-bound genes and genes with reduced expression in brm or ref6.

(C) Venn diagrams showing no statistically significant overlap between BRM-REF6 co-bound genes and genes with induced expression in brm or in ref6 or brm ref6.

(D) A Venn diagram showing no statistically significant overlap between REF6-dependent BRM target genes and genes with induced expression in brm ref6.
3.2.7 BRM and REF6 Coordinately Regulate a Wide Range of Cellular Activities and Biological Processes

The data presented above clearly demonstrate that BRM and REF6 co-localize at over a thousand genes across the *Arabidopsis* genome. Binding of BRM to a subset of these co-target genes (i.e. the 199 genes described above), depends on REF6, suggesting a concerted action of these two global regulators. Functional classification of these 199 REF6-dependent BRM target genes, based on Gene Ontology categories, indicated that these two proteins coordinately regulate a range of biological processes and cellular activities (Figure 29A and 29B). With regard to the biological processes, genes involved in responses to environmental and endogenous stimuli, including stress, light, radiation, salicylic (SA) and ABA, were highly enriched. Tests were also carried out to determine whether mutations of BRM or REF6 would cause altered plant responses to ABA. In one such tests, cotyledon greening was chosen as an indicator of seedling response to ABA (Han et al., 2012). Indeed, it was shown that *ref6* mutants displayed reduced cotyledon greening compared to wild-type under ABA treatment (Figure 29C and 29D), indicating that these mutants were more sensitive to ABA. *brm* mutants also displayed hypersensitivity to ABA (Figure 29C and 29D), which is consistent with a previous study (Wu et al., 2012).

In terms of cellular processes, genes involved in transcription, cellular transport and cellular signaling were enriched. According to the genome annotation at The *Arabidopsis* Information Resource (TAIR), approximately 80 out of the 199 genes (40%) have been experimentally studied and their functions have been elucidated (Figure 29B). These experimentally characterized functions provide direct evidence and mechanisms for roles of BRM and REF6 in regulating a wide range of cellular, developmental, and regulatory processes.
Figure 29: Functional Categorization of BRM and REF6 Co-Target Genes.

(A) Gene Ontology analysis of the 199 REF6-dependent BRM target genes. Numbers, on the top of each blue bars are P values (hypergeometric test) for GO category enrichment generated by comparing the percentage of the corresponding categories in genes that showed decreased BRM occupancy in ref6 mutant with those in the whole genome.

(B) Representative REF6-dependent BRM target genes with known functions in various cellular processes and response/regulatory pathways.

(C) and (D) Both brm and ref6 mutants are hypersensitive to ABA. Seedlings were grown on MS plates in the absence or presence of ABA for 11 days. The percentage of green cotyledons was determined from four independent replicates, each with an average of >100 seeds. Error bars indicate the standard deviations among these replicates. Asterisks indicate significant differences compared to wild-type as determined by Student’s t test (n=4, P <0.001).
Chapter 4 Discussion

4.1 BRM Antagonizes PcG Activity

In both animals and plants, a group of proteins that counteract PcG function has been described and referred to as TrxG proteins (Schwartz and Pirrotta, 2007; Bemer and Grossniklaus, 2012). Several putative TrxG proteins have been proposed in Arabidopsis, including the H3K4 methyltransferase ATX1 (Alvarez-Venegas et al., 2003), the SAND-domain DNA binding protein ULTRAPETALA1 (ULT1) (Carles and Fletcher, 2009), the chromatin remodeling ATPase PICKLE (PKL) (Aichinger et al., 2009), the H3K27me3 demethylase REF6 (Lu et al., 2011), and the SWI2/SNF2 ATPases SPLAYED (SYD) and BRM (Wu et al., 2012). A challenge for this field is to understand the specific roles of the putative TrxG proteins and their functional relationship in antagonizing PcG.

4.1.1 BRM Is Necessary for Preventing High Levels of H3K27me3 on Many Genes

The nature of the antagonism between SWI/SNF-type chromatin remodeling ATPases and PcG proteins has been investigated in several studies in animals and several models have been proposed (Tamkun et al., 1992; Shao et al., 1999; Kia et al., 2008; Wilson et al., 2010; Ho et al., 2011). Interestingly, a recent report in Arabidopsis showed that BRM could overcome the Polycomb repression on AG and AP3 loci during the initiation of floral development (Wu et al., 2012). However, how this is done and to what extent BRM is required to antagonize the PcG function in plants remains unclear. The genome-wide study performed in this thesis work shows that BRM deficiency leads to an increase in H3K27me3 levels at several hundred genes across the genome during vegetative development in Arabidopsis. This appears to be a result, at least in some cases, of an “inappropriate” invasion of the PcG complex at loci where BRM would normally be located. Therefore, a model of antagonism between BRM and PcG is being proposed, wherein the binding of BRM prevents or decreases PcG binding to a group of genes.
Figure 30: A Model of BRM Preventing Inappropriate PcG binding at the SVP Locus to Promote the Vegetative Growth

