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Functional analysis of Arabidopsis chromatin modification and remodeling regulators (CHR5 and JMJ15) in gene expression

Yuan Shen

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THÈSE DE DOCTORAT

Soutenance prévue le 28/05/2014

par

Yuan SHEN

**Functional analysis of *Arabidopsis* chromatin
modification and remodeling regulators
(CHR5 and JMJ15) in gene expression**

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ABBREVIATIONS

ABI3	Abscisic acid Insensitive3
ARP	Actin-Related Protein
BAH	Bromo-Adjacent Homology
CBP	CREB-Binding Protein
CLF	Curly Leaf
CHD	Chromodomain Helicase DNA binding domain
ChIP	Chromatin ImmunoPrecipitation
CMT	Chromomethylase
CRC	Cruciferin C
DCL3	Dicer-like3
DDM1	Decreased in DNA Methylation 1
DME	DEMETER
DML2, 3	DEMETER like 2, 3
DNA	DeoxyriboNucleic Acid
DNMT1	DNA Methyltransferase 1
DRM2	Domains Rearranged Methyltransferase2
ES	Embryonic Stem
E(z)	Enhancer of zeste
EMF	Embryonic Flower
FAD	Flavin Adenine Dinucleotide
FIE	Fertilization Independent Endosperm
FIS2	Fertilization Independent Seed 2
FLC	Flowering Locus C
FT	Flowering Locus T
GNAT	Gcn5-related N-terminal Acetyltransferase
GUS	β -Glucuronidase
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HIS	High-level expression of Sugar-Inducible gene
HMT	Histone Methyltransferase
HR	Homologous Recombination
IBM1	Increased in BONSAI Methylation 1
JMJ	Jumonji domain containing protein
KYP	KRYPTONITE
LEA	Late Embryogenesis Abundant
LEC1, 2	Leafy Cotyledon1, 2

LHP1	Like Heterochromatin Protein 1
LSD1	Lysine Specific Demethylase1
MBD	Methyl CpG-Binding Domain
MEA	MEDEA
MEE27	Maternal Effector Embryo arrest 27
MET1	Methyltransferase1
MSI1-5	Multicopy Suppressor of IRA 1-5
NFR	Nucleosome Free Region
NuRD	Nucleosome Remodeling and Deacetylase
PHD	Plant Homeodomain
PIC	Pre-initiation complex
PIE1	Photoperiod-Independent Early flowering1
PKL	PICKLE
POL	Polymerase
PRC1, 2	Polycomb Repressive Complex1, 2
PTGS	PostTranscriptional Gene Silencing
PTM	PostTranslational Modification
RdDM	RNA-directed DNA Methylation
RDR2	RNA-Dependent RNA Polymerase 2
REF6	Relative Early Flowering 6
RNA	Ribonucleic Acid
ROS1	Repressor of Silencing 1
RPD3	Reduced Potassium Dependency 3
SAM	Shoot Apical Meristem
SET	Su(var)3-9 E(z) TRX
SIR2	Silent Information Regulator 2
SSP	Seed Storage Protein
SWI/SNF2	Switch/Sucrose Non-Fermenting
SWN	SWINGER
Su(var)3-9	Suppressor of variegation
TE	Transposable Elements
TF	Transcription Factors
TRX	Trithorax
TSS	Transcription Start Site
UBP	Ubiquitin Protease
VRN	Vernalization

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CHAPTER 1

GENERAL INTRODUCTION

Epigenetics and epigenetic regulation

The term "epigenetics" (epi meaning over or above) was coined by C. H. Waddington in 1942 as a portmanteau of the words epigenesis and genetics. He used it as a conceptual model of how genes might interact with their surroundings to produce a phenotype. Then Robin Holliday defined "epigenetics" as "the study of the mechanisms of temporal and spatial control of gene activity during the development of complex organisms". Thus epigenetic can be used to describe anything other than DNA sequence that influences the development of an organism. Recently, a consensus definition of the epigenetic trait, "stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence", was made at a Cold Spring Harbor meeting (Berger et al., 2009). In general, epigenetics is a fascinating new field in the genetic sciences and a brief history of major discoveries in epigenetics is shown in Figure 1.

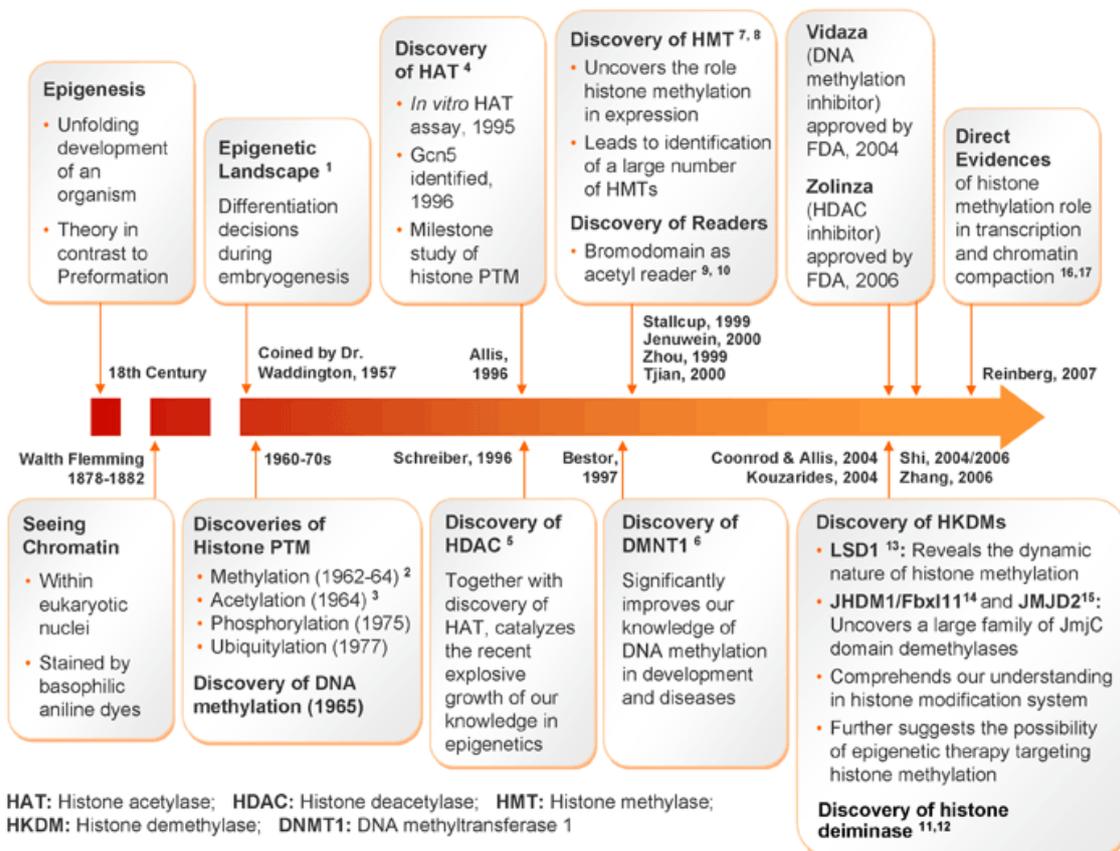


Figure 1. Timeline of epigenetics study (<http://www.epigenetic.us/disco.htm>)

Epigenetic regulation is the process by which a gene's activity is modulated through covalent modifications to the DNA, the histones around which it wraps, or the physical packaging of the chromatin in which it is embedded. Mechanisms of epigenetic regulation are likely to have originated as a defense against parasitic DNAs, such as transposons and viruses, but they are also used to control the expression of many genes essential for development or environmental responses. In this chapter, I will focus on the mechanism and role of epigenetic regulation in plant gene expression and development.

1. 1 Chromatin organization

Chromatin is the combination or complex of DNA and its associated proteins, which makes up the contents of the nucleus in eukaryotes. The basic unit of chromatin is the nucleosome, constituted of 147 bp of double stranded super helical DNA wrapped around an octamer formed by two copies each of four basic proteins called histones (H2A, H2B, H3, H4) (Figure2) (Richmond and Davey, 2003). Repeating nucleosomes with intervening "linker" DNA are packed into a higher-order structure of 30 nm filaments. The 30 nm fiber is arranged into loops along a central protein scaffold to form chromosome (Figure 2).

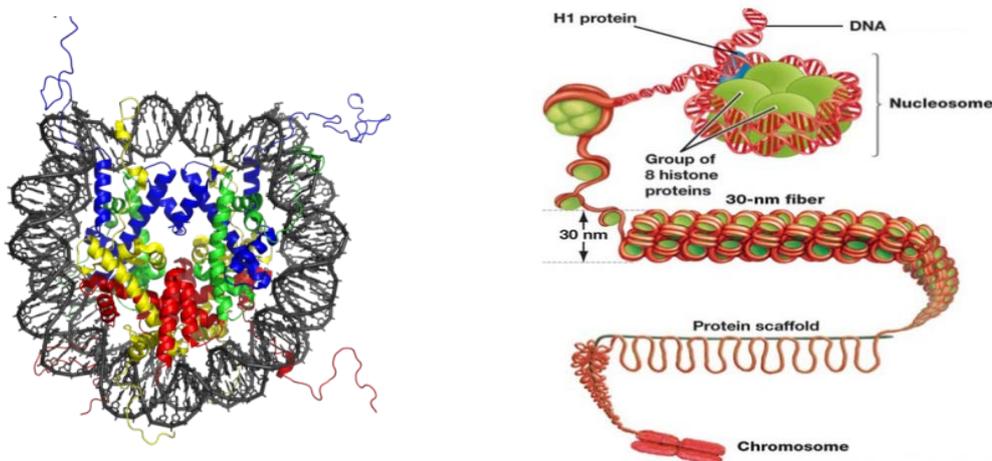


Figure 2. Nucleosome structure. Left: The crystal structure of the nucleosome core particle consisting of H2A (yellow), H2B (red), H3 (blue) and H4 (green) core histones and DNA. Right: The major structure in DNA compaction: nucleosome, 30 nm fiber and chromosome.

Chromatin can be roughly divided into two states: (i) active or open state called euchromatin and (ii) silent or condensed chromatin state called heterochromatin. Euchromatin is generally gene rich, transcriptionally active and contains only few repetitive elements. Constitutively expressed genes in plants and other organisms typically reside within euchromatic regions and often have nucleosome free regions within their promoters (Rando and Ahmad, 2007; Zhang et al., 2007b). On the other hand, constitutive heterochromatin is rich in repetitive DNA, such as transposons and other duplicated sequences, permanently condensed, generally capable of silencing genes (Elgin and Grewal, 2003).

1. 2 Nucleosome positioning

Nucleosome positioning is a dynamic process. Owing to the rapid progress of high-throughput array and sequencing techniques, it is possible to detect the global nucleosome positioning map in diverse organisms and identify the positions of individual nucleosomes at a specific time. For instance, the genome-wide nucleosome positioning maps of some model organisms including yeast, worms, flies and humans have been completed (Yuan et al., 2005; Lee et al., 2007; Mavrich et al., 2008; Schones et al., 2008; Valouev et al., 2008; Li et al., 2011). These results show that although most genomic DNA is occupied by nucleosomes, some functional regions, such as promoters, enhancers and terminators, are depleted of nucleosomes. In addition, nucleosomes at most genes are organized in basically the same pattern: a nucleosome free region (NFR) near the transcriptional start site (TSS), flanked by two well-positioned nucleosomes (the -1 and +1 nucleosomes) and followed by a nucleosomal array that packages the gene (Figure 3). The -1 nucleosome located upstream of TSS covers a region from -300 to -150 which regulates the accessibility of promoter regulatory elements in that region. The -1 nucleosome will process changes during transcription such as histone replacement, acetylation and methylation, as well as transcription repositioning and ultimately eviction after pre-initiation complex (PIC) formation (Li et al., 2011). The +1 nucleosome displays the strongest

positioning which often includes histone variants (H2A.Z and H3.3) and histone modifications such as methylation and acetylation. During transcription the +1 nucleosome might be evicted for PIC assembly. Nucleosomes at the 5' end of the gene are generally better localized than those in the middle. It is reported that nucleosome positioning is mainly determined by the intrinsic DNA sequence, while the NFR is determined mainly by the binding of transcription factors (TF) (Ozonov and van Nimwegen, 2013; Struhl and Segal, 2013).

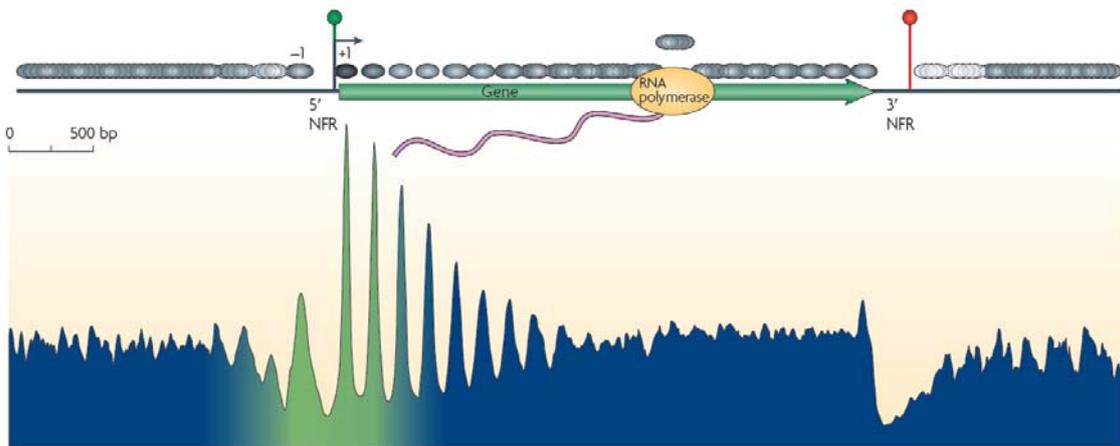


Figure 3. Nucleosomal landscape of yeast genes. The distribution of nucleosomes in a gene is shown. The peaks and valleys represent similar positioning relative to TSS. The green shading represents high H2A.Z levels and histone modification (Jiang and Pugh, 2009).

Nucleosome positioning plays both positive and negative roles in transcription via modulating accessibility of DNA to proteins. To initiate the transcription, the transcription start site must be made available to the transcription machinery. During elongation, RNA polymerase II (Pol II) must overcome the transcriptional barriers imposed by nucleosomes in chromatin. Therefore, nucleosomes positioned at promoter region influence the initiation of transcription, and relocation of nucleosomes has a dramatic effect on transcription rates (Parthasarthy and Gopinathan, 2006; Choi et al., 2009; Hodges et al., 2009).

1. 3 Histone modification

Histones are small alkaline proteins (11 to 21.5 kDa), which are highly conserved from yeast to humans. Histones are composed of a globular carboxyl (C)-terminal domain and a protruding amino (N)-terminal “tails” that are mainly targeted by numerous posttranslational modifications (PTM). These modifications are usually limited to several amino acids, for example lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, and lysine ubiquitination and sumoylation. Histone modifications can be abbreviated as the histone name, the position of the mark, and the nature and number of the marks. For example, H3K4me3 is a trimethylation of Histone H3 Lysine 4 (K4) of. At least eight types of histone modifications have been characterized to date (Table 1) (Kouzarides, 2007).

Chromatin Modifications	Residues Modified	Functions Regulated
Acetylation	K-ac	Transcription, Repair, Replication, Condensation
Methylation(lysines)	K-me1 K-me2 K-me3	Transcription, Repair
Methylation(arginines)	K-me1 K-me2a K-me2s	Transcription
Phosphorylation	S-ph T-ph	Transcription, Repair, Condensation
Ubiquitylation	K-ub	Transcription, Repair
Sumoylation	K-su	Transcription
ADP ribosylation	E-ar	Transcription
Deimination	R>Cit	Transcription
Proline Isomerization	P-cis>P-trans	Transcription

Table 1. Overview of different classes of modification identified on histones (Kouzarides, 2007).

Histone modifications at many sites are conserved in plants, however there also exist a few unique histone modification sites in *Arabidopsis* (Zhang et al., 2007a). For example, acetylated H4K20, H2BK6, H2BK11, H2BK27, H2BK32, and H2AK144 and monoubiquitinated H2BK143 are found in *Arabidopsis* but not in human or yeast cells, whereas H3K79me, highly conserved and functioning in telomeric silencing in non-plant systems, is not modified in *Arabidopsis* (Zhang et al., 2007a). Although most of the known histone modifications occur on the N-terminal tails of histones, exceptions include monoubiquitination of the C-terminal tails of

H2A and H2B and acetylation of H2AK144. Figure 4 shows the main known histone acetylation (ac), methylation (me) and ubiquitination (ub) sites in *Arabidopsis*.