In wild-type plants, BRM is physically present at target chromatin sites, preventing PcG binding and maintaining the expression of the target loci. Without BRM, PcG is allowed to access certain genomic sites, resulting in increased H3K37me3 levels, which suppresses gene expression. For example, SVP is highly expressed in wild-type seedlings and its downstream target FT is repressed, therefore, vegetative growth is promoted. Conversely, the expression of SVP is repressed by mistargeting PRC2 in brm mutants. As a result, FT is de-repressed leading to the early flowering phenotype. Red stars: the H3K27me3 mark.
including SVP (Figure 30). Taken together, the genome-wide data and the data showing increased PcG binding upon loss of BRM indicate that BRM keep certain genes active by preventing them from being targeted by PcG in Arabidopsis. Notably, the fact that CLF presence was not increased at some genes upon loss of BRM (Figure 5D) suggests another possible way of antagonism between BRM and PcG: BRM might inhibit the H3K27 methyltransferase activity of PcG proteins, rather than prevent their genomic targeting.

In addition to chromatin remodeler BRM, plants might employ transcription factors to counteract PcG activities. A recent study showed that the binding of transcription factor AG to the promoter of zinc finger repressor KNUCKLES (KNU) causes the eviction of the PcG proteins from the locus, leading to the induction of KNU (Sun et al., 2014). Thus, both transcription factor and chromatin remodeling protein could be involved in counteracting PcG. It will be interesting to determine whether and how these two machineries work together in antagonizing PcG function.

### 4.1.2 BRM Might Co-Operate with PcG in Regulating Gene Expression

Genome-wide analysis of H3K27me3 occupancy in brm mutant indicates that BRM not only antagonizes PcG function during plant development, but also cooperates with PcG at certain loci (Figure 4D and 4F-H). For example, the level of H3K27me3 is decreased at WRKY23 whose expression is up-regulated in both brm (this study) and fie mutant (FIE is a PcG subunit) (Bouyer et al., 2011), suggesting that both BRM and PcG are required for repressing the expression of WRKY23. Furthermore, the data here show that the decreased H3K27me3 level observed at the WRKY23 locus in brm mutant could be due to, at least partly, the decrease in CLF binding. Therefore, this observation suggests that BRM may work with PcG proteins at certain common loci and thus, represses the target’s expression. WRKY23 was recently found to be essential for proper root development and the over-expression of WRKY23 results in the reduction of root length (Grunewald et al., 2012). It will be interesting to test whether the increased transcription of WRKY23 could explain the short root phenotype of brm (Hurtado et al., 2006). Consistent with this
observation, the synergistic relationship between BRM and PcG reported here was observed in a study using human embryonic stem cell which showed that an embryonic stem cell specific SWI/SNF complex acts synergistically with PRC2 at all four Hox loci (Ho et al., 2011). The mechanism by which BRM cooperates with PcG is currently unknown. One possibility would be that BRM directly interacts with PcG and facilitates the targeting of PcG to genes. Indeed, it was found that BRM co-localizes with H3K27me3 at the WRKY23 locus in wild-type seedlings (see Figure 5D and Figure 9), suggesting that BRM might interact with PcG proteins. However, no study thus far has demonstrated a direct physical interaction between BRM and PcG proteins. It is possible that these two complexes might interact transiently or indirectly. Nevertheless, the apparent, synergistic relationship between BRM and PcG, as found in both animals and plants, suggest its biological relevance and warrants further studies.

4.1.3 BRM Is a Direct Activator of SVP

The proper transition from vegetative growth to flowering is a critical step for the reproductive success of angiosperm plants and must be precisely controlled. BRM has been proposed as a repressor of flowering as suggested by the early flowering phenotype and elevated FT expression of brm mutants (Farrona et al., 2004; Farrona et al., 2011). However, it is unclear whether BRM acts directly or indirectly to repress FT. SVP has been demonstrated to be a direct repressor of FT (Lee et al., 2007a; Li et al., 2008), serving as a key repressor of floral transition. The precise regulation of SVP is obviously of critical importance for our understanding of flowering control. However, no direct upstream activator(s) of SVP has been identified thus far. In this thesis work, several lines of evidence are provided demonstrating that BRM represses FT by maintaining a high level of SVP expression (Figure 30). First, loss of BRM activity results in decreased expression of SVP (Figure 10A-E), which is associated with increased H3K27me3 level (Figure 4F and 4G) and increased occupancy of CLF at the SVP locus (Figure 5D). Second, BRM binds to the SVP locus in vegetative tissues, where SVP is highly expressed (Figure 9B). Together, these observations suggest that BRM represses the
floral transition through activating SVP transcription. This is further supported by genetic evidence, in which brm-1 svp-31 double mutant displays a similar early flowering phenotype as both brm-1 and svp-31 single mutants (Figure 13A and 13B).