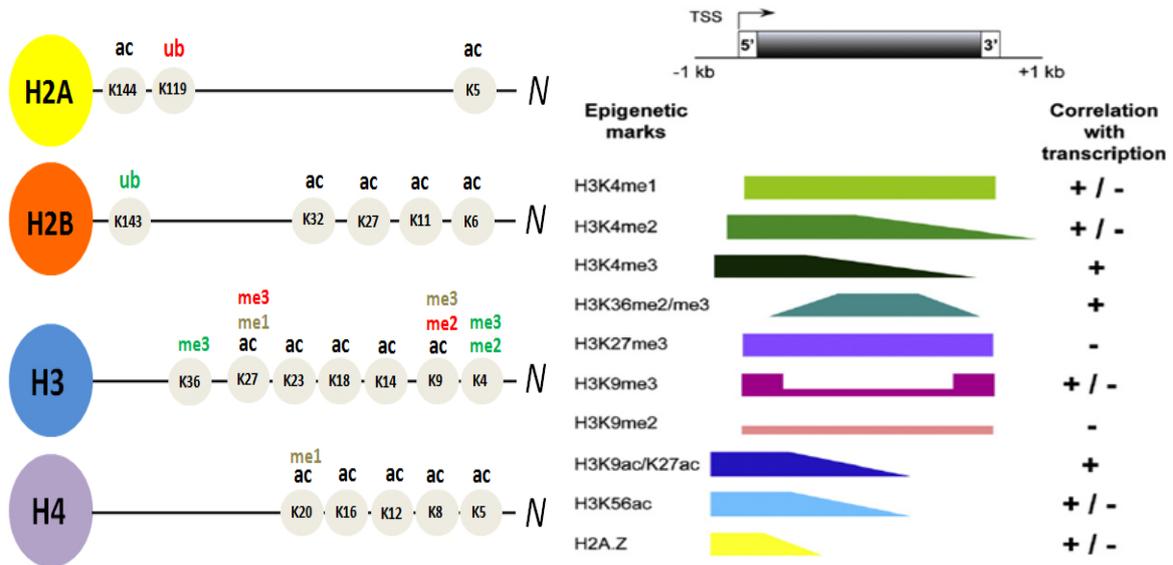


Figure 4. Major histone modification in *Arabidopsis*. Left: Histone acetylation (ac), methylation (me) and ubiquitination (ub) sites on core histones are shown. Green is generally associated with transcriptional activation. Red is often related to transcriptional repression. Right: Genome wide distribution pattern of histone modifications from a transcription perspective (Lauria and Rossi, 2011)

Genome wide analyses show that histone modifications particularly on H3 are related to gene expression (Figure 4). In *Arabidopsis* it is found that H3K9ac and H3K27ac are almost exclusively located within genes and invariably correlate with transcriptional activation, with both marks being enriched towards 5' end of genes and peaking around transcriptional start site (TSS) (Charron et al., 2009; Zhou et al., 2010). The distribution of H3K56ac is similar to that of H3K9ac and H3K27ac, but it is not correlated with active transcription and seems to be a mark of transcriptional competence (Tanurdzic et al., 2008). Histone methylation is more complicated, as it can either activate or repress gene expression depending on the location of the lysine and the number of methyl groups added. Here below I will discuss the role of histone acetylation/deacetylation, histone methylation/demethylation and histone ubiquitination/deubiquitination function in plant gene expression.

1.3.1 Histone acetylation and deacetylation

Among these post-transcriptional modifications, histone acetylation was first reported and most well characterized. Histone acetylation is a dynamic, reversible process that is highly conserved among eukaryotes. It involves the transfer of an acetyl group from acetyl-CoA to ϵ - amino group of lysine residues in all core histones, mainly at the tails but also at a few residues within the globular domain (Berger, 2007). The lysine acetylation neutralizes the positive charge of histone and therefore decreases their affinity for negatively charged DNA. Histone acetylation can also be recognized by histone modifications readers such as bromo-domain proteins (Filippakopoulos and Knapp, 2012), which promote the recruitment of additional ATP-dependent chromatin remodelers and chromatin modifier complexes, establishing a relaxed chromatin that facilitates the recruitment of RNA polymerases and gene expression (Bannister and Kouzarides, 2011). Histone acetylation which relaxes chromatin structure is often associated with active gene transcription. On the other hand, histone deacetylation which induces chromatin compaction is related to gene repression (Berger, 2007). The level of histone acetylation is catalyzed by the activity of both histone acetyltransferases (HATs) and histone deacetylases (HDACs).

1.3.1.1 Plant histone acetyltransferases (HATs)

Based on primary homology with their yeast and mammalian counterparts, plant HATs are classified into four types, the GCN5-RELATED N-TERMINAL ACETYLTRANSFERASES (GNAT), MOZ1 YBF2/SAS3 SAS2 TIP60 (MYST), CREB-BINDING PROTEIN (CBP/P300), and TATA-BINDING PROTEIN-ASSOCIATED FACTOR II 250 (TAFII250) types (Pandey et al., 2002). The *Arabidopsis* genome is predicted to encode 12 histone acetyltransferases, including three GNAT family genes (*HAG1/AtGCN5*, *HAG3/ELP3*, and *HAG2*, respectively), two MYST family genes (*HAG4* and *HAG5*), five p300/CBP family genes (*HAC1*, *HAC2*, *HAC4*, *HAC5* and *HAC12*), and two TAF1 genes (*HAF1* and *HAF2*). *AtGCN5* is shown to have a histone H3 acetyltransferase activity *in vitro*

(Earley et al., 2007) and the global H3 acetylation is reduced in *Atgcn5* mutants by Western blot (Bertrand et al., 2003). *In vivo* AtGCN5 is found to affect the acetylation of H3K9, H3K14, H3K27, and H4K12 on the target promoters (Benhamed et al., 2006). AtGCN5 is involved in many plant development pathways such as meristem function, leaf cell differentiation, leaf and floral organogenesis, and responses to environmental conditions such as light and cold (Bertrand et al., 2003; Benhamed et al., 2006; Kim et al., 2009; Kornet and Scheres, 2009; Servet et al., 2010), in accordance with the fact that *AtGCN5* is required for both long-term developmental gene and short-term inducible gene expression (Benhamed et al., 2008). AtELP3/ELO3/HAG3 as a component of the Elongator complex is reported to interact with MINIYO (IYO) to activate RNA polymerase II (RNAPII) transcriptional elongation (Nelissen et al., 2010; Sanmartin et al., 2011). AtELP3 is found to be involved in auxin-related process by regulating H3K14 acetylation of auxin activated genes (Nelissen et al., 2010). The *Arabidopsis* MYST-family HAM1 and HAM2 proteins were proved to specifically acetylate H4K5 *in vitro* (Earley et al., 2007) and to function redundantly to regulate gametophyte development and flowering transition *in vivo* (Latrasse et al., 2008; Xiao et al., 2013). *Arabidopsis* CBP/p300-like protein AtHAC1/PCAT2 is found to possess a HAT activity on core histones *in vitro* (Bordoli et al., 2001) and to be implicated in the control of flowering time and ethylene signaling pathway (Deng et al., 2007; Han et al., 2007; Li et al., 2014). Finally, TAF1 is found to participate in light signal by regulating H3 and/or H4 acetylation at some light-responsive loci (Bertrand et al., 2005).

1.3.1.2 Plant histone deacetylases (HDACs)

Similar to HATs, the HDACs in *Arabidopsis* are encoded by 18 genes and can be classed into three types, including Reduced Potassium Dependency 3/Histone DeAcetylase 1 (RPD3/HDA1), Silent Information Regulator 2 (SIR2) and the plant-specific Histone Deacetylase 2 (HD2) (Pandey et al., 2002). There are 12 RPD3/HDA1 genes, many of which have been characterized. For instance,

HDA19/HD1 down-regulation or overexpression results in a significant change of H3 and H4 acetylation (Tian et al., 2003; Zhou et al., 2005; Fong et al., 2006). Similarly, HDA6 is also found to deacetylate multiple lysines on H3 and H4 *in vitro*, however the total level of histone acetylation is only slightly affected in *hda6* mutants (Probst et al., 2004; Earley et al., 2006). HDA19 and HDA6 play redundant roles in embryonic and flower development, responses to environmental conditions such as JA/ethylene-mediated defense, ABA-mediated responses to drought or salinity (Zhou et al., 2005; Tanaka et al., 2008; Chen and Wu, 2010). Recently, several lines of evidence suggest that HDA6 and HDA19, like their counterparts in other eukaryotes, operate histone deacetylation within a large multi-protein complex (Perrella et al., 2013). HDA18 is found to have *in vitro* histone deacetylase activity and to be implicated in root epidermal patterning (Xu et al., 2005; Alinsug et al., 2012; Liu et al., 2013a). HDA15 negatively regulates chlorophyll biosynthetic and photosynthetic genes in dark by decreasing histone acetylation and RNAPII-associated transcription (Liu et al., 2013b). HDA14 is shown to deacetylate α -tubulin and partially retained on GTP/taxol-stabilized microtubules by direct association with the PP2A-A2 phosphatase (Tran et al., 2012). The SIR2 family contains two members (*SRT1* and *SRT2*) in *Arabidopsis* genome, which catalyze deacetylation via a reaction depending on NAD⁺ (Dali-Youcef et al., 2007). *SRT2* is a negative regulator of basal defense (Wang et al., 2010) and functions in mitochondrial energy metabolism by deacetylating organellar proteins (Konig et al., 2014). The HD2 family proteins are found exclusively in plants. AtHD2A, AtHD2B and AtHD2C are proposed to function in transcriptional gene repression during seed development, plant defense, and response to abiotic and biotic stresses (Sridha and Wu, 2006; Bourque et al., 2011; Colville et al., 2011; Luo et al., 2012; Grandperret et al., 2013; Yano et al., 2013). The main function and sublocalization of *Arabidopsis* HDACs is shown in Table 2.

Name	Accession	Localization	Function	Reference
RPD3/HDA1				
HDA1 (HDA19)	AT4G38130	Nucleus	embryonic and flower development, stress response, light response and basal defense	(Zhou et al., 2005)(Benhamed M, 2006)(Tanaka et al., 2008)(Choi et al., 2012)
HDA2	AT5G26040	Nucleus?	Unknown	(Alinsug et al., 2012)
HDA5	AT5G61060	Cytoplasm Nucleus ER	Binds 14-3-3 proteins; Involved in light response.	(Alinsug et al., 2012)
HDA6 (RPD3B)	AT5G63110	Nucleus Chloroplast envelope	embryonic and flower development, Jasmonate and salt signaling, silence rRNA genes, repress transgenes and transposable elements	(Probst et al., 2004)(Wu et al., 2008)(Chen et al., 2010)
HDA7	AT5G35600	Nucleus	Unknown	(Alinsug et al., 2009)
HDA8	AT1G08460	Cytoplasm	Unknown	(Alinsug et al., 2009)
HDA9	AT3G44680	Nucleus	Flowering	(Kim et al., 2013)
HDA10	AT3G44660	Nucleus	Unknown	
HDA14	AT4G33470	Mitochondria Cytosol	Association with PP2A-A2 phosphatase and microtubules	(Alinsug et al., 2012)(Tran et al., 2012)
HDA15	AT3G18520	Nucleus Cytosol	Negative control of chlorophyll synthesis Enhances Agrobacterium transformation	(Alinsug et al., 2012)(Crane and Gelvin, 2007)(Liu et al., 2013)
HDA17	AT3G44490	Nucleus	Unknown	
HDA18	AT5G61070	Nucleus Cytosol	Differentiation of hair and non- hair cells in the root epidermis	(Xu et al., 2005)(Alinsug et al., 2012)(Liu et al., 2013)
SIR2				
Sir1	AT5G55760		?	
Sir2	AT5G09230	Nucleus	Plant defense, mitochondrial energy metabolism	(Wang et al., 2010)(Koenig AC et al., 2014)
HD2				
HDT1(HD2A)	AT3G44750	Nucleus	Seed dormancy	(Colville et al., 2011)(Zhou et al., 2004)
HDT2(HD2B)	AT5G22650	Nucleus	Seed dormancy	(Yano et al., 2013)
HDT3(HD2C)	AT5G03740	Nucleus	ABA and salt stress signaling; Seed dormancy	(Sridha and Wu, 2006)(Colville et al., 2011)
HDT4(HD2D)	AT2G27840	Nucleus	ABA and salt stress signaling; Seed dormancy	(Luo et al., 2012b) (Luo, 2012)

Table 2. Summary of HDAC proteins characterized in plants (modified from Grandperret et al., 2013).

1.3.2 Histone methylation and demethylation

Histone methylation mainly occurs on lysines and arginines residues of histone N-terminal tails. Histone lysines can be mono-, di- or tri-methylated and arginines can be mono- or di-methylated, where the two methyl groups can be added to one (asymmetrical) or the two (symmetrical) amine groups of arginine (Bedford and Clarke, 2009; Black et al., 2012). Histone methylation has important roles in many biological processes, such as transcription, cell cycle, DNA repair, stress response, and heterochromatin formation (Mosammamaparast and Shi, 2010). In *Arabidopsis*, histone H3 methylation at K4, K9, K27 and K36 have been well characterized in recent years.

In *Arabidopsis*, H3K4 methylation is associated exclusively with genes and promoters (two thirds of all genes) and is absent from heterochromatic regions (Zhang et al., 2009). H3K4me1 is abundant in the body of genes, while H3K4me2 and H3K4me3 are enriched in promoter and 5' end regions, with H3K4me3 being further upstream of H3K4me2 (Zhang et al., 2009). Only H3K4me3 is associated with active gene transcription while H3K4me1 and H3K4me2 are not well correlated with gene transcription (Zhang et al., 2009). H3K4me3 promotes transcription through interaction with effectors including transcription factors to recruit RNAPII to target genes (Lauberth et al., 2013).

H3K27 methylation is a repressive mark. In plant, H3K27me1 is enriched at constitutive heterochromatin, while H3K27me3 is preferentially localized to the transcribed regions of genes, with an increase towards the 5' end (Zhang et al., 2007c; Roudier et al., 2011). About 17% of coding genes show H3K27me3, indicating that H3K27me3 is a major repressive mark for gene expression in *Arabidopsis* (Zhang et al., 2007c). Several well-known *Arabidopsis* developmental genes, including flower timing gene *FLOWERING LOCUS C (FLC)*, floral organ patterning gene *AGAMOUS (AG)*, homeobox gene *SHOOT MERISTEMLESS (STM)*, imprinted genes *MEDEA (MEA)* and *PHERES1 (PHE1)* and embryo identity genes *LEAFY COTYLEDON1 (LEC1)*, *LEAFY COTYLEDON 2 (LEC2)*, *ABSCISIC ACID-INSENSITIVE 3 (ABI3)*, *FUSCA3 (FUS3)* have been reported to be repressed by H3K27me3 (Schubert et al., 2006; Turck et al., 2007; Bouyer et al., 2011; Lafos et al., 2011). The expression level of H3K27me3-marked genes is very low suggesting transcription repression by H3K27me3 is alleviated only in the place where their expression is needed (Zhang et al., 2007c).

Histone H3K9 methylation in *Arabidopsis* predominantly occurs at H3K9me1 and H3K9me2, while some H3K9me3 can be detected mainly located in genes peaking at 5' and 3' ends as a mild activating transcription mark (Charron et al., 2009). H3K9me2 is abundant in pericentromeric heterochromatin as well as in transposons and repeated sequence region, consistent with its role in repression of

transposon elements (Bernatavichute et al., 2008). It is found that H3K9me2 and DNA methylation form a self-reinforcing loop in the maintenance of genome-wide transcriptional gene silencing and genome stability in *Arabidopsis* (Bernatavichute et al., 2008; Zhou et al., 2010; Du et al., 2012; Stroud et al., 2014).

H3K36me3 peaks in the first half of the coding region in *Arabidopsis*, in contrast to the 3' end localization reported in mammals (Wang et al., 2008; Roudier et al., 2011). In fact, H3K36me3 distribution in *Arabidopsis* is similar to that of H3K79me3 in other organisms (Wang et al., 2009; Zhou et al., 2011). In addition *Arabidopsis* lacks H3K79me3 modification and the H3K79 methyltransferase, it is possible that H3K36me3 in plants functions equivalently to H3K79me3 in mammals. Furthermore, H3K36me2 in *Arabidopsis* peaks at the 3' end of expressed genes, suggesting it could play a role similar to that attributed to H3K36me3 in other organisms (Oh et al., 2008; Roudier et al., 2011).