Although the data in this thesis support a scenario that BRM represses flowering mainly through SVP, some other evidence suggests that BRM may also repress flowering through other pathways or genes. Indeed, the expression of CONSTANS (CO), an activator of FT in the photoperiod pathway, was increased in brm mutants (Farrona et al., 2011).

In addition to CO and FT, elevated expression of FLC in brm mutants was also reported previously (Farrona et al., 2004; Farrona et al., 2011). Since FLC is a repressor of FT expression (Helliwell et al., 2006), it seems difficult to understand why the expression levels of both FLC and FT were increased in brm mutants. The results presented in this thesis provide an explanation for this apparent discrepancy: mutation of BRM results in reduced expression of SVP and consequently lower abundance of the SVP-FLC repressor complex, ultimately leading to activation of FT, regardless of the increased expression of FLC.

It is also relevant to note that down-regulation of BAF60/SWP73A was recently reported to cause increased FLC expression and delayed floral transition (Jégu et al., 2014). The apparently opposing flowering time phenotype of brm mutants and the BAF60 knockdown line is puzzling. It is unknown whether and how BAF60 regulates SVP expression. It might be possible that the presence of BAF60 in a SWI/SNF complex inhibits the activity of BRM, thus reduction of BAF60 could allow BRM to activate SVP expression, which, in turn, leads to delayed floral transition. Alternatively, it might also be possible that BRM and BAF60 are present in distinct complexes that differ in their regulatory activities and target genes, e.g., BRM activates SVP, while BAF60 represses FLC.

Finally, it was shown here that loss of BRM activity leads to precocious flowering at low temperature (16 °C) (Figure 15), suggesting that BRM also modulates flowering in response to ambient temperature. It is tempting to speculate that BRM participates in the
activation of the SVP transcription at lower temperature by preventing H3K27me3 deposition, similar to its function at high temperature. Further experiments are required to better understand how BRM participates in the ambient temperature pathway.

4.1.4 A Potential Pathway Consists of BRM-miR166-PHB-Seed Storage Protein

The genome-wide H3K27me3 profiling data also reveal that BRM is involved in the regulation of a number of other important developmental genes including, most noticeably, members of the miR166 family (Figure 4G and 4F). It is well established that members of the miR166 family target the transcripts of PHABULOSA (PHB) and PHAVOLUTA (PHV) genes, controlling the level and tissue of their expression to allow their proper functions in plant development (McConnell et al., 2001; Emery et al., 2003; Mallory et al., 2004). More recently, our lab uncovered a new role for miR166 in repressing the seed maturation program during vegetative development (Tang et al., 2012). It was shown that PHB directly targets the promoter of seed maturation gene LEAFY COTYLEDON 2 (LEC2) and activates its expression (Tang et al., 2012). Based on these results, the authors concluded that miR166 represses seed maturation genes by controlling PHB message RNA, whose product is an upstream activator of seed maturation genes. An earlier study in our lab demonstrated the involvement of BRM in repression of seed maturation genes in leaves (Tang et al., 2008) – a brm mutation was isolated in a reporter-assisted genetic screen for Arabidopsis mutants exhibiting ectopic expression of seed storage protein genes (Tang et al., 2008; Lu et al., 2010; Tang et al., 2012). However, the underlying mechanism remains unclear. The data presented in this thesis suggest a potential link between the two early studies (Tang et al., 2008; Tang et al., 2012): i.e. they suggest that BRM promotes the accumulation of miR166, which in turn represses seed maturation genes by reducing the PHB level in developing seedlings. Further investigation will be necessary to test this hypothesis. In conclusion, the work in this thesis demonstrates that BRM promotes vegetative development by harnessing PcG proteins (by mainly preventing their activities) at key developmental genes.
4.2 BRM and REF6: Partners in Regulating Gene Expression

Both SWI2/SNF2 chromatin remodeling complexes and H3K27 demethylases are thought to be capable of activating gene expression. Although earlier findings in animal models suggested that these two machineries might co-localize and cooperate in gene activation (Miller et al., 2010; Tie et al., 2012), the interdependence between them has not been comprehensively addressed on a genome scale. Here, I show that BRM interacts with a plant-unique H3K27 demethylase REF6 and that they share a large number of common target genes. Furthermore, I demonstrate that the targeting of BRM to hundreds of genes, many of which are involved in responses to various endogenous and exogenous stimuli, is REF6 dependent.