1.3.2.1 Plant histone methyltransferases (HMTs)

The homeostasis of the histone methylation is maintained by histone methyltransferases (HMTs) (Liu et al., 2010). HMTs usually contain a SET domain, named after the three *Drosophila* proteins: Suppressor of variegation (Su(var)3-9), Enhancer of Zeste (E(z)) and Trithorax (TRX). In *Arabidopsis*, 49 genes encoding putative SET domain-containing proteins have been identified and are divided into five categories (www.chromDB.org) (Ng et al., 2007a; Gendler et al., 2008; Thorstensen et al., 2011). Different classes of SET domain proteins, their histone methyltransferase specificity, interaction partners, and interacting domains are showed in Table 3.

CHAPTER 1—GENERAL INTRODUCTION

Class	Name (Arabidopsis)	SDG number (Arabidopsis)	AtGID	HMTase specificity	Method*	Interacts with	Interacting domain	References	
I-E(Z)	MEA	SDG5	At1g02580	H3K27me3	ChIP	FIE, FIS2	N-terminal (FIE)	(Makarevich et al., 2006); (Schubert et al., 2006); (Yadegari et al., 2000); (Spillane et al., 2000); (Wang et al., 2006)	
	CLF	SDG1	At2g23380	H3K27me3	ChIP	FIE, BLI	CXC(BLI)	(Makarevich et al., 2006); (Schubert et al., 2006); (Schatlowski et al., 2010); (Wang et al., 2006)	
	SWN	SDG10	At4g02020	H3K27me3	ChIP	FIE, FIS2, EMF2	C5 (EMF2)	(Makarevich et al., 2006); (Schubert et al., 2006); (Wang et al., 2006); (Chanvattana et al., 2004)	
II-ASH1	ASHH1	SGD26	At1g76710	H3; H4	ON			(Zhao et al., 2005); (Berr et al., 2009)	
	ASHH2/ESF	SDG8	At1g77300	H3K4me3; H3K36me2/me3	EH, ChIP, G	H3K4me1,H3 K4me2, H3K4me3	CW	Kim et al. 2005; (Ko et al., 2010); (Zhao et al., 2005); (Grini et al., 2009); (Hoppmann et al., 2011)	
	ASHH3	SDG7	At2g44150	–					
	ASHH4	SDG24	At3g59960	–					
	ASHR3	SDG4	At4g30860	H3K4me2; H3K36me2	CH	Aborted micro-spores (AMS)	PHD, SET	(Cartagena et al., 2008);(Thorstensen et al., 2008)	
III-TRX	ATX1	SDG27	At2g31650	H3K4me3	P, ChIP	PI5P, WDR5a, TBFII,CLF	PHD/ePHD	(Alvarez-Venegas et al., 2003); (Alvarez-Venegas and Avramova, 2005);(Alvarez-Venegas et al., 2006);(Jiang et al., 2009);(Jiang et al., 2011);(Ding et al., 2011);(Saleh et al., 2007)	
	ATX2	SDG30	At1g05830	H3K4me2	ChIP			(Saleh et al., 2008); (Pien et al., 2008)	
	ATX3	SDG14	At3g61740	–					
	ATX4	SDG16	At4g27910	–					
	ATX5	SDG29	At5g53430	–					
	ATXR3	SDG2	At4g15180	H3K4me1/me2/ me3	EH, RH, ChIP, G			(Berr et al., 2010); (Guo et al., 2010)	
	ATXR7	SDG25	At5g42400	H3K4me1/me2/	ON, ChIP			(Berr et al., 2009); (Tamada et al., 2009)	
	IV	ATXR5	SDG15	At5g09790	H3K27me1	EH, ChIP	PCNA	PIP Box	(Jacob et al., 2009)
ATXR6		SDG34	At5g24330	H3K27me1	EH, ChIP	PCNA	PIP Box	(Jacob et al., 2009)	
V-SU(VAR) 3-9	SUVH1	SDG32	At5g04940	–					
	SUVH2	SDG3	At2g33290	H3K9me1/me2; H4K20me; H3K27me2	EH	CG methylation	YDG/SRA	(Johnson et al., 2008); (Naumann et al., 2005); (Ay et al., 2009)	
	SUVH3	SDG19	At1g73100						
	SUVH4/KYP	SDG33	At5g13960	H3K9me1/me2	P, ChIP, MS	CHG methylation	YDG/SRA	(Wang et al., 2006); Ebbs (Ebbs and Bender, 2006)	
	SUVH5	SDG9	At2g35160	H3K9me1/me2	EH, P, ChIP	CG, CHG, CHH methylation	YDG/SRA	(Ebbs and Bender, 2006); (Rajakumam et al., 2011)	
	SUVH6	SDG23	At2g22740	H3K9me1/me2	ChIP	CHG, CHH methylation	YDG/SRA	(Johnson et al., 2008);(Wang et al., 2006); (Ebbs and Bender, 2006)	
	SUVH7	SDG17	At1g17770	–					
	SUVH8	SDG21	At2g24740	–					
	SUVH9	SDG22	At4g13460	–			CHH methylation	YDG/SRA	(Johnson et al., 2007)
	SUVR1	SDG13	At1g04050	–					
	SUVR2	SDG18	At5g43990	–					
	SUVR3	SDG20	At3g03750	–					
	SUVR4	SDG31	At3g04380	H3K9me2/me3	EH, ChIP, MS	Ubiquitin, H2Bub1	WYLD	(Thorstensen et al., 2006);(Veiseth et al., 2011)	
SUVR5	SDG6	At2g23740	H3K9me2, H3K27me2	ChIP	AtSWP1	Not determined	(Krichevsky et al., 2007b; Krichevsky et al., 2007a)		

Table 3. Plant SET domain-containing proteins (modified from Thorstensen et al., 2011).

Like animal homologs, plant E(z) proteins are part of Polycomb Repressive Complex 2 (PRC2) that suppresses genes by mediating H3K27 trimethylation. PRC2 core complex in *Drosophila* is composed of four components (Figure 5): E(z), Su(z)12, a DNA/Protein binding C2H2 Zn-finger protein; Extra Sex Combs (ESC), a protein with a WD40 beta-propeller; Nucleosome remodeling factor 55 (N55), a WD40 domain protein (Schwartz and Pirrotta, 2007). In *Arabidopsis*, the PRC2 proteins are conserved in small families, which associate in different compositions to target different loci (Pien and Grossniklaus, 2007; Hennig and Derkacheva, 2009). The homologs of E(z) are *CURLY LEAF (CLF)*, *SWINGER (SWN)* and *MEDEA (MEA)* while *EMBRYONIC FLOWER 2 (EMF2)*, *REDUCED VERNALIZATIONRESPONSE 2 (VRN2)* and *FERTILIZATION INDEPENDENT SEED 2 (FIS2)* are related to Su(z)12 (Luo et al., 1999; Gendall et al., 2001; Chanvivattana et al., 2004). The WD-40 protein ESC is encoded by *FERTILIZATION INDEPENDENT ENDOSPERM (FIE)* (Ohad et al., 1999). N55 has 5 homologs named MULTICOPY SUPPRESSOR OF IRA 1 to 5 (MSI1-5) (Kohler et al., 2003; Guitton and Berger, 2005).

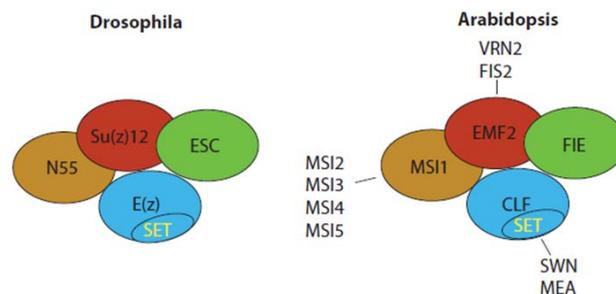


Figure 5. PRC2 complex core components in *Drosophila* and *Arabidopsis*.

According to the Su(z)12 components, there are three distinct *Arabidopsis* PRC2 complexes PRC2-FIS, PRC2-EMF and PRC2-VRN. Mutations of the PRC2-FIS partners *MEA*, *FIS2*, *FIE* and *MSI1* lead to autonomous endosperm development, and the complex is thought to control embryonic development by repressing central seed development regulators (Ohad et al., 1999; Kohler et al., 2003; Weinhofer et al., 2010). The PRC2-EMF complex contains CLF/SWN and functions in floral transition, floral organ development and vegetative growth (Aichinger et al.,

2009; Hennig and Derkacheva, 2009; Bouyer et al., 2011). PRC2-VRN complex also contains CLF/SWN which regulates flowering time mediated by vernalization (Wood et al., 2006; De Lucia et al., 2008). *clf* mutant induces early flowering and pleiotropic phenotypes, while *swn* mutant shows a wild type like phenotype. However, the *clf swn* double mutant is severely impaired and develops to a callus-like structure, and H3K27me3 is probably globally mitigated (Chanvivattana et al., 2004; Aichinger et al., 2009), suggesting that *CLF* and *SWN* have partial redundant functions in plant development.

In the *Arabidopsis* ASH1 class, four ASH1 HOMOLOG (ASHH) and three ASH1 RELATED (ASHR) members are identified (Baumbusch et al., 2001). ASHH1/SDG26 can methylate H3 and H4 *in vitro* and *ashh1* mutant shows a delayed flowering phenotype (Xu et al., 2008; Berr et al., 2009). ASHH2 is considered as a major H3K36me2/me3 HMT in *Arabidopsis* (Zhao et al., 2005; Xu et al., 2008), and *ashh2* mutant results in a pleiotropic phenotype (Dong et al., 2008; Cazzonelli et al., 2009; Grini et al., 2009; Berr et al., 2010a; Tang et al., 2012). The TRX class of SET domain proteins in *Arabidopsis* consists of two subgroups: *ARABIDOPSIS TRITHORAX* (ATX1-5) and *ARABIDOPSIS TRITHORAX RELATED* (ATXR1-7) (Baumbusch et al., 2001). It is shown that ATX1 mediates H3K4 trimethylation and ATX2 mediates H3K4 dimethylation on a few loci. *atx1* mutants display an early flowering phenotype and alter leaf morphogenesis. ATXR3/SDG2 functions in many processes including gametophyte development, flowering time, leaf and root growth (Berr et al., 2010b; Guo et al., 2010; Yun et al., 2012; Yao et al., 2013). In *sdg2* mutants, there is a global genome-wide reduction in H3K4me3, suggesting it is a major H3K4 trimethyltransferase in *Arabidopsis* (Berr et al., 2010b; Guo et al., 2010). ATXR5 and ATXR6 have an H3K27 monomethyltransferase activity. The double mutations *atxr5 atxr6* show partial heterochromatin decondensation and transcriptional activation of repressed heterochromatic elements, accompanied with decreasing H3K27me1 *in vivo* (Jacob et al., 2009). The SU(VAR)3-9 class contain 14 proteins in *Arabidopsis* which are divided into two subgroups: the SU(VAR)3-9

Homologs SUVH1-9 and the SU(VAR)3-9 Related proteins (SUVR) SUVR1-5 (Baumbusch et al., 2001). In general, members of this class have a H3K9 methyltransferase activity and are associated with inactive genes and highly condensed constitutive heterochromatin. *KRYPTONITE (KYP/SUVR4)* is the earliest and best studied member of this class. It is found in two independent genetic screens which are relative to reactivation of loci that were transcriptionally silenced by DNA methylation (Jackson et al., 2002; Malagnac et al., 2002). Mutations in *KYP* lead to a major decrease of heterochromatic H3K9me2 but not significant effect on H3K9me1, revealing that *KYP* is a major H3K9me2 methyltransferase in *Arabidopsis* (Jackson et al., 2004). *SUVH5* and *SUVH6*, two close *KYP/SUVH4* homologs, were demonstrated to methylate H3K9 *in vitro* and are partially redundant with *KYP* (Ebbs et al., 2005; Ebbs and Bender, 2006). *SUVH2* and *SUVH9* function in RNA-directed DNA methylation (RdDM) pathway, in which *SUVH2* and *SUVH9* bind to methylated DNA and facilitate the recruitment of Pol V to RdDM loci (Johnson et al., 2008; Johnson et al., 2014; Liu et al., 2014). *SUVR4* requires the H3K9me1 peptide as substrate *in vitro*, whereas its two close homologs *SUVR1* and *SUVR2* do not have detectable HMTase activity (Thorstensen et al., 2006). *SUVR4* as well as H3K27 monomethyltransferase *ATXR5* and *ATXR6* are found to involve in rRNA metabolism (Pontvianne et al., 2012).

1.3.2.2 Plant histone demethylases (HDMs)

Histone methylation was considered as irreversible until the discovery of Lysine Specific Demethylase 1 (LSD1), which was shown to remove methyl groups from H3K4 (Shi et al., 2004). Histone demethylases can be divided into two classes harbouring distinct mechanisms: amine oxidation by LSD1 and hydroxylation by Jumonji C (JmjC) domain-containing proteins (Liu et al., 2010). LSD1 family proteins need flavin adenine dinucleotide (FAD) and only acts on mono- or di-methylated but not tri-methylated lysines. The *Arabidopsis* genome encodes 4 Lysine-Specific Demethylase 1 (LSD1) homologs: *LSD1-LIKE 1 (LDL1)*, *LDL2*, *LDL3*

and *FLOWERING LOCUS D (FLD)*. FLD, LDL1 and LDL2 are involved in transition from vegetative to reproductive phase with partial redundancy by repressing *FLC* expression (Jiang et al., 2007). In *ldl1ldl2* and *ldl1 fld* double mutants, H3K4me2 on *FLC* locus is elevated suggesting an H3K4 demethylases activity of the proteins (Jiang et al., 2007). FLD is shown to interact with the HDA6 in flowering control (Yu et al., 2011), indicating the crosstalk between H3K4 demethylation and histone deacetylation in transcription repression.

1.3.2.3 JmjC proteins and their functions in plant development

The *jumonji (jmj)* gene was first identified in mouse by gene trap approach and was named after the morphology of the neural plates in mutant mice, which looks like a cruciform or “jumonji” in Japanese (Takeuchi et al., 1995). Structure analysis indicates that Jumonji proteins contain a conserved domain, named JmjC domain, consist of conserved 2-oxoglutarate-Fe (II)-binding site found in the dioxygenase super family (Clissold and Ponting, 2001). In recent years, a number of JmjC domain-containing demethylases have been identified in animals, which remove mono-, di- and trimethylated lysines in the presence of Fe (II) and α -ketoglutarate as cofactors (Tsukada et al., 2006; Couture et al., 2007; Ng et al., 2007b). JmjC proteins can also demethylate arginine residues, and other protein substrates or nucleotides (Chang et al., 2007). Phylogenetic analyses of sequences from mammals show that JmjC proteins can be divided into several subfamilies, including JARID/KDM5, JMJD1/JHDM2/KDM3, JMJD2/KDM4, JMJD3/UTX/KDM6, JHDM1/FBX/KDM2 and the JmjC domain-only group. *Arabidopsis* genome encodes 21 JmjC domain-containing proteins which show both conservation and divergence with animal homologs in evolution (Figure 6) (Sun and Zhou, 2008). For example, JARID/KDM5, JMJD1/JHDM2/KDM3, JMJD2/KDM4 and JmjC domain-only subfamilies are conserved among plant and animals, while JMJD3/UTX/KDM6 and JHDM1/FBX/KDM2 groups have not been found in *Arabidopsis* and plants contain a special group of JmjC proteins with additional protein modules (Lu et al., 2008).