4.2.1 Both BRM and REF6 Overlap with Active Histone Marks

REF6 is an H3K27 demethylase, and thus, can be classified as a transcriptional activator. The genome-wide association data for REF6, as presented in this thesis, revealed that among the differentially expressed genes in ref6 mutant seedlings, 42% (32 genes) were bound by REF6 and were therefore considered as REF6 directly regulated genes (Figure 31). The vast majority of these genes (97%) were REF6-activated genes (Figure 31, Appendix B), supporting the predicted role of REF6 mainly as a transcriptional activator. Consistent with its role, REF6 tends to localize to regions enriched with histone marks usually associated with highly expressed genes such as H3K4me2, H3K4me3, H3K9ac, and H3K18ac, but not to regions marked with repressive histone marks such as H3K27me1, H3K27me3, and H3K9me2 (Table 1). These data are also in line with findings in mammals that UTX interacts with H3K4 methyltransferase and promotes gene activity (Cho et al., 2007; Issaeva et al., 2007; Lee et al., 2007b). On the other hand, among the differentially expressed genes in brm-1 mutant, about 21% (53 genes) were direct BRM targets (Figure 31). Out of the 53 genes, 81% were activated and 19% were repressed by BRM (Figure 31, Appendix B), suggesting that BRM mainly acts as an activator. Consistent with this finding, I also noticed that BRM target sites greatly overlap regions with active modifications (Table 1). These findings concerning the role
of BRM in modulating gene activity in Arabidopsis are consistent with the documented transcriptome data from yeast and animals, as well as the positive roles of SWI/SNF complexes in transcription (Clapier and Cairns, 2009; Ho and Crabtree, 2010). SWI/SNF complexes are considered as nucleosome remodelers. They hydrolyze ATP to conduct reactions affecting the positioning and density of nucleosomes, which in turn, affect the access of DNA-binding factors to their cognate sites. DNA-binding proteins can be transcriptional repressors or activators. In this context, it seems difficult to comprehend the apparent overlap between BRM and active histone marks. The simplest explanation could be that BRM binding is facilitated by these histone modifications at a subset of its targets. Indeed, such a role for histone acetylation has been demonstrated through its direct interaction with the BRM bromodomain (Hassan et al., 2001; Farrona et al., 2007; Chatterjee et al., 2011).

4.2.2 REF6 Facilitates the Recruitment of BRM to Chromatin

How SWI2/SNF2 chromatin remodelers are recruited to specific sites on chromatin still remains a crucial question. Previous studies in mammals and Drosophila have shown that UTX and JMJD3 interact with SWI2/SNF2 proteins BRM and BRG1 (Miller et al., 2010; Lee et al., 2012; Shpargel et al., 2012; Tie et al., 2012), but the consequences of this interaction are not yet fully understood. The genome-wide studies described here clearly indicate that histone H3K27 demethylase REF6 is a factor that facilitates the recruitment of BRM to hundreds of genes in Arabidopsis. DNA motif analysis shows that BRM and REF6 share common cis elements and support the dependence of BRM recruitment on REF6. At this point, the key question is how REF6 contributes to the recruitment of BRM. One possibility is that the common DNA motifs for BRM and REF6 provide binding sites for a transcription factor(s) that, in turn, recruits REF6, and finally brings BRM to target loci. It is noteworthy, however, that REF6 contains four zinc fingers at its C-terminus, which presumably have DNA-binding capability (Lu et al., 2008). Therefore, another possibility is that REF6 directly binds to DNA motifs, without any help from additional DNA-binding factors, and then recruits BRM. The observation that not just
Figure 31: Directly Regulated Genes by REF6 or BRM

A Venn diagram (left) showing a statistically significant overlap between genes mis-regulated in ref6 mutant and genes bound by REF6. A statistically significant overlap between genes mis-regulated in brm mutant and genes bound by BRM was also shown in A Venn diagram (right).
one, but two common consensus DNA motifs were found, from the 199 REF6-dependent BRM target genes (Figure 24E), is apparently in support of the first scenario. However, the second possibility cannot be completely eliminated. Further work is required to sort out which possibility is correct. Meanwhile, the finding that the enrichment of BRM at some REF6-associated sites is substantially but not completely lost in the absence of REF6 (Figure 24D), suggesting that there may be other factor(s) involved in the recruitment of BRM. Consistent with this finding, several transcription factors have also been identified as required in facilitating the recruitment of SWI/SNF. For example, a forkhead box O (FOXO) transcription factor DAF-16 in *C. elegans* has a role in recruiting SWI/SNF to target promoters (Riedel et al., 2013). In *Arabidopsis*, several transcription factors, including AN3, SEPALLATA3 (SEP3) and LFAFY (LYF), were also reported to recruit SWI/SNF remodeler to some chromatin sites (Wu et al., 2012; Vercruyssen et al., 2014). These observations together suggest that the recruitment of SWI/SNF to chromatin is complex and may require more than one factor, which is consistent with the roles of BRM in diverse aspects and phases of plant growth and development. Potential cross-talks between REF6 and other factors (for example, AN3, SEP3 and LFY found in *Arabidopsis*) in recruiting SWI/SNF complexes will be important issues to investigate in the future.