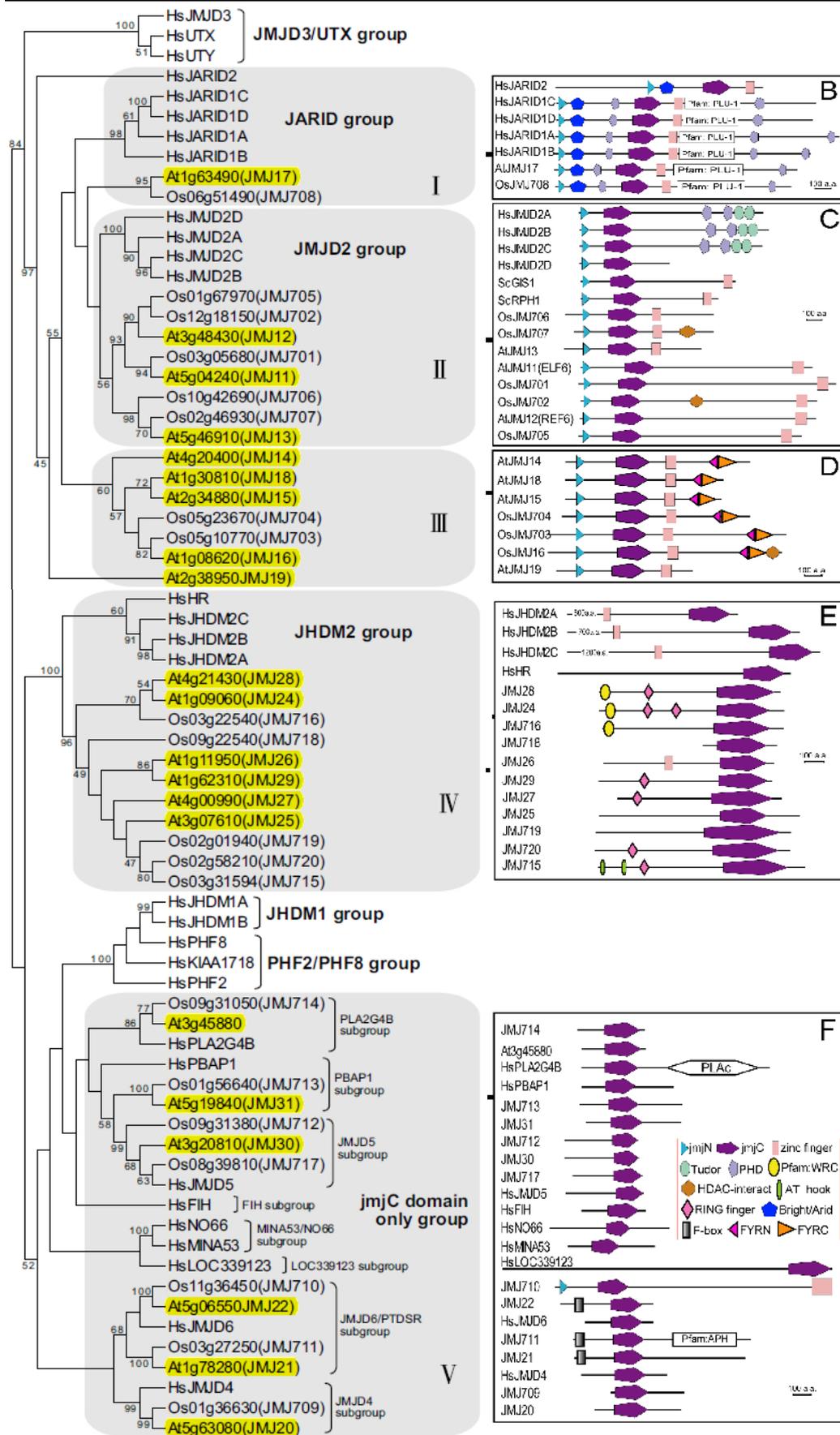


Figure 6. Phylogenetic relationship and structure of jmjC domain-containing proteins. *Arabidopsis* JmjC proteins are labeled in yellow (adapted from Sun and Zhou 2008).

JMJD2/KDM4 group

The JMJD2/KDM4 subfamily is the first reported JmjC proteins in animals, which consist of three ~130 kDa proteins (KDM4A-C) and KDM4D, a half size protein lacking double PHD and Tudor domains (Klose and Zhang, 2007). JMJD2/KDM4 proteins are demethylases which of di- and trimethylated H3K9 and H3K36 as well as trimethylated H1.4K26 (Berry and Janknecht, 2013). In *Arabidopsis*, *Early Flowering 6* (*ELF6/JMJ11*), its close homolog *Relative of Early Flowering 6* (*REF6/JMJ12*) and *JMJ13* belong to this group (Sun and Zhou, 2008). *ELF6* and *REF6* play divergent roles in the control of flowering time, as mutations in *ELF6* show an early flowering phenotype and *ref6* mutants display a late flowering phenotype (Noh et al., 2004). The function of *ELF6* as a floral repressor is related to *FT* repression (Jeong et al., 2009). The activity of SDG8 (a specific H3K4/H3K36 HMT) on *FLC* can be balanced by *REF6*, indicating *REF6* may be involved in H3K36 demethylation (Ko et al., 2010). Interestingly, *ELF6* and *REF6* can change H3K9 methylation status on some brassinosteroid related genes, suggesting that *ELF6* and *REF6* may act as H3K9 demethylases (Yu et al., 2008). Recently *REF6* is found to specifically demethylate H3K27me3 and H3K27me2 on hundreds of genes involved in plant development (Lu et al., 2011a), probably reflecting the effect of different potential co-factors on substrate specificity of the enzyme.

JMJD1/JHDM2/KDM3 group

JMJD1/JHDM2/KDM3 subfamily contains JHDM2A, JHDM2B, JHDM2C and HR in mammals, which possess JmjC and modified zinc-finger domain and have the ability to demethylate mono- and dimethylated H3K9 (Mosammaparast and Shi, 2010). In *Arabidopsis*, *JMJ24*, *JMJ25*, *JMJ26*, *JMJ27*, *JMJ28* and *JMJ29* belong to this group (Lu et al., 2008; Sun and Zhou, 2008). *JMJ25* is also named as *IBMI* (Increased in BONSAI Methylation 1), as mutation of *IBMI* displays ectopic H3K9 methylation at the BONSAI locus, leading to non-CG DNA hypermethylation and gene silencing (Saze et al., 2008b). Loss of function of *IBMI* causes multiple

developmental defects, including small and narrow leaves, pollen grain abortion, floral organ and embryo abnormalities and decreased reproduction (Saze et al., 2008b). Genome-wide profiling has revealed that *ibm1* mutation displays ectopic CHG DNA methylation and H3K9me2 accumulation in thousands of genes, especially at long transcribed genes, whereas transposable elements (TEs) are unaffected (Miura et al., 2009; Inagaki et al., 2010). These results suggest that *IBM1* protects protein coding genes from repression via H3K9 and non-CG DNA methylation (Saze et al., 2008b; Miura et al., 2009; Inagaki et al., 2010). In addition, aberrant phenotypes in *ibm1* mutants in both DNA methylation and plant development can be suppressed by mutations in the H3K9 HMTase *KYP/SUVH4* and the CHG methylase *CMT3*, showing the interplay between H3K9 methylation and DNA methylation in regulating gene expression (Saze et al., 2008b). The relationship between H3K9me2 and DNA methylation will be discussed in 1.5. Furthermore, it is found that *IBM1* not only protects genes from silencing via the direct association to prevent the coupling of histone and DNA methylation, but also targets components of RdDM pathway, *RNA-DEPENDENT RNAPOLYMERASE 2 (RDR2)* and *DICER-LIKE 3 (DCL3)*, hence indirectly participating in RdDM-directed repression (Fan et al., 2012).

JmjC domain-only group

This group contains several JmjC domain-containing proteins that, apart from the JmjC domain, contain no other recognizable protein domains. This group establishes its own branch based on homology within the JmjC domain. It is proposed that the proteins in this group might have been diverged in eukaryotes to carry out functions that are independent of histone demethylation (Klose and Zhang, 2007). For instance, JMJD6 was initially suggested to demethylate both asymmetrically and symmetrically dimethylated H3 arginine 2 (H3R2me2) and H4 arginine 3 (H4R3me2) (Chang et al., 2007), however the activity was challenged by another study showing that JMJD6 has lysyl hydroxylation activity (Webby et al., 2009). JMJD5 (also called KDM8) has

been reported to demethylate H3K36me₂ to regulate genes that control cell cycle and circadian rhythm (Hsia et al., 2010; Jones et al., 2010; Ishimura et al., 2012). Similarly, the activity of JMJD5 on histone demethylation is also questioned considering the biochemical assays *in vitro* and main function as a protein hydroxylase *in vivo* (Del Rizzo et al., 2012; Youn et al., 2012).

In *Arabidopsis*, *JMJ20*, *JMJ21*, *JMJ22*, *JMJ23*, *JMJ30* and *JMJ31* belong to this group. *JMJ20* and *JMJ22* are found to act as histone arginine demethylases that play redundantly positive roles in seed germination (Cho et al., 2012). *In vitro*, *JMJ20* could demethylate H3R2me₂, H4R3me₁, and H4R3me_{2s}. *In vivo*, *JMJ20*/*JMJ22* are induced upon phytochrome B activation, and *JMJ20* /*JMJ22* promote the expression of gibberellin anabolic genes *GA3ox1*/*GA3ox2* by direct binding to and reducing repressive H4R3me_{2s} levels on these genes (Cho et al., 2012).

Similarly to the homolog of JMJD5, *JMJ30* is involved in the pace of circadian clock by regulating the center oscillators *CCA1*, *LHY* and *TOC1* expression via a potential negative feedback loop between *CCA1/LHY* and *JMJ30* (Lu et al., 2011b). Interestingly, human JMJD5 is able to rescue the circadian phenotype of *jmj30* mutants and vice versa, suggesting that this gene has conserved function in both *Arabidopsis* and humans in circadian clock (Jones et al., 2010). However, the histone demethylase activity of *JMJ30* in *Arabidopsis* still remains to be determined.

JARID/KDM5 group

KDM5 proteins contain five conserved domains: JmjN, AT-rich, JmjC, PHD and C5HC2-zinc-finger (Figure 6). This group catalyzes the demethylation of H3K4me₃ and H3K4me₂, and is constituted by *KDM5A*, *KDM5B*, *KDM5C*, *KDM5D* and *JARID2* in mammalian cells (Blair et al., 2011). However, *Arabidopsis* genome only encodes one member *JMJ17* whose molecular activity and biological function remains to be discovered (Sun and Zhou, 2008).

Plant specific JmjC proteins

This group contains JmjN, JmjC, a C5HC2-zinc-finger, and FYRN/FYRC domains at their C-termini (Figure 6). Interestingly, FYRN/FYRC domains are usually found together in the H3K4 methyltransferase Trithorax and its homologs. In *Arabidopsis*, *JMJ14*, *JMJ15*, *JMJ16*, *JMJ18* and *JMJ19* belong to this group. *In vitro* *JMJ14* effectively demethylates H3K4me₃ and to a lesser extent H3K4me₂ and H3K4me₁ (Jeong et al., 2009; Lu et al., 2010; Yang et al., 2010). This demethylase activity is confirmed by *in vivo* assay in *Nicotiana benthamiana* (Lu et al., 2010). In *Arabidopsis*, *JMJ14* is shown to demethylate H3K4me₃ and H3K4me₂ at the *FT* locus, which is consistent with the fact that *jmj14* mutants display a lower level of *FT* expression and an earlier flowering time (Jeong et al., 2009; Lu et al., 2010). *JMJ14* is also required for DRM2-mediated RdDM pathway (Deleris et al., 2010). Mutation of *JMJ14* causes reduced DNA methylation in non-CG contexts at RdDM targets such as *MEA-ISR*, *FWA*, *AtSN1*, but not *Ta3* which is methylated by CMT3. Accompanied with reduced DNA methylation, there is an increase in H3K4me₃ at RdDM targets in *jmj14* mutants. However, *JMJ14* does not impact *de novo* DNA methylation (Deleris et al., 2010). Furthermore, genome-wide DNA methylation analyses reveal that *JMJ14* and LSD1 genes *LDL1* and *LDL2* cooperate to maintain RdDM pattern by counteracting H3K4 methylation (Greenberg et al., 2013). Another study searching for components of RNA silencing shows that *JMJ14* participates in silencing sequences targeted by RdDM (Searle et al., 2010). Although in *jmj14* mutants target sequences show significant increases of RNA transcripts and decreases of non-CG DNA methylation, endogenous small RNA abundance is not affected in *jmj14* mutants. Further analysis indicates that *JMJ14* acts downstream from Argonaute effector complex in RdDM (Searle et al., 2010). Similarly, *JMJ14* is also identified in a screen for mutants defective in posttranscriptional gene silencing (PTGS) (Le Masson et al., 2012). *jmj14* mutants release transgene PTGS that is correlated with an increase in promoter methylation and retardation of transcription (Le Masson et al., 2012).

JMJ18 is found to be involved in the control of flowering time (Yang et al., 2012b). Mutations in *JMJ18* resulted in a weak late-flowering, while *JMJ18* overexpressors exhibited an obvious early-flowering phenotype (Yang et al., 2012b). *In vitro*, *JMJ18* displays demethylase activity toward H3K4me3 and H3K4me2. In *Arabidopsis*, *JMJ18* directly represses *FLC* expression by demethylating H3K4 methylation on *FLC* loci, thereby promoting the expression of downstream flowering activator *FT* to stimulate flowering (Yang et al., 2012b).

JMJ15 has been first identified as MEE27 (Maternal Effector Embryo arrest 27) in a screen for mutants defective in the female gametophyte development (Pagnussat et al., 2005), suggesting it may play a role in reproductive development. In tobacco cells, *JMJ15* is capable of demethylating H3K4me3, H3K4me2 and H3K4me1 (Liu et al., 2010). Similarly, *JMJ15* is also proved to be a histone demethylase of H3K4me3 by MALDI-TOF mass spectrometry *in vitro* (Yang et al., 2012a).

1.3.3 Histone ubiquitination and deubiquitination

In addition to acetylation and methylation, histones can also be modified through ubiquitination (Jason et al., 2002). Although core histones, linker histones and several histone variants have been reported to be ubiquitinated (Hicke, 2001), most studies are so far focused on H2A and H2B monoubiquitination (Zhang, 2003; Weake and Workman, 2008). Similar to animals, H2A monoubiquitination (H2Aub1) in plant is mediated by Polycomb Repressive Complex1 (PRC1) and is required for the repression of genes when they are not necessary in a specific differentiation status (Bratzel et al., 2010; Molitor and Shen, 2013; Feng and Shen, 2014). In contrast to H2Aub1 for gene silencing, H2B monoubiquitination (H2Bub1) is mainly associated with gene activation (Zhang, 2003; Weake and Workman, 2008). The E2 enzyme *Rad6* and E3 enzyme *Bre1* and their homologs of the ubiquitination system are responsible for H2Bub1 in different eukaryotic organisms. In *Arabidopsis*, there are two *Bre1* homologs, named *HUB1* and *HUB2*, and three *Rad6* homologs named *ATUBC1*,

ATUBC2 and *ATUBC3*. Histone ubiquitination is also a reversible process. Deubiquitination enzymes or ubiquitin proteases (UBPs) specially cleave the peptide bond between the ubiquitin and substrate. In *Arabidopsis*, from over 27 putative proteins encoding UBPs (Liu et al., 2008), only *UBP26/SUP32* has so far been reported to participate in H2B deubiquitination (Sridhar et al., 2007).

1.4 DNA methylation

Unlike the situation in mammals, where methylation in differentiated cells is found almost exclusively in CG dinucleotides, cytosine methylation in plants often occurs in three sequence contexts: CG, CHG and CHH (where H is either T, A or C) (Henderson and Jacobsen, 2007; Zilberman et al., 2007). Bisulfite sequencing of the *Arabidopsis* genome shows that approximately 5% of all cytosines are methylated, with 55% of that methylation in CG, 23% in CHG and 22% in CHH contexts, respectively (Cokus et al., 2008; Lister et al., 2008). Depending on the location in the genome, DNA methylation can be broadly classified as either genic or non-genic. The genic pattern is methylation in the transcribed region or gene body (excluded from both ends and assuming a bell-like shape with a slight bias toward the 3' end) (Feng and Jacobsen, 2011). This genic methylation is found in ~1/3 of all protein coding genes in *Arabidopsis* and takes place predominantly in the CG context (Zhang et al., 2006; Cokus et al., 2008; Lister et al., 2008). It is found that genic methylation somewhat positively correlates with gene transcription, with the highest methylation level observed in genes with moderate transcription activities (Tran et al., 2005; Zilberman et al., 2007). The non-genic pattern is methylation on transposable elements (TEs) and other repetitive DNA, which are mostly found in pericentromeric heterochromatic regions but also exist in small patches between genes in the euchromatic arms (Feng and Jacobsen, 2011). Non-genic methylation happens in all three contexts and is generally associated with transcriptional repression (Zhang et al., 2006; Cokus et al., 2008; Lister et al., 2008). Some studies have shown that non-genic

DNA methylation represses protein-coding gene transcription when the mark is present in the gene's regulatory regions or promoters (Chan et al., 2005)

In *Arabidopsis*, methylation at CG dinucleotides is catalyzed by DNA *METHYLTRANSFERASE 1* (*MET1*), the ortholog of mammalian DNA *Methyltransferase 1* (*DNMT1*) (Kankel et al., 2003). The CG site is symmetrical on the opposite strand and MET1 can bind to the methylation strand and methylated the newly synthesized strand to maintain the DNA methylation pattern. Methylation at CHG is mediated mainly by *CHROMOMETHYLASE3* (*CMT3*) (Lindroth et al., 2001). This site is also symmetrical and can potentially be regenerated by a semi-conservative mechanism. Methylation in the non-symmetric CHH context is maintained mainly by *DOMAINS REARRANGED METHYLTRANSFERASE 2* (*DRM2*) and *CHROMOMETHYLASE2* (*CMT2*) (Cao and Jacobsen, 2002a; Stroud et al., 2014). In addition to these well-characterized context preferences, there is a degree of redundancy for maintenance of non-CG methylation between CMT3 and DRM2 (Cao and Jacobsen, 2002b; Stroud et al., 2013), as well as CMT2 at some loci (Zemach et al., 2013; Stroud et al., 2014). Among all the methyltransferase, only DRM2 is required for *de novo* DNA methylation in all three contexts (Cao and Jacobsen, 2002a).

There also exist mechanisms to remove cytosine methylation. DNA demethylation can be achieved either passively or actively. Passive demethylation takes place during DNA replication by replacing methylated cytosines with unmethylated ones (Saze et al., 2008a), whereas active demethylation occurs in a base excision repair pathway initiated by DNA glycosylases. Four DNA glycosylases are known in *Arabidopsis*: REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DEMETER-LIKE2 (DML2) and DML3 (Gong et al., 2002; Penterman et al., 2007b; Ortega-Galisteo et al., 2008; Zhu, 2009). ROS1 is a DNA repair protein shown to repress DNA methylation at numerous endogenous loci including many transposons (Gong et al., 2002; Penterman et al., 2007a; Zhu et al., 2007). DME is required for genomic imprinting during female gametophyte development, in the fertilized egg cell

and during endosperm formation (Gehring et al., 2006; Morales-Ruiz et al., 2006; Schoft et al., 2011). The two Demeter-like genes DML2 and DML3 are also required for appropriate distribution of DNA methylation marks within the genome (Ortega-Galisteo et al., 2008).

1.5 The link between DNA methylation and histone modification

In *Arabidopsis*, DNA methylation is correlated with specific histone modifications that vary depending on the context and genomic location of the DNA methylation. For instance, genic DNA methylation is largely co-incidental with H3K4me1 (Zhang et al., 2009). In contrast, non-genic DNA methylation is negatively correlated with H3K4me2/me3 (Greenberg et al., 2013).

The relationship between H3K9me2 and CHG DNA methylation is established by the CMT3 pathway: CMT3 binds to H3K9me2 through its eponymous chromodomain, as well as bromo-adjacent homology (BAH) domain (Lindroth et al., 2004; Rajakumara et al., 2011). Loss of CHG methylation in *kyp suvh5 suvh6* (three major H3K9 methyltransferases) triple mutants mimics the loss of CHG methylation in *cmt3* mutants genome-wide (Stroud et al., 2013), suggesting that KYP, SUVH5 and SUVH6 repress transcription by directing CHG methylation via CMT3 DNA methyltransferase. The H3K9 demethylase gene *ibm1* mutations display ectopic CHG DNA methylation and aberrant DNA methylation in *ibm1* mutants can be suppressed by mutants in *KYP* or *CMT3* (Saze et al., 2008b). All of these illustrate the tight correlation between H3K9me2 and DNA methylation. In addition, DRM2 maintains CHH methylation through a small interfering RNA (siRNA)-driven signal in a process known as RNA-directed DNA methylation (RdDM) (Law et al., 2010). DRM2-dependent RdDM relies on two plant specific RNA polymerases: RNA Polymerase IV and V (Pol IV and V). It is found that Pol IV occupancy requires a factor, *SAWADEE HOMEODOMAIN HOMOLOG1 (SHH1)*, which is a dual histone modification sensor, preferentially binding to histones containing H3K9 methylation

as well as lacking in H3K4me2/me3 (Law et al., 2011; Law et al., 2013). On the other hand, mutations of *JMJ14*, *LDL1* and *LDL2* (three H3K4 demethylases) reduce non-CG DNA methylation at RdDM targets accompanied by an increase in H3K4 methylation (Deleris et al., 2010; Greenberg et al., 2013), suggesting that H3K4 methylation antagonizes DNA methylation.

1.6 Chromatin remodeling

ATP-dependent chromatin remodeling is an essential aspect of chromatin dynamics that leads to conformational changes in nucleosome assembly and positioning (Figure 7). Chromatin remodeling process is mediated by a large family of SWI/SNF related enzymes in an energy-dependent manner. The term SWI/SNF stems from the two independent screening in yeast that characterize the genes affecting the mating-type switching (SWI) and sucrose fermentation (Sucrose Non-Fermenting, SNF) pathway (Neugeborn and Carlson, 1984; Stern et al., 1984). There are four main conserved classes of SWI/SNF chromatin remodelers that utilize ATP hydrolysis to alter histone-DNA contacts and share a conserved ATPase domain. The ATPase domain is comprised of two parts, the DExx and HELICc regions, which are separated by a linker. The four classes are distinguished according to the unique domains outside the ATPase motif and their specific associated subunits (Figure 7).

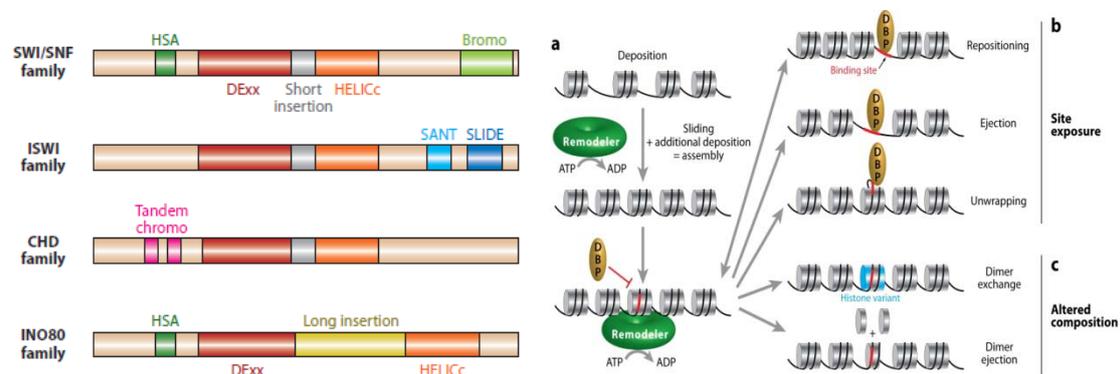


Figure 7. The chromatin remodeling families and chromatin remodeling functions (Clapier and Cairns, 2009). Left: Chromatin remodeler families. All remodeler families contain a SWI2/SNF2-family ATPase subunit. The differences among each family are the unique

domains residing within, or adjacent to, the ATPase domain. Right: The different outcomes of ATP-dependent chromatin remodeling. Remodelers can assist in chromatin assembly generating room for additional deposition (a). Remodeler action on a nucleosome array results in two categories: (b) site exposure, in which a site (red) for a DNA-binding protein (DBP) becomes accessible by nucleosomal sliding (repositioning), or nucleosomal eviction (ejection) or localized unwrapping, and (c) altered composition, in which the nucleosome content is modified by dimer replacement or through dimer eviction.

SWI/SNF chromatin remodelers have been mostly conserved through evolution across yeast, animals and plants kingdoms (Flaus et al., 2006; Narlikar et al., 2013). Based on the presence of ATPase/helicase domain, there are 42 potential SWI/SNF2 chromatin remodelers (<http://www.chromdb.org/index.html>) in *Arabidopsis*. Those that have been studied are indicated in Figure 8.

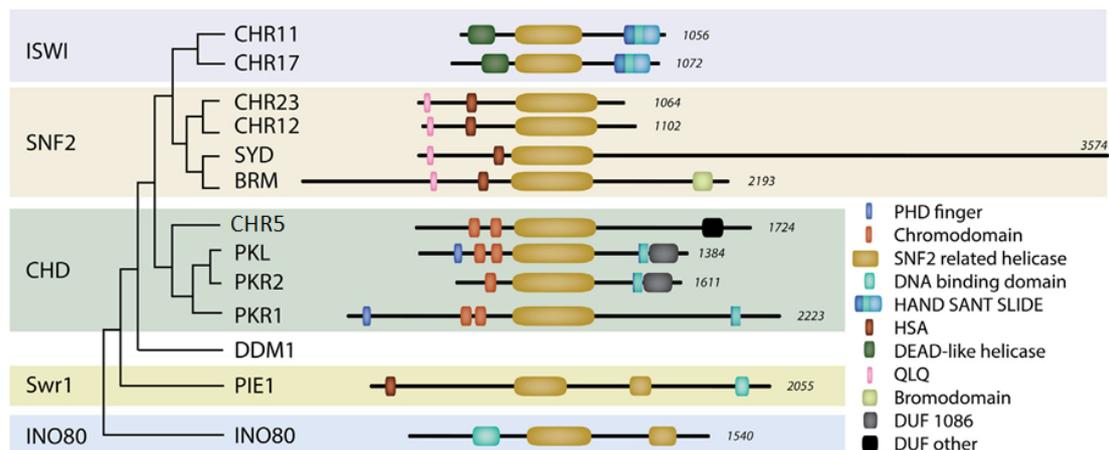


Figure 8. Phylogenetic relationship and domain structure of the major SNF2 family members in *Arabidopsis* (Gentry and Hennig, 2014).

1.6.1 SWI/SNF class—SYD, BRM and MINU1/2 in *Arabidopsis*

The SWI/SNF class contains a bromodomain at the C-terminal, that binds to acetylated N-terminal histone region, besides a helicase-SANT (SWI3, ADA2, NcoR, TFIIB) domain and an ATPase domain (Hassan et al., 2002; Martens and Winston, 2003). This is consistent with the fact that HAT complexes stabilize SWI/SNF nucleosome binding at the promoter region (Hassan et al., 2001). This family has many activities, for instance it slides and evicts nucleosome at many loci but lacks

roles in chromatin assembly (Clapier and Cairns, 2009). Proteins in this class include *SMARCA4* (*BRG1*) and *SMARCA2* (*BRM*) in human, *Brahma* in *Drosophila* and *Swi2/Snf2* and *Sth1* in *S. cerevisiae*. These proteins are mostly identified as transcriptional activators and several studies reveal the sequential recruitment of HAT and SWI/SNF complexes to promote transcription (Yudkovsky et al., 1999; Dilworth et al., 2000). However, there is also evidence showing that SWI/SNF complex directly repress transcription of some genes (Martens and Winston, 2003).

Arabidopsis *SPLAYED* (*SYD/CHR3*), *BRAHMA* (*BRM/CHR2*), *MINU1/CHR12* and *MINU2/CHR23* belong to the SWI/SNF class. *SYD* and *BRM* (3574 and 2193 amino acids, respectively) are large proteins; *MINU2* and *MINU3* (1132 and 1054 amino acids, respectively) are significantly smaller. Only *BRM* has a C-terminal region that resembles a bromodomain domain, which is found to target remodeling complexes to hyperacetylated chromatin in yeast (Kasten et al., 2004; Jerzmanowski, 2007).

Loss of function alleles of *SYD* were first identified by genetic screening for mutants that enhanced the *lfy* phenotype (Wagner and Meyerowitz, 2002). *LEAFY* (*LFY*) is a meristem identity gene that is required for switching from vegetative to reproductive development (Weigel et al., 1992). *syd* null mutants exhibit pleiotropic developmental defects including stem cell maintenance, patterning (alteration of leaf polarity, flower morphogenesis and ovule development), developmental transitions (precocious onset of reproductive development) and growth (small stature, slow growth and reduced apical dominance) (Wagner and Meyerowitz, 2002). *SYD* represses *LFY* activity prior to the floral transition, whereas it functions as a co-activator with *LFY* in the transcriptional regulation of class B and C floral homeotic genes (Wagner and Meyerowitz, 2002). *SYD* is required for reproductive shoot apical meristem (SAM) by directly regulating the expression of *WUS* (Kwon et al., 2005).

BRM was originally identified by a genetic screen for mutants that exacerbated the defect of cotyledon separation in *cuc2* (Kwon et al., 2006). *syd* and *brm* null mutants exhibit both similar and distinct developmental defects, indicating partially functional redundancy. For example, both single mutants share short stature and delayed growth. The double mutant *syd brm* reveals embryo lethality but the single not (Bezhani et al., 2007). Although both are required for flower patterning and cotyledon separation, BRM and SYD may control different molecular events. During the formation of cotyledon separation, BRM upregulates the transcription of all three *CUC* genes, whereas SYD only upregulates the expression of *CUC2* (Kwon et al., 2006). During the initiation of flower patterning, the temporal recruitment of SYD to *AP3* and *AG* is similar to LFY, whereas the temporal binding of BRM is different (Wu et al., 2012). In addition, BRM has also some specific role. For example, *brm* mutants strongly inhibit the primary root elongation and promote more lateral roots and secondary root branches. Some studies reveal that the phenotype of *brm* mutant is similar to that of *swi3c*, suggesting that they may work in the same complex, which is confirmed by yeast two hybrid assays (Farrona et al., 2004; Sarnowski et al., 2005; Hurtado et al., 2006; Archacki et al., 2009).

MINU1 and MINU2, other two SNF2/SWI smaller members, also have redundant roles in plant development. No significant phenotype has yet been characterized in single mutant, however, the double mutants show embryonic lethality which is associated with increased expression of *WUSCHEL* and *WOX5* (Sang et al., 2012).

1.6.2 ISWI class—CHR11 and CHR17 in *Arabidopsis*

The ISWI class takes its name from the *Drosophila* ISWI (Imitation Switch). In *Drosophila*, this unique member can belong to three different complexes: NURF (nucleosome-remodeling factor), CHRAC (chromatin-accessibility complex) and ACF (ATP-dependent chromatin assembly and remodeling factor) (Tsukiyama and Wu, 1995; Ito et al., 1997). All have chromatin remodeling activities and participate

in transcription regulation (Corona and Tamkun, 2004). The ISWI members possess a SANT adjunct to a SLIDE domain (SANT-like ISWI) at the C-terminus, both of which together form a nucleosome recognition module that binds to an unmodified histone tail and DNA component of nucleosomal substrates (Boyer et al., 2004). Many ISWI complexes (ACF, CHRAC) optimize nucleosome spacing to promote chromatin assembly and repress the gene transcription. However, certain complexes (NURF) can randomize spacing, which can assist in RNAPII transcription (Clapier and Cairns, 2009, 2012).

CHR 11 and *CHR17* encode two ISWI proteins in *Arabidopsis*. Both have conserved domains, HAND, SANT and SLIDE in the C-terminal. CHR11 is highly expressed in sporophytic and gametophytic tissues during reproductive development (Huanca-Mamani et al., 2005). Therefore, CHR11 is shown to be required for haploid nuclear proliferation during megagametogenesis and cell expansion during the sporophytic phase (Huanca-Mamani et al., 2005). One study shows that CHR11 and CHR17 physically interact with RLT1 and RLT2, two plant-specific DDT-domain containing proteins, together in preventing vegetative-to-reproductive transition by regulating several key genes such as *FT*, *SEP3* (Li et al., 2012). It is also found that the SLIDE domain of CHR11 and the DDT domain of RLT1 together with an adjacent sequence are responsible for the interaction (Dong et al., 2013).

1.6.3 INO80 class —INO80 and PIE1 in *Arabidopsis*

INO80 class of chromatin remodelers was first identified from *S. cerevisiae* which is required for growth in absence of inositol (Ebbert et al., 1999). There are two INO80 members *Ino80* and *Swr1* in yeast, and three homologous genes *INO80*, *SRCAP* (SNF2-related CREB-activator protein) and *p400* in humans. One of the structural characteristics of this class is a large insertion in the middle of ATPase domain, to which the helicase-related (AAA-ATPase) Rvb1/2 proteins and one actin-related protein (ARP) bind. INO80 complex has different functions including transcription activation and DNA repair (Shen et al., 2000). Although SWR1 is highly related to

INO80, it is unique in reorganizing nucleosome by recruitment and exchange of H2A.Z variant (Krogan et al., 2003; Mizuguchi et al., 2004).

Arabidopsis encodes one homolog of Ino80 protein. *Ino80* mutant reduces the homologous recombination (HR) frequency to 15% of that in wild type. However it does not seem to affect other DNA repair pathways (Fritsch et al., 2004). This is unlike the situation of yeast Ino80 that is involved in both HR and non-HR DNA repair events. Like yeast Ino80, *Arabidopsis* INO80 regulates a subset of genes not functionally related to HR, suggesting its dual role in transcription and DNA repair (Fritsch et al., 2004). *Swr1* is required for replacement of histone H2A with the H2A.Z variant in yeast. *PIE1* (*PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1*) encodes the single representative of *SWR1* gene in *Arabidopsis*. *pie* is originally identified as a suppressor of FRIGIDA-dependent late flowering (Noh and Amasino, 2003). Similar to homolog in yeast, PIE1 as part of SWR complex is responsible for deposition of H2A.Z at multiple loci (Choi et al., 2007; March-Diaz et al., 2008). It is also found that PIE1 plays important roles in somatic DNA repair and during meiosis (Rosa et al., 2013). *Arabidopsis* characterized SWI/SNF2 remodelers are summarized in Table 4.