It is interesting to note that motifs targeted by BRM in plants, as described here, apparently show no similarities to those in mammals (Ho et al., 2011; Morris et al., 2014) or *C. elegans* (Riedel et al., 2013), suggesting that BRM homologs in different organisms have adopted distinct partners in diverse cellular contexts to facilitate their genomic localization. This is somewhat surprising and unclear why these differences exist. In addition, it is also worth mentioning that genomic motifs have not yet been reported for any H3K27 demethylases in animal model organisms.

One question still remains as to how REF6 activates gene expression in concert with BRM. In mammalian cells, several recent studies have uncovered an H3K27-demethylase activity-independent role of UTX in regulating gene expression (De Santa et al., 2009; Miller et al., 2010; Lee et al., 2012; Shpargel et al., 2012; Vandamme et al., 2012; Wang et al., 2012). Whether REF6 has a demethylase activity-independent function is currently
unknown. However, it was observed that among the 199 REF6-dependent BRM target genes, half of them do not show increase in H3K27me3 levels in ref6 mutant. The data thus suggest that REF6 might carry histone demethylase activity-independent functions at these loci, likely through recruiting BRM as a downstream effector. Consequently, this recruitment potentiates local chromatin remodeling to enable the binding of downstream transcription components and thereby induces transcription. On the other hand, another half of 199 genes show increased H3K27me3 levels and decreased BRM occupancy in ref6 mutant, which suggests that REF6 contains both the ability to demethylate H3K27 and the ability to recruit BRM at this group of loci. Whether the ability to demethylate H3K27 and the ability to recruit BRM are independent or intrinsically linked to each other at these loci will be an interesting issue to be addressed in the future.

4.2.3 Roles of BRM and REF6 in Stress Responses and Other Signaling and Developmental Processes

Both BRM and REF6 are targeted to several thousands of genes in the Arabidopsis genome (Figure 21). GO term analysis revealed that both proteins associate with a large number of genes involved in responses to stresses and plant hormones. Targeting of BRM-REF6 to stimuli/stress related-genes would predict that both brm and ref6 mutants should display enhanced or compromised tolerance to stress. Previous studies have shown the involvement of BRM in ABA and GA pathways (Wu et al., 2012; Archacki et al., 2013), while the role of REF6 in BR signal pathway has also been reported (Yu et al., 2008). Furthermore, most of the common target genes of BRM-REF6 are involved in plant responses to stress (Figure 21), suggesting that these two proteins may act together, at least partially, in stress responses. Supporting this implication, similar to brm mutants, ref6 mutants are also hypersensitive to the drought stress hormone ABA (Figure 29C). Considering the fact that plants are sessile, the finding in this thesis is in agreement with the notion that plants have evolved a chromatin-mediated flexible mechanism to respond to environment (Chinnusamy and Zhu, 2009; Boyko and Kovalchuk, 2011; Luo et al., 2012). Under normal growth conditions, the co-occupancy of BRM and REF6 on stress-
related genes may enable plants to rapidly respond to stress signals. Conversely, this mechanism might ensure that the response pathway to stress is quickly turned off as soon as the stress signals vanish. Thus, BRM-REF6 may play an important role during plant in facilitating stress responses during plant development.

In addition to their possible common roles concerning stress responses as discussed above, BRM and REF6 each may also play unique roles in diverse signaling and developmental processes. This is reflected by their respective unique subsets of target genes and associated cis elements (Figure 22B). Specifically, the data here revealed that several BRM unique motifs are also binding motifs of many other transcription factors that are involved in leaf development and photomorphogenesis (Figure 22B). These data suggest that BRM may functionally cooperate with these transcription factors. Future detailed dissection of the potential physical and functional interplays, between BRM and the signaling and developmental related transcription factors, will greatly enhance our understanding of the roles of BRM in plant growth and development. In summary, the genome-wide data reported in this thesis reveal concerted actions of BRM and REF6 during vegetative development, and connect these two important epigenetic regulators to key signaling and developmental processes in Arabidopsis.
References


Appendices

Appendix A: Primers Used in This Thesis

Primers pairs for genotyping T-DNA mutants

*brm-1*-LP: 5’-AATATACGCTTGCTGCATTGG-3’
*brm-1*-RP: 5’-AGTTTATACCGTTGCATCCCC-3’
*clf-29*-LP: 5’-AAGAAACTTGCTAGTTCCGCC-3’
*clf-29*-RP: 5’-GAGGCATTGACTTTGATTTGC-3’
*sfp-31*-LP: 5’-TCCAATAACCACCAACCACACAG-3’
*sfp-31*-RP: 5’-TGATATCCAGATTACATCTTTTTT-3’
*ref6-1*-LP: 5’-TCATATACAAGGCGTGTCGTC-3’
*ref6-1*-RP: 5’-CAGTTGCAACTCTTGAGAAGG-3’
LB: 5’-GCGTTGACCCTTTGCTGCAACT-3’

Gene-specific primers pairs for qRT-PCR

AT1G06980-F: 5’-AGGACACTCTCCTCGCCGGAAG-3’
AT1G06980-R: 5’-TCGACGCCGACGACACTACT-3’
AT1G54740-F: 5’-CATGCGGAGGGTGGAGACCGA-3’
AT1G54740-R: 5’-TTCTCGCGCTTGACCAACCAC-3’
AT3G22160-F: 5’-ACAACCACCACCTGCGGGA-3’
AT3G22160-R: 5’-CGGACCCGAAAGCCATACGC-3’
AT4G37540-F: 5’-TCCAACGTCCTGCTTTGTTTCAG-3’
AT4G37540-R: 5’-TCACAGGAGATCATCAACGACG-3’
SVP-F: 5’-TTCCATTTCAGTCTTGTCA-3’
SVP-R: 5’-GAATCTTTTCTCTCGCCATCAC-3’
BEL1-F: 5’-AATCCTACAAGCAGCATGCTCC-3’
BEL1-R: 5’-CTTCCCTTTTACTCGGACAGC-3’
TCP2-F: 5’-TTCTCGGTCACTTAAGGACCACG-3’
TCP2-R: 5’-GGAAATAGTGGTGTTGAGTGA-3’
AT5G33390-F: 5’-ATATCCAAATCCCAACAAAT-3’
AT5G33390-R: 5’-AACTAGGTACGGGTCTTGTC-3’
WRKY23-F: 5’-ACTACCCGTCGTCACAAAGC-3’
WRKY23-R: 5’-CAAAGTCTTGATGCTGCTGAG-3’
BRM-F: 5’-TTTAGAAAGGAAAGGATTAGGC-3’
BRM-R: 5’-GCCGTTCGATAAACCCTCA-3’
AP1-F: 5’-GAAGGCCATACAGGAGCAAA-3’
AP1-R: 5’-ACTGCTCCTGTGAGCCTCA-3’
FT-F: 5’-CTTGGCAGGCAAACAGTGTATGCAC-3’
FT-R: 5’-GCCACTCCTCCCTCTGACAATTGAGTGA-3’
FIE-F: 5’-CGTTTCTTGATGTCTTCTCGT-3’
FIE-R: 5’-ACGACTCTCTCCTATCTTCATCAG-3’
EMF2-F: 5’-CAGAAGACTGAAGTAACTGAAGAC-3’
EMF2-R: 5’-AAATGAGGAGATCGTGTTG-3’
VRN2-F: 5’-GCAGAAATAACACCAGAGGAGAC-3’
VRN2-R: 5’-CCACCGTTCTCCATCTTCAGC-3’
CLF-F: 5’-ATTATTGCATGACCCTTTGAG-3’
CLF-R: 5’-CATGTCTTGCCTTGATTTAC-3’
SWN-F: 5’-CAGGGAGATGATAATGATGAGG-3’
SWN-R: 5’-GACCCAGCAGACTTTGAGAG-3’
SMZ-F: 5’-AGGGAGAAGGAGCCATGAAGTTGTTG-3’
SMZ-R: 5’-GCTCTTCAGAGGTTTCTGATGTCCATG-3’
AGL24-F: 5’-CAGAGTCCGGTGACCACAAATG-3’
AGL24-R: 5’-ATGGAAGCCTAACCACAAAC-3’
AT1G76590-F: 5’-TCGATTCTTTCCGTTTCTGCTC-3’
AT1G76590-R: 5’-TCTCTTCCATGCTCCCTCTCAAA-3’
SNRK2.8-F: 5’-TGGAGAGGTACCCTAGTGAAGG-3’
SNRK2.8-R: 5’-TTGGCCTCGCTCGATGAGTT-3’
NPF6.4-F: 5’-GGAGCCAAAGATGGCTGTCA-3’
NPF6.4-R: 5’-TTAAACCGCGCTACACCAT-3’
CYP707A1-F: 5’-CCAAACTCCACTCCCTCGG-3’
CYP707A1-R: 5’-CGAAACTTAGCAGCGCTTCGACTC-3’
YUC3-F: 5’-TGGCTCGGGACAGAAGACTA-3’
YUC3-R: 5’-TTTATGATTCCGGGGACGATTTT-3’
NAC079-F: 5’-CAACCTTGCCCTAAAAACCGCTAAG-3’
NAC079-R: 5’-ACCGATTCGGATTAACGTCG-3’
AAP2-F: 5’-CAAGTTCAGATTTTGTGGGTGTTGA-3’
AAP2-R: 5’-GCTTATGGAAGCTGCTATCGT-3’
AT5G51670-F: 5’-GGCCAGCCTCCGTTCTTCTT-3’
AT5G51670-R: 5’-ATATCGCCCATTTCTCCATCTCT-3’
AAP4-F: 5’-GGTTCCAAGATTTGTTGGGCTG-3’
AAP4-R: 5’-TAGATGCTGCGATTGCTTACC-3’
BRM-F: 5’-TTTAGAAAAGGAAAAGGATTAGGC-3’
BRM-R: 5’-GCCGTTCCGATAACCTCA-3’
REF6-F: 5’-TGAGTCAGAAGATAATGGCG-3’
REF6-R: 5’-CTATTCGTTTGGCTTGTGAC-3’
GAPDH-F: 5’-CTTGGAAAGGAGCTAAGGAC-3’
GAPDH-R: 5’-ATGTGTTTCCCTGCACCTTCT-3’