Family	Protein	AGI code	Mutant phenotype	Aliases
SNF2	SYD	At2g28290	Floral organ defects, loss of SAM. Slow growth. Ovule growth arrest	CHR3
	BRM	At2g46020	Increased arrest in early development. Slowed growth, delayed development	CHR2, CHA2
	MINU1	At3g06010	Embryonic lethality. Stem cell maintenance defects	CHR12
	MINU2	At5g19310		CHR23
ISWI	CHR11	At3g06400	Reduced embryo size	
	CHR11, CHR17	At3g06400, At5g18620	Early flowering, reduced adult size	
SWR1	PIE1	At3g12810	Early flowering in short day	CHR13
INO80	INO80	At3g57300	Reduced homologous recombination	
CHD	PKL	At2g25170	Thick opaque root. Reduced apical dominance, shortened hypocotyl	CHD3, CHR6,
	PKR1	At5g44800	Exacerbates <i>pkl</i> hypocotyl shortening	CHR4
	PKR2	At4g31900	Exacerbates <i>pkl</i> hypocotyl shortening and root phenotype	CHR7
	CHR5	At2g13370		PKR3

Table 4. Summary of the major SWI/SNF2 chromatin remodelers and their mutant phenotypes in *Arabidopsis* (Gentry and Hennig, 2014).

1.6.4 CHD class

CHD chromatin remodeling proteins is named based on the three characteristic domains: an N-terminal pair of Chromodomains, a central Helicase-like ATPase motor, and a C-terminal DNA binding domain (Lusser and Kadonaga, 2003). The chromodomain is a well conserved ancient structural motif, since it can be found in a vast array of organisms as diverse as protists, plants, amphibians, and mammals (Eissenberg, 2001). Yeast has only one CHD protein (Chd1), whereas other high organisms such as fly, human and plants have several CHD proteins, some of which can be in large functional complex. Based on the structure and function, CHD proteins have been broadly divided into three separate subfamilies (Hall and Georgel, 2007). Subfamily I proteins (CHD1 and CHD2 in humans) are the prototypical examples of the family and do not contain additional identified functional domains. Subfamily II (CHD3 and CHD4) is characterized by the presence of plant homeodomain (PHD)-zinc-finger domain and in the absence of a clear DNA-binding domain (Hall and Georgel, 2007). Subfamily III (CHD 5-9 in humans) is less defined structurally and far less studied compared to the other two subgroups.

Besides CHD proteins, the chromodomain has also been identified in other chromatin associated proteins such as HP1 (Brasher et al., 2000) and Polycomb (Ingram et al., 1999; Brehm et al., 2004). The chromodomain of human CHD1 binds to H3K4me2 or me3 (Flanagan et al., 2005; Sims et al., 2005). In contrast, the chromodomain of Mi-2 (CHD3) seems to recognize DNA rather than methylated histone tails (Brehm et al., 2004). The ATPase domain is required for the remodeling of nucleosomes through histone displacement (Durr and Hopfner, 2006) or histone octamer sliding (Becker and Horz, 2002). The ATP hydrolysis energy is transduced into the conformational stress necessary to remodel chromatin (Pazin and Kadonaga, 1997). Thus the ATPase domain serves as a DNA-translocating motor to break histone-DNA contacts and release histones from nucleosomes (Durr and Hopfner, 2006). Of the 3 defined domains for CHD proteins, the DNA-binding domain is by far the least conserved. This domain is fairly well defined in CHD1, whereas CHD3/4, as

part of the Nucleosome remodeling and deacetylase (NuRD) complex, may also gain DNA binding ability through interactions with other subunits of the complex. Moreover, the PHD domains of CHD3/4 can bind histone H3 and affect interactions with nucleosomes (Bienz, 2006). Some CHD remodelers have the ability to slide or evict nucleosomes to promote transcription, however, others play repressive roles such as vertebrate Mi-2/NuRD complex, which contains histone deacetylases (HDAC1/2) and methyl CpG-binding domain (MBD) proteins (Hall and Georgel, 2007).

1.6.4.1 The function of CHD1 protein

The subfamily I member CHD1 was initially thought to be integral to transcriptional activity. As mentioned above, the chromodomain of human CHD1 was found to specifically bind to H3K4me3. Presently, there is *in vitro* evidence both for (Pray-Grant et al., 2005) and against (Sims et al., 2005) Chd1 binding to H3K4me in budding yeast *S. cerevisiae*. The fission yeast *S. pombe* Chd1 orthologues Hrp1 and Hrp3 may interact and bind to H3K4me via the chromodomains of Hrp3, which have all the consensus residues required for methyl-lysine binding (Opel et al., 2007). In *Drosophila melanogaster*, the chromodomain of CHD1 is critical for chromatin remodeling activity but is less important for localization to chromatin (Morettini et al., 2011). The crystal structural and biochemical studies show an autoregulated domain organization of *S. cerevisiae* Chd1 (Narlikar et al., 2013). The chromodomain contacts the two lobes of ATPase domain and inhibits the ATPase motor (Hauk et al., 2010). In contrast, the DNA binding element, containing homology to SANT and SLIDE domains, contributes to positively regulate ATPase activity (McKnight et al., 2011). *In vitro* assays reveal that Chd1 has the ability to assemble, remodel, slide and promote regular spacing of nucleosomes (Lusser et al., 2005; Stockdale et al., 2006).

Chd1 plays important roles in transcription processes including initiation, elongation and termination (Alen et al., 2002; Simic et al., 2003; Biswas et al., 2007; Quan and Hartzog, 2010). It has been shown that human CHD1 associates with the pre-initiation transcription complex through interactions with Mediator *in vitro* (Lin et

al., 2011). Yeast Chd1 is found to be a factor required for remodeling the nucleosomal PHO5 promoter and for transcriptional activation of the gene (Ehrensberger and Kornberg, 2011). In fly and yeast, CHD1 is localized to a transcriptionally active gene and physically interacts with elongation factors and RNA Polymerase II (Simic et al., 2003; Srinivasan et al., 2005). Yeast Chd1 is also an essential factor for chromatin structure at the ends of genes at transcription, and transcriptional termination fails to occur in its absence (Alen et al., 2002).

CHD1 is a chromatin remodeler which participates in diverse biological processes. In *Drosophila*, although *chd1*-mutant zygotes are viable and display only a mild notched-wing phenotype, *chd1*-null male and female are sterile (McDaniel et al., 2008). In fact, maternal Chd1 in *Drosophila* is required for incorporation of H3.3 into the male pronucleus during early development after fertilization. Failure to incorporate H3.3 may render the paternal genome unable to participate in mitosis in the zygote, resulting in non-viable haploid embryos (Konev et al., 2007). In mice, Chd1 is essential for embryonic stem (ES) cell pluripotency and the formation of induced pluripotent stem cells (Gaspar-Maia et al., 2009). *Chd1* mice mutants show an increase in the heterochromatic mark H3K9me3 and the ES cells display a tendency towards neuronal differentiation (Gaspar-Maia et al., 2009). CHD1 is also found to work as a tumor suppressor, as deletion or mutation of *Chd1* is associated with prostate cancer, with cells displaying an increase in invasiveness (Huang et al., 2012).

Recent study points to a critical role for Chd1 in nucleosome positioning *in vivo*. As mentioned above, nucleosomes typically show a stereotypic organization over most genes: a nucleosome free region just upstream of the TSS followed by a regular nucleosomal array over the coding region. However, in the absence of *S. cerevisiae* Chd1, the nucleosome positioning is largely lost over gene bodies (Gkikopoulos et al., 2011). Specially, NFR and +1 nucleosome over the transcribed regions are minimally affected, but downstream nucleosomes (particularly those starting at the +3 nucleosome) were dramatically delocalized in *chd1* mutants (Figure

9) (Gkikopoulos et al., 2011). This is consistent with *in vitro* data that Chd1 is able to space nucleosome arrays on plasmid DNA *in vitro* (Lusser et al., 2005). A similar situation is observed in *S. pombe*, as the deletion of Chd1 homologs, Hrp1 and Hrp3, interrupts nucleosome spacing (Figure 9) (Hennig et al., 2012; Pointner et al., 2012; Shim et al., 2012).

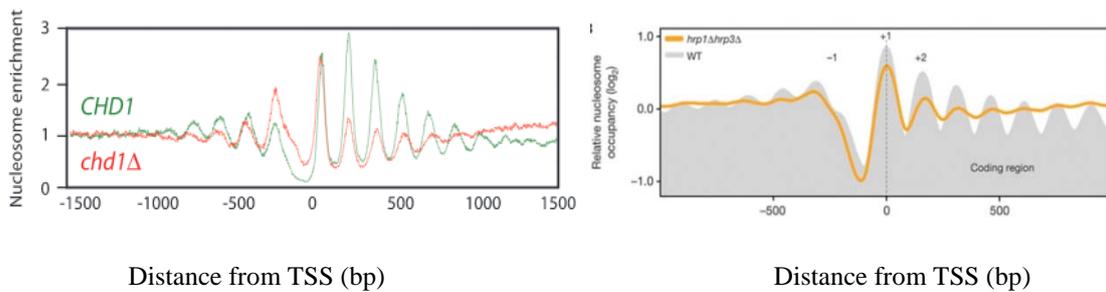


Figure 9. Nucleosome organization is disrupted upon deletion of CHD1. Left: nucleosome organization in budding yeast *S. cerevisiae* (Gkikopoulos et al., 2011). Right: nucleosome positioning in fission yeast *S. pombe* (Hennig et al., 2012).

In *S. cerevisiae*, loss of *Chd1* impairs nucleosome reassembly coupled with H2B monoubiquitination (Lee et al., 2012). Loss of Chd1 results in a substantial loss of H2Bub levels, and nucleosomal occupancy is reduced in gene bodies in both *chd1* and *h2b* mutants, suggesting that Chd1 may positively regulate gene expression through promoting nucleosome reassembly coupled with H2B monoubiquitination (Lee et al., 2012). Consistent with a role for Chd1 in maintaining chromatin structure at active genes, Chd1 is also reported to prevent cryptic transcription within gene bodies in *S. pombe*, as the deletion of *hrp1* and *hrp3* causes strong, genome-wide accumulation of antisense transcripts (Hennig et al., 2012; Shim et al., 2012). Furthermore, Chd1 is also involved in histone exchange in gene body in *Drosophila* and yeast (Radman-Livaja et al., 2012; Smolle et al., 2012). It is possible that irregular spacing of nucleosomes in *chd1* mutants renders it prone to dissociation as a result of collisions between and adjacent nucleosomes. Transient dissociation of histones provides an opportunity for exchange with the soluble pool of nascent histones (Radman-Livaja et al., 2012; Smolle et al., 2012; Narlikar et al., 2013).

Genome-wide mapping of *S. cerevisiae* Chd1 binding on native chromatin shows that Chd1 is highly enriched in NFRs, although it acts in activated gene body (Gkikopoulos et al., 2011; Zentner et al., 2013). In light of these data, Chd1 plays essential roles in regulating nucleosome dynamics during transcription at different levels as shown in Figure 10.

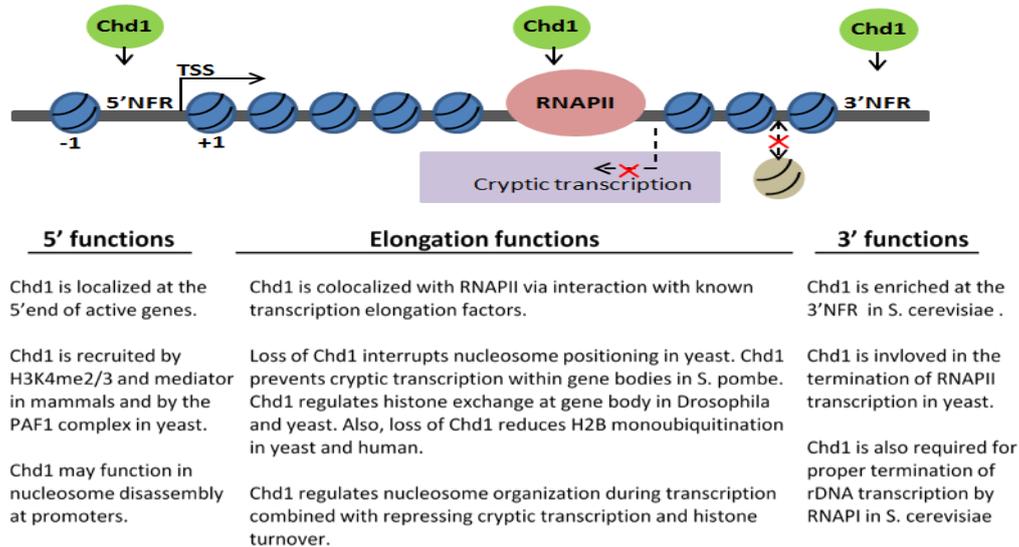


Figure 10. A model for Chd1 in regulating nucleosome dynamics during transcription.

1.6.4.2 Plant CHD proteins—PKL, PKR1, PKR2 and CHR5

Phylogenetic analysis reveals that there is only one CHD1 protein (CHR5) in the genome of *Arabidopsis*, no CHD2 subfamily protein exists in plants, in contrast, three members belong to CHD3 subfamily (Figure 11) (Hu et al., 2012). In plant, CHD1 protein (CHR5) has not been studied.

Among plant CHD3 members, PICKLE (PKL) is the most studied. The hallmark of *pkl* seedlings is a green, tuberous and opaque region at the primary root, referred to as the “pickle root” (Figure 12) (Ogas et al., 1997). Pickle primary roots can express numerous embryonic differentiation characteristics such as expression of storage protein genes and accumulation of storage lipids and undergo spontaneous somatic embryogenesis (Ogas et al., 1997; Henderson et al., 2004). This pickle root phenotype is related to fail to repress embryo identity genes, such as *LEC1*, *LEC2*,

ABI3, and *FUS3* after seed germination (Figure 12) (Ogas et al., 1999; Zhang et al., 2008; Aichinger et al., 2009; Zhang et al., 2012).

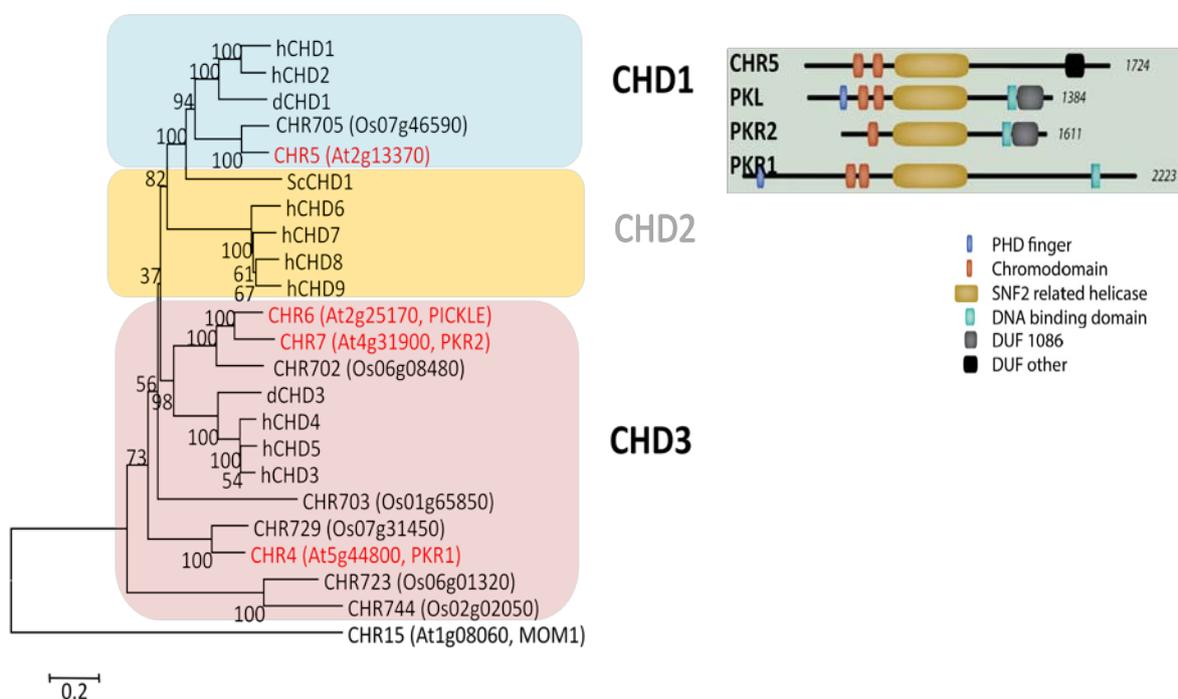


Figure 11. Relationship and structure of CHD proteins. Left: phylogenetic tree of CHD proteins (Hu et al., 2012). Right: structure and size of plant CHD proteins (Gentry and Hennig, 2014).