Oligonucleotide probes for detecting microRNAs
miR156: 5’-TGACAGAAGAGAGTGAGCCCTTC-3’
U6: 5’-GAGAAGATTAGCATGGCCCCTTC-3’

Primers for the XVE::aMIRBRM transgene construct
BRMmiR-s-I: 5’-GATACAAATTTGCGGTACGGCGTTCTCTCTTTTGTATTCC-3’
BRMmiR-a-II: 5’-GAACGCGCTACCGCAAATTTGTATCAAAGAGAATCAATGA-3’
BRMmiR*s-III: 5’-GAACACCGGTACCGCATATTTGCACAGGTCGTGATATG-3’
BRMmiR*a-IV: 5’-GAAACAAATATGCGGTACGGGTCTACATATATTCCT-3’
BRMmiR-A: 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCGCGATTAAGTTGGGGTAAC-3’
BRMmiR-B:
5’-
GGGGACCACTTTGTACAAAGAAAGCTGGGTCGGGATAAAATCCACACAGG AAACAG-3’

Primers for generating constructs used in BiFC and Co-IP
REF6-attB-F: 5’-GGGGACAAGTTTGTACAAAGAAAGCTGGGTCGGGATAAAATCCACACAGG AAACAG-3’
ATGGGCTTCAAGGACTGCTGGT-GAT-3’
REF6-attB-R: 5’-GGGGACCACTTTGTACAAAGAAAGCTGGGTCGGGATAAAATCCACACAGG AAACAG-3’
CCTTTGTGCTTCTTCTAAACCGGA-3’
REF6-ZnF-attB-F: 5’-GGGGACAAGTTTGTACAAAGAAAGCTGGGTCGGGATAAAATCCACACAGG AAACAG-3’
ACCAGGTCAACAGCCAAACG-3’
BRM-attB-F: 5’-
GGGGACAAGTTTGTACAAAGAAAGCTGGGTCGGGATAAAATCCACACAGG AAACAG-3’
BRM-attB-R: 5’-GGGGACCACTTTGTACAAAGAAAGCTGGGTCGGGATAAAATCCACACAGG AAACAG-3’
TAAATGGGCTAGGCGTCTTTTACCAG-3’
BRM-attB-F5: 5’-
GGGGACAAGTTTGTACAAAGAAAGCTGGGTCGGGATAAAATCCACACAGG AAACAG-3’
BRM-attB-R5: 5’-GGGGACCACTTTGTACAAAGAAAGCTGGGTCGGGATAAAATCCACACAGG AAACAG-3’
BRM-attB-F6: 5’-
GGGGACAAGTTTGTACAAAGAAAGCTGGGTCGGGATAAAATCCACACAGG AAACAG-3’
BRM-attB-R6: 5’-
GGGGACAAGTTTGTACAAAGAAAGCTGGGTCGGGATAAAATCCACACAGG AAACAG-3’

Primers for generating ProREF6:REF6-GFP vector
REF6-P-attB-F: 5’-GGGGACAAGTTTGTACAAAGAAAGCTGGGTCGGGATAAAATCCACACAGG AAACAG-3’
CACCTAAAAAAGCAGCTGGGTCGGGATAAAATCCACACAGG AAACAG-3’
REF6-attB-R: 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTC
CCTTTTGTGGTCTTCTTAACCGAA-3’