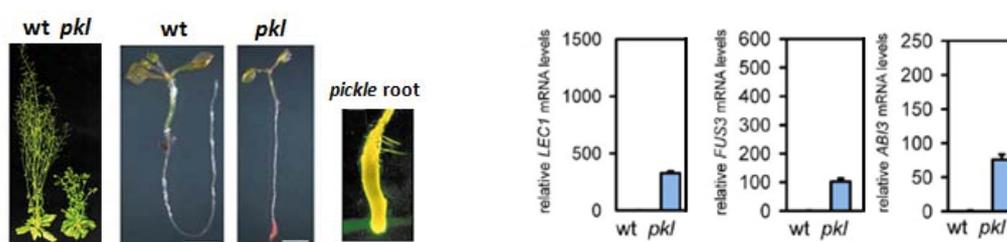


Figure 12. The phenotype of *pkl*. Left: “pickle root” phenotype (Ogas et al., 1997); Right: embryo identity genes are upregulated in *pkl* seedlings (Aichinger et al., 2009).

PKL contributes to other developmental process in addition to repression of embryonic traits. PKL plays a role in repression of ectopic stipules and meristems in leaf tissue (Hay et al., 2002). PKL is involved in repressing meristematic genes in carpel tissue (Eshed et al., 1999). Loss of *PKL* results in hypersensitivity to cytokinin with respect to callus growth and greening (Furuta et al., 2011). PKL is also required for proper root development and has been found to work in two somewhat opposing

ways: PKL is a negative regulator of auxin-mediated lateral root initiation by regulating *ARF7/9* transcription (Fukaki et al., 2006) and yet also promotes root growth by increasing expression of root stem cell and meristem marker genes (Aichinger et al., 2011). Recently, PKL is found to be a repressor of photomorphogenesis by interacting with HY5 to regulate cell elongation-related genes in hypocotyls (Jing et al., 2013).

1.6.4.3 The mechanism of PKL regulating embryonic genes

In contrast to animal CHD3 proteins functioning in histone deacetylation, comparative genomic analyses reveal that PKL acts to promote trimethylation of histone H3 lysine 27 (H3K27me3) (Zhang et al., 2008; Zhang et al., 2012). As mentioned above, H3K27me3 is a repressive mark for gene expression in both plants and animals (Zheng and Chen, 2011). PRC2 is the main factor responsible for catalyzing trimethylation of H3K27 (Schwartz and Pirrotta, 2007; Schmitges et al., 2011). In *Arabidopsis*, mutants of PRC2 genes substantially reduce H3K27me3 levels and exhibit profound developmental defects including the derepression of embryonic traits (Chanvivattana et al., 2004; Schubert et al., 2006; Bouyer et al., 2011). Presently, it is controversial how PKL represses embryo identity genes. Some results support that PKL directly associates and represses the embryonic genes by regulating H3K27me3 on the loci. In particular, PKL was found to present at the promoters of *LEC1* and *LEC2* during germination (Zhang et al., 2008; Zhang et al., 2012). Whereas, other results suggest that PKL represses embryonic traits in an indirect way. In this case, PKL, like a transcriptional activator, binds to and up-regulates PRC2 genes rather than embryonic genes (Aichinger et al., 2009).

1.7 Embryo development

Seed development is an intriguing, specific and crucial phase of angiosperm plant life cycle, which begins with a double fertilization process that occurs within the ovule and ends with a dormant seed primed to become the next plant generation. Embryo

development consists of the morphogenesis and maturation phases as shown in Figure 13. Morphogenesis refers to the differentiation of cell fates in initially equivalent cells, which results in an embryo established with the specification of the shoot root axis and the formation of discernible major organ and tissue systems (Capron et al., 2009).

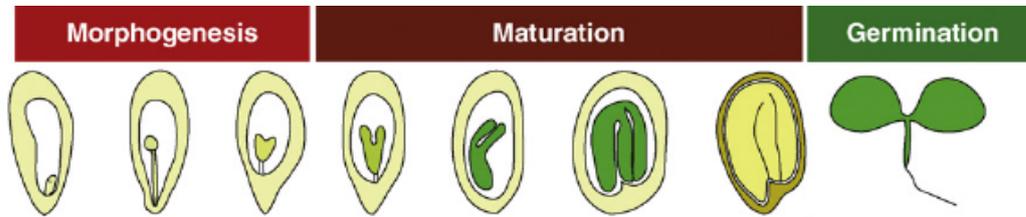


Figure 13. Embryo development in seed plants (Braybrook and Harada, 2008).

Maturation, the second phase of zygotic embryogenesis, occurs exclusively in seed plants. Upon entering the maturation stage, embryo ceases cell division and continues growing by cell expansion filling up the entire seed. One of the characteristics of this phase is the synthesis and accumulation of carbohydrates, lipids, and seed storage proteins (SSP), which will provide nutrients to the growing seedling before the photosynthetic capacity is fully acquired (Miquel and Browse, 1998). The embryo also accumulates late embryogenesis abundant (LEA) proteins, dehydrins, and osmocompatible solutes, serving to protect the embryo from desiccation (Ingram and Bartels, 1996). Developmental arrest and the ability to withstand drying enable the embryo to remain in a quiescent or dormant state until the seed encounters a favorable set of conditions that promote germination (Bewley, 1997).

1.7.1 LEC1/AFL transcription factors

Genetic studies have uncovered a number of factors that act as positive regulators of seed developmental programs. Among them, *LEC1/AFL* (*ABI3*, *FUS3*, *LEC2*), as the main regulators, have been characterized at greater depth. The *LEC* and *FUS3* were originally identified as regulators of cotyledon identity, as recessive mutations in these genes partially convert the cotyledons into vegetative leaves (Figure 14), hence, the name *LEAFY COTYLEDONS* (Meinke, 1992; Meinke et al., 1994). The name

FUSCA is derived from Greek meaning ‘to darken’, and is designated to mutant loci with dark seeds (Figure 14) (Keith et al., 1994). The dark color of the seed is due to the accumulation of anthocyanins in the embryo. Recessive mutations in *abi3* were originally identified in screens aiming to identify mutants insensitive to ABA during germination (Koornneef et al., 1984). The *LEC1/AFL* genes, as the master regulators of late embryogenesis and seed maturation, share overlapping but not identical mutant phenotypes (Parcy et al., 1997; Vicient et al., 2000; Harada, 2001). Mutants of these genes exhibit reduced dormancy and precocious germination of immature seeds. *LEC1/AFL* can trigger the expression of genes of seed storage protein (SSP) and the oil body protein, as a consequence, the seeds of the mutants are desiccation intolerant and non dormant except *lec2* embryo (Giraudat et al., 1992; Keith et al., 1994; Meinke et al., 1994; Stone et al., 2001). *Lec2* mutants are desiccation tolerant, do not germinate precociously, and show a pattern of storage product accumulation closer to wild type than to other mutants (Keith et al., 1994; Meinke et al., 1994). Only *abi3* mutants show ABA insensitivity and defects in chlorophyll degradation (Giraudat et al., 1992). The *lec1*, *lec2* and *fus3* mutations cause partial conversion of cotyledons into leaf-like state by the development of trichomes and the partial leaf ultrastructure (Keith et al., 1994; Meinke et al., 1994). The differences of *lec1* and *fus3* embryos are restricted mostly on the frequency of stomata and the position of anthocyanin accumulation (Meinke, 1992; Keith et al., 1994). In general, the analyses of *LEC1/AFL* mutations indicate these genes, as the main positive regulators during seed maturation, have redundant and distinctive roles in embryo formation, cotyledon identity, seed storage protein expression and desiccation tolerance.



Figure 14. Seed phenotype of mutants in *lec1*, *abi3*, *fus3* and *lec2* (Meinke, 1992; Keith et al., 1994; To et al., 2006).

1.7.1.1 Structure and targets of *LEC1/AFL* genes

LEC1/AFL genes encode two different classes of transcriptional factors. *ABI3*, *FUS3* and *LEC2* belong to the B3-domain transcription factor family, which are only found in plants. The conserved B3 domains have similar DNA binding specificities and recognize the RY/Sph I cis-regulatory motif (purine-pyrimidine, CATGCA as the core sequence), which is found in the many seed storage protein genes (Kroj et al., 2003; Monke et al., 2004; Roschttardt et al., 2009). It explains why these regulators have partially redundant roles, as they are likely to share downstream targets. In contrast, *LEC1* encodes a protein homologous to HAP3 subunit of CCAAT-binding protein complex (CBF), which is conserved among eukaryotes (Lotan et al., 1998; Lee et al., 2003). *LEC1* is able to activate the expression of cruciferin C (CRC) seed storage protein when co-expressed with ABRE-binding bZIP transcription factors (Yamamoto et al., 2009). In general, *LEC1/AFL* proteins act through direct interactions with regulatory elements present in the promoters of maturation-related genes to control the various features of seed development.

1.7.1.2 Expression profiles of *LEC1/AFL* genes

In agreement with their embryonic mutant phenotypes, *LEC1/AFL* genes are primarily expressed in embryos as shown in Figure 15. *LEC1* is expressed in seed (in both embryo and endosperm). Specially, *LEC1* mRNA is present at the protoderm and is detected throughout the developing embryo by the bent-cotyledon stage (Lotan et al., 1998). *LEC2* is expressed throughout the embryo in early stages but primarily to the hypocotyl and the embryo axis at later stages (Kroj et al., 2003; To et al., 2006). *ABI3* is expressed broadly in embryo tissues throughout embryogenesis except the root meristem (To et al., 2006). *FUS3* transcripts can be detected in apical region and suspensor of the globular early heart stage, as well as in the vasculature, root tip, aleurone of the mature embryo (Gazzarrini et al., 2004; Tsuchiya et al., 2004). *LEC2* and *ABI3* are also expressed in vegetative tissues, where *ABI3* is involved in lateral meristem development (Rohde et al., 2000; Stone et al., 2001; Kroj et al., 2003; To et

al., 2006). *LEC1/AFL* genes also have distinctive temporal expression profiles (Figure 15). *LEC1* is expressed at early stages of embryogenesis, followed by *LEC2* (Stone et al., 2001; Kroj et al., 2003). *FUS3* and *ABI3* expression levels peak after the maximum of *LEC2*, with *FUS3* levels decreasing before maturation and *ABI3* levels remaining high throughout the late embryo development (Parcy et al., 1994; Luerssen et al., 1998).

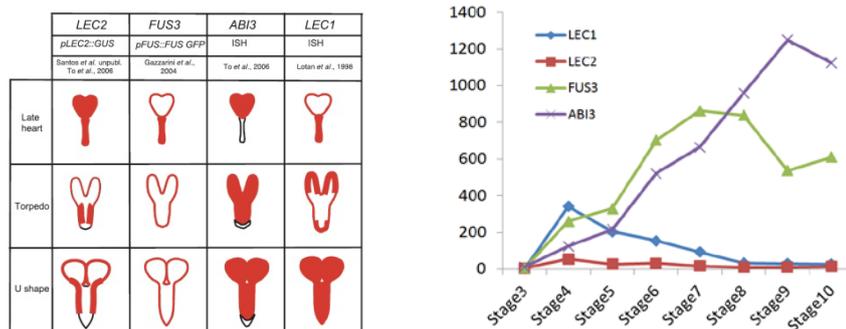


Figure 15. Spatial and temporal expression pattern of *LEC1/AFL* in developing embryos. Left: Tissue-specificity of *LEC1/AFL* genes in embryo which are indicated in red. (Santos-Mendoza et al., 2008). Right: temporal expression pattern from the website <http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi>. (3 d, globular; 4 d, heart; 5 d, torpedo; 7 d, walking stick; 9 d, curled cotyledons; 10 d, green cotyledons).

1.7.2 A network of interacting *LEC1/AFL* factors

Genetic analysis has revealed the existence of a complex interacting network among the *LEC1/AFL* genes. According to a previously established model (To et al., 2006), *LEC1* and *LEC2* act upstream of *ABI3* and *FUS3*, and promote their expression. *LEC1* and *LEC2* also regulate each other. *ABI3* and *FUS3* also mutually regulate their own expression (Figure 16) (To et al., 2006). However, these functional interactions may be of direct or indirect effects and the molecular mechanism still remains to be elicited. It was reported that *LEC2* binds specially to the RY sequence on the promoter of *AGAMOUS-Like15* (*AGL15*) *in vitro* and activates *AGL15* expression *in vivo* (Figure 16) (Braybrook et al., 2006). *AGL15* is a MADS domain transcriptional regulator accumulating in embryos (Wang et al., 2002). By using ChIP-chip approach, it was shown that *AGL15* directly targets to *LEC2*, *FUS3*, *ABI3* and activates their

expression (Figure 16) (Zheng et al., 2009). Similarly, it was confirmed that FUS3 directly binds to *LEC1*, *ABI3*, *AGL15* and itself *in vivo*, and upregulates these target gene transcription levels (Figure 16) (Wang and Perry, 2013). The cross-regulation between these embryonic regulators partially explains the complex and overlapping phenotypes displayed by each mutant.

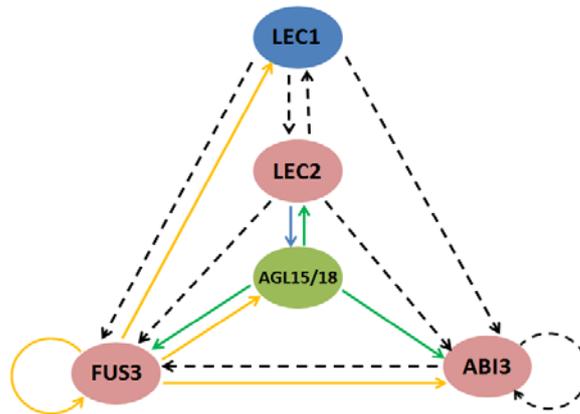


Figure 16. A model for LEC1/AFL regulatory interaction network during embryogenesis.

1.7.3 Epigenetic regulation of *LEC1/AFL* genes

Genetic analysis has revealed the importance of chromatin modification in the control of the expression of *LEC1/AFL* genes. As discussed above, PKL, the CHD3 chromatin remodeling factor, acts as a transcriptional repressor for *LEC1/AFL* genes during germination and vegetative phases. *pkl* mutant displays embryonic trait in the primary roots (Ogas et al., 1997). PKL-dependent repression of *LEC1/AFL* genes, to some extent, is associated with regulation of H3K27me3 levels (Zhang et al., 2008; Aichinger et al., 2009; Zhang et al., 2012). VP1/ABI3-LIKE (VAL, also known as HSI) 1-3 proteins also play negative roles in seed developmental programs (Suzuki et al., 2007). *VAL* genes encode B3 transcription factors, containing PHD-like and CW domains often associated with chromatin factors (Perry and Zhao, 2003; Bienz, 2006). Double mutants of *val1val2* show strong embryonic phenotype including callus proliferation in shoot and root regions as well as arrested shoot apical meristem development that prevents formation of leaves and progression to vegetative development (Suzuki et al., 2007). The embryonic phenotype of *val1val2* is associated

with de-repression of *LEC1/AFL* genes in seedlings. The *VAL* genes are independently identified as sugar signaling factors (*HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE2 (HSI2/VAL1)*, *HSI2-LIKE1 (HSL1/VAL2)*, and *HSI2-LIKE2 (HSL2/VAL3)*) (Tsukagoshi et al., 2005; Tsukagoshi et al., 2007). Interestingly, in the *hsi2 hsl1* background the expression of *AFL* genes can be induced by sugar, suggesting that the *AFL* genes may be involved in sugar signaling (Tsukagoshi et al., 2007). Recently, it is reported that H3K27me3 levels of *LEC1*, *FUS3*, *ABI3* are reduced in *val1val2*, accompanied with the decreased levels of H2Aub, indicating that *VAL* proteins interact with Polycomb Repressive complex1 (PRC1) and recruit PRC1-mediated H2Aub to initiate repression of embryonic genes after germination (Yang et al., 2013).