**Primers used for ChIP-qPCR analyses**

AT1G06980-F: 5’-AGGACACTCCTCCTGCGGGAAG-3’
AT1G06980-R: 5’-TCGACGCGACGACACTACT-3’
AT1G54740-F: 5’-GTATCTATCTTGTACCCCTTCT-3’
AT1G54740-R: 5’-CATCCCTTTGCTTGTGGT-3’
AT3G22160-F: 5’-ACAACCACCACCTGCGGTA-3’
AT3G22160-R: 5’-CGGACCCGAAAGCCCATAGCC-3’
AT4G37540-F: 5’-ATTTCGATGCTCTCAAACCGA-3’
AT4G37540-R: 5’-GAAGGCAAGGACGAGGATGC-3’
SVP-F-1: 5’-CAACGGCGGACGAACTTGAG-3’
SVP-R-1: 5’-GAGGAAAGAAAGACCTGGAGC-3’
BEL1-F: 5’-AATCTCAACAGCCTGAGCT-3’
BEL1-R: 5’-CTTTCCCTTACTGCAAAGACTC-3’
TCP2-F: 5’-TGTGGACTTTCAAGGACCAGC-3’
TCP2-R: 5’-GGAAATGAGTGTGTTGTGATG-3’
AT5G33390-F: 5’-ATATCCAAATCCTCCAATA-3’
AT5G33390-R: 5’-AACTAGTTACGGGTCTTGTC-3’
miR156D-F: 5’-TCAATCTTTGTTAGTTGCTG-3’
miR156D-R: 5’-ATTAGTCCAGAAACCGATGAA-3’
WRKY23-F: 5’-ACTACCCGTCGTCACAAAAGC-3’
WRKY23-R: 5’-CAAATCCTTCTGCTGCTGAG-3’
ACTIN 2/7-F: 5’-CGTTTCGCTTTTCCCTTATGTTGT-3’
ACTIN 2/7-R: 5’-CTTGAAGAAAGAAGAAGATGATA-3’
AG-F: 5’-ATGCTGAAGTCGCACTCATCGTCT-3’
AG-R: 5’-GAGCAAGACAGGACGAGAAAGAAGGACCASTG-3’
SMZ-F: 5’-CGAAGATCGTGATGCTGATGTTG-3’
SMZ-R: 5’-CCGACGACATGCTGACACG-3’
AGL24-F: 5’-CTTTGCGATGCTGATGTTG-3’
AGL24-R: 5’-GATCTCCGAGCCTACTGATAAT-3’
AT1G76590-F: 5’-TCGATTTCTTCCGTGGTTGCTCTC-3’
AT1G76590-R: 5’-TCTCTTCCATGCTTCCCTCTCAAA-3’
SNRK2.8-F: 5’-TGGAGAGGTACGAAATAGTGAAAG-3’
SNRK2.8-R: 5’-TTGGCCTCGCTCGATGAAC-3’
NPF6.4-F: 5’-GGAGCCAAAGATGGCTCTGA-3’
NPF6.4-R: 5’-TAAACCGGCCTAACC-3’
CYP707A1-F: 5’-AAGGTTACAATTTCATGACCAGTA-3’
CYP707A1-R: 5’-TGTTCGTGTGTTAGCTCTTCTT-3’
YUC3-F: 5’-TGGCTCGCGGACAAAGACTA-3’
YUC3-R: 5’-TTTGATTCTCCCGGGACGATTTT-3’
NAC079-F: 5’-CAAATCTGCAATGAAAACGCTAAG-3’
NAC079-R: 5’-GCAGATCCACCATTCAATC-3’
AAP2-F: 5’-CAAGTTCAAGATTTGTTGGTTGTT-3’
AAP2-R: 5’-GCTTATGGAAGCTGCTATCGT-3’
AT5G1670-F: 5’-GGCCAGCCCTCGTTCTT-3’
AT5G1670-R: 5’-GATCGCCATTCCATCTCTCTC-3’
AAP4-F: 5’-GGTTCAAGATTTTGTTGGGCTG-3’
AAP4-R: 5’-TAGATGCTGGATTTGCGG-3’
TA3-F: 5’-GATTTACTGTAAGAACAATGGCATTGAGAG-3’
TA3-R: 5’-TCCAAAATTTCTGAGGCTTGTGTAACC-3’
AT2G17550-F: 5’-CCGTACTTTGGATGAGATGTTGT-3’
AT2G17550-R: 5’-AGAAGAAGAAAGAATTGGGGAGAG-3’
### Appendix B: List of Genes Directly Regulated by REF6 or BRM

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Curriculum Vitae

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Fu Jiarui Scholarship
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Submitted Manuscripts


**Published Manuscripts**