LEC1/AFL genes are epigenetically repressed after germination by both PRC1 and PRC2 via histone modifications. As mentioned before, PRC2 mutants *clf swm* and *pie* result in neoplastic, callus-like structures during vegetative phase because of the elevated expression of *LEC2*, *FUS3*, *ABI3* (Chanvivattana et al., 2004; Bouyer et al., 2011). *Arabidopsis* PRC1, constituted of two RING1 proteins (AtRING1a and AtRING1b), Like Heterochromatin1 (LHP1), and three BMI proteins (AtBMI1a, AtBMI1b and AtBMI1c), functions in the modification of histone H2A mono-ubiquitination (H2Aub) (Sanchez-Pulido et al., 2008; Bratzel et al., 2010; Chen et al., 2010). Similar to PRC2 mutants (*clf swm*, *pie*), the double mutants *Atring1a Atring1b*, *Atbmi1a Atbmi1b* show the embryonic traits in somatic tissues, which is associated with increased *LEC1/AFL* gene expression, indicating that PRC1 represses the embryonic program after germination (Bratzel et al., 2010; Chen et al., 2010). Recently, it is shown that H3K27me3 at *LEC1/AFL* genes are strongly reduced in *val1 val2* and *atbmi1a atbmi1b atbmi1c*, while H2Aub level at these genes is significantly decreased in *val1 val2* but not in *emf1* and *clf swm*, indicating that *VAL* proteins and AtBMI1-mediated H2Aub first initiate repression of seed maturation genes and then the repression is maintained by PRC2-mediated H3K27me3 (Figure 17) (Yang et al., 2013).

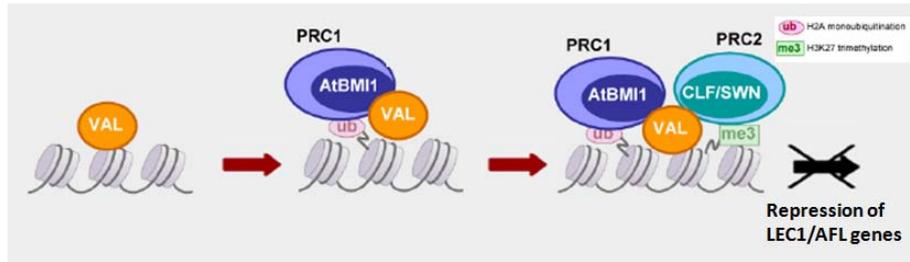


Figure 17. Proposed model for the PcG-mediated repression of seed maturation genes after germination (Yang et al., 2013).

On the other hand, the histone deacetylase *hda6 hda19* double mutants show growth arrest and somatic embryo formation, and this embryonic phenotype can be suppressed by the *lec1*, *abi3* and *fus3* mutations suggesting that *LEC1*, *ABI3* and *FUS3* expressions are repressed by HDA6 and HDA19 (Tanaka et al., 2008). Recently, it is found that HDA19 interacts with HSL1 (VAL2) *in vitro* and *in vivo* (Zhou et al., 2013). HDA19 could directly bind to the chromatin of the seed maturation genes and the ectopic expression of these genes in *hda19* seedlings is associated with increased levels of H3ac, H4ac and H3K4me3 and decreased levels of H3K27me3, similar to that of *hsl1*. These results suggest that HDA19 and HSL1 may act together to repress seed maturation gene expression during germination. The embryonic lethal phenotype of *hsl1 hda19* double mutants also indicates that HDA19 and HSL1 may play a vital role during embryogenesis (Zhou et al., 2013).

LEC2 and *FUS3* expressions are also regulated by microRNA, as mutation of *DICER-LIKE1* leads to premature expression of the maturation program during early embryogenesis accompanied with elevated levels of *LEC2* and *FUS3*, indicating the microRNA processing machinery negatively regulates *LEC2* and *FUS3* transcription during early embryogenesis (Willmann et al., 2011).

Recently, it is found that *BRM*, a SNF2 chromatin remodeling factor, has a role in repressing *ABI5* expression after seed germination and during vegetative development and *ABI3* during seed germination. *BRM* is strongly associated with the region of *ABI5* locus occupied by the +1 nucleosome. Loss of *BRM* activity leads to

destabilization of a nucleosome at *ABI5* locus, indicating that BRM represses *ABI5* transcription by promoting high occupancy of the +1 nucleosome and by directing this nucleosome from a more favorable predicted position to a position more proximal to the TSS (Han et al., 2012).

In general, the above data have shown that epigenetic modifications play important roles in the repression of *LEC1/AFL* genes especially during and after seed germination. However, little is known concerning how these transcription factors are activated during seed development. In particular, the mechanism of initiation and establishment the activate state of *LEC1/AFL* genes during embryogenesis and maturation process is not understood and remains to be elucidated.

1.8 Objective and Organization of this thesis

Chromatin remodeling and histone modification play important roles in the establishment and dynamic regulation of gene expression states. However, little is known regarding to the regulatory mechanism of chromatin modification and remodeling that control gene expression involved in plant development and responses to environmental cues. My thesis work concerns the analysis of a chromatin remodeling factor CHR5 and a histone demethylase gene JMJ15 for their function in regulating chromatin structure or in resetting chromatin modifications that control the expression of plant developmental and stress responsive genes.

The first result chapter (Chapter 2) aims to analyze the temporal and spatial expression pattern and function of CHR5 in late embryogenesis and seed maturation process. The key points of this part were (1) the expression pattern of CHR5 in plant development; (2) characterization of *chr5* T-DNA mutants; (3) the effect of CHR5 mutations on the *LEC1/AFL* transcription during embryogenesis; (4) the antagonistic role between CHR5 and PKL in the control of *LEC1/AFL* gene expression; (5) the chromatin mechanism of *LEC1/AFL* gene regulation by CHR5 and PKL during seed maturation program.

The objective of the second part of results (Chapter 3) is to investigate the biological and molecular function of JMJ15 in plant development. To examine the biological function, JMJ15 tissue expression pattern and *jmj15* mutants (gain-of-function and loss-of-function mutants) were characterized. The effects of the mutations on plant growth and development were evaluated. For the molecular function, the transcriptome of *jmj15* overexpression mutants was analyzed by microarray followed by RT-PCR validation. The de-regulated genes were analyzed for GO function category classification, for comparison with microarray data from *atx1/sdg2* mutants, and for enrichment for histone H3K4 modification.

Finally a general discussion section is provided that discusses the main results and outlines the perspectives of the work.

CHAPTER 2

Functional analysis of a chromatin remodeling factor *CHR5* in *Arabidopsis*

Introduction

CHR5 belongs to the CHD1 subfamily of chromatin remodeling factors, the function of which is not yet studied in plants. *LEC1/AFL* genes, as the master transcriptional factors that control seed development, have been found to be expressed during embryo development but repressed during vegetative phase by several negative epigenetic regulators including PKL (a CHD3 chromatin remodeler). However, the mechanism of activation of *LEC1/AFL* gene expression during embryogenesis is unknown.

In this part, I first studied the expression pattern of CHR5 by using transgenic plants containing *proCHR5-GUS* construct and RT-PCR approaches. The results showed that CHR5 was highly specifically expressed during late embryogenesis. To study CHR5 function in gene expression and plant development, I obtained 6 lines of *chr5* T-DNA insertion mutants and the complementation of a mutant allele with the *proCHR5-CHR5-HA* fusion construct was obtained. The analysis showed that *CHR5* was involved in the regulation of *LEC1/AFL* gene expression during embryo development and seed maturation process. In order to analyze the relationship between CHR5 and PKL in seed maturation gene expression, the double mutant *chr5 pkl* was generated and analyzed. The results indicate that CHR5 and PKL function antagonistically in the control of *LEC1/AFL* transcription during seed developmental and vegetative phases. The chromatin modifications of *LEC1/AFL* loci in *chr5* and *pkl* mutants were further detected, also confirming the opposite role of CHR5 and PKL in *LEC1/AFL* expression. Finally, the mechanism by which *CHR5* regulates *LEC1/AFL* genes was studied. The key points of this were (1) if CHR5 directly associates with *LEC1/AFL* genes during embryogenesis; (2) how CHR5 regulates the transcription level of *LEC1/AFL* genes—nucleosome positioning, histone modification or others. ChIP experiment proved that CHR5 directly binds to the promoter region of *FUS3* and *ABI3*. At the same time, CHR5 was shown to reduce nucleosome occupancy near the transcription start site (TSS) of *FUS3*.

This part of the results is written in an article that is currently submitted for publication.

Chromodomain, Helicase and DNA-binding CHD1 and CHD3 proteins act antagonistically to regulate seed maturation program in *Arabidopsis*

Running title: Regulation of embryo genes by CHD proteins

Chromodomain, Helicase and DNA-binding CHD1 and CHD3 proteins act antagonistically to regulate seed maturation program in *Arabidopsis***2.1 Abstract**

Chromatin modification and remodeling are the basis for epigenetic regulation of gene expression. LEAFY COTYLEDON1 (*LEC1*), LEAFY COTYLEDON 2 (*LEC2*), ABSCISIC ACID-INSENSITIVE 3 (*ABI3*), and FUSCA3 (*FUS3*) are key regulators of seed maturation and are expressed during embryo development but epigenetically repressed after seed germination. The CHD3 protein PICKLE (*PKL*) is involved in the epigenetic silencing of the genes. However, the chromatin mechanism that establishes the active state of the genes during embryogenesis is not clear. We show that the *Arabidopsis* CHD1-related gene, *CHR5*, displayed a similar expression pattern as *ABI3* and *FUS3* during embryo development. The mutation of the gene led to decreased expression of *LEC1*, *ABI3* and *FUS3* in developing seeds and reduced seed storage protein accumulation. Analysis of double mutants revealed an antagonistic function between *CHR5* and *PKL* in embryo gene promoter activity and seed storage protein accumulation. *CHR5* was directly associated with the promoters of *ABI3* and *FUS3* and *chr5* mutations led to increased nucleosome occupancy near the transcriptional start site. The results suggest that *CHR5* is involved in nucleosome occupancy to regulate embryo identity genes expression, which is counterbalanced by *PKL* during embryo development.

2.2 Introduction

Chromatin modification and remodeling are the biochemical basis for epigenetic regulation of gene expression. The nucleosome is the basic repeating unit of chromatin and is composed of an octamer of histones and a segment of 147 bp DNA. Histone modification such as histone lysine acetylation and methylation play important roles for gene activity. Histone lysine acetylation is generally involved in gene activation, whereas histone lysine methylation may have either a positive or a negative effect on gene expression (Berger, 2007; Mosammaparast and Shi, 2010). In plants tri-methylation of H3 lysine 4 (H3K4me3) is enriched at the 5' end of actively transcribed genes, whereas tri-methylation of H3 lysine 27 (H3K27me3) marks repressed developmental and stress responsive genes (Liu et al., 2010; Li et al., 2013).

Chromatin remodeling involves altering histone-DNA contacts, sliding nucleosomes, and exchanging or removing histones and entire nucleosomes. Chromatin remodelers which are ubiquitous in eukaryotic cells (Flaus et al., 2006), are compositionally and functionally diverse, but they all share the presence of a subunit that belongs to the SNF2-like family of ATPases, among which are the Chromodomain, Helicase/ATPase and DNA-binding domain (CHD) proteins (Lusser and Kadonaga, 2003). Besides *Saccharomyces cerevisiae* that has only one CHD protein (Chd1), other higher eukaryotes have several CHD members that are divided into CHD1, CHD2 and CHD3 subfamilies (Hall and Georgel, 2007). The two chromodomains in CHD1 proteins are essential for recognition of di- or tri-methylated lysine 4 of histone H3 (H3K4me_{2/3}) (Flanagan et al., 2005; Sims et al., 2005; Vermeulen et al., 2010). CHD1 proteins are implicated in gene expression at different levels (Ho and Crabtree, 2010). Recent results suggests that CHD1 proteins have both chromatin assembly and remodeling functions to direct the positioning of nucleosomes required for gene transcription (Gkikopoulos et al., 2011; Torigoe et al., 2013). In addition to the double chromodomains and the helicase/ATPase, CHD3 subfamily proteins contain one or two Plant Homeodomain (PHD) fingers at the N-terminus. CHD3 members in *Drosophila melanogaster* and mammalian cells are the central components of the Nucleosome Remodeling and histone Deacetylase (NuRD) or Mi-2 complexes regulating transcriptional repression (Hall and Georgel, 2007; Ramirez and Hagman, 2009). However, other studies revealed that CHD3 members

can also function as a transcriptional coactivator (Saether et al., 2007; Murawska et al., 2008).

Several CHD3 members are identified in plants (Hu et al., 2012). The *Arabidopsis thaliana* (*Arabidopsis*) CHD3 protein PICKLE (PKL) was initially found to repress embryonic traits after seed germination (Ogas et al., 1997). PKL functions as a transcriptional repressor of embryo identity genes such as *LEAFY COTYLEDON1* (*LEC1*), *LEAFY COTYLEDON 2* (*LEC2*), *ABSCISIC ACID-INSENSITIVE 3* (*ABI3*), and *FUSCA3* (*FUS3*) in seedlings (Ogas et al., 1999; Zhang et al., 2008; Aichinger et al., 2009; Zhang et al., 2012). In contrast to animal CHD3 proteins, PKL is found to promote trimethylation of histone H3 lysine 27 (H3K27me₃) over target genes rather than histone deacetylation (Zhang et al., 2008). But other results suggest that the PKL protein may be a transcriptional activator required for the expression of genes encoding Polycomb Repressive Complex2 (PRC2) that catalyzes trimethylation of H3K27 (Aichinger et al., 2009). Recent data revealed that the rice (*Oryza sativa*) CHD3 protein, CHR729, is a bifunctional chromatin regulator that recognizes and modulates H3K4 and H3K27 methylation over repressed tissue-specific genes (Hu et al., 2012). By contrast, only one CHD1 gene is found in the genome of *Arabidopsis* or rice (Hu et al., 2012). Its function in plant gene expression and development remains unknown.

LEC1 and the B3-domain ABI3, FUS3, and LEC2 (referred to as AFL) are key transcriptional regulators of zygotic embryo development (Giraudat et al., 1992; Lotan et al., 1998; Stone et al., 2001). These factors activate the seed maturation gene expression program in a complex network (Kagaya et al., 2005; Santos-Mendoza et al., 2008; Suzuki and McCarty, 2008; Monke et al., 2012; Wang and Perry, 2013). These genes are expressed specifically during embryo development and epigenetically repressed after seed germination primarily by PRC2-mediated H3K27me₃ (Makarevich et al., 2006; Aichinger et al., 2009; Berger et al., 2011). *Arabidopsis* PRC2 mutants with reduced H3K27me₃ display derepression of *LEC1* and *AFL* genes and embryonic traits in seedlings (Chanvivattana et al., 2004; Schubert et al., 2006; Bouyer et al., 2011). Recent data indicate that the E3 H2A monoubiquitin ligase activity of the Polycomb Repressive Complex1 (PRC1) that recognizes and binds to

H3K27me3 is also required for the post-germination repression of the genes (Bratzel et al., 2010; Yang et al., 2013).

Besides the epigenetic repression of the embryo identity or seed maturation genes after seed germination, little is known about the chromatin mechanism that establishes the active state of these genes during embryogenesis and seed development. In this work we show that the *Arabidopsis* CHD1 gene, known as *CHR5*, is expressed during embryo development and seed maturation and is directly involved in the activation of *ABI3* and *FUS3* expression. In addition, we show that *CHR5* and *PKL* have antagonistic function in *ABI3* and *FUS3* expression in developing seeds. *CHR5* binds to the promoter of *ABI3* and *FUS3* and regulates nucleosome occupancy near the transcriptional start sites of the genes, whereas *PKL* represses the expression of *ABI3* and *FUS3* genes during seed maturation. The data suggest that interplay between CHD proteins regulates seed maturation gene expression program.

2.3 Results

2.3.1 *CHR5* is expressed during late embryogenesis

Analysis of microarray data suggested that *CHR5* is highly expressed in developing and mature seeds, shoot apex and floral organs (<http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi?primaryGene=AT2G13370&modeInput=Absolute>). To confirm the data, we produced >10 independent transgenic plants expressing the GUS reporter gene under the control of a 2 kb promoter region of *CHR5*. Most of the transgenic lines displayed a similar GUS staining pattern that was detected in seeds, seedlings, flower buds and floral organs such as carpel, stigma, stamen and pollen (Figure S1). In young seedlings GUS staining was mostly detected in root tip, shoot apex, and vasculature (Figure S1). In developing seeds GUS expression started at about late globular-triangular stages and gradually increased till the mature stage of embryogenesis (Figure 1A). To further study *CHR5* expression in developing seeds, we analyzed *CHR5* transcript levels by RT-qPCR using mRNA isolated from siliques from stage 3 to stage 10 as defined at <https://www.genomforschung.uni-bielefeld.de/GF-research/AtGenExpress-SeedsSiliques.html>. Stages 1 to 4 correspond to the zygote to globular stages of embryo development. The *CHR5* transcript was undetectable before stage 4 (Figure 1B). After stage 4, the *CHR5* mRNA level increased gradually till the embryo maturation stage (Figure 1B). This confirmed the GUS staining data and indicated that *CHR5* is activated during embryo development, which roughly corresponded to that of the late *AFL* genes (i.e., *FUS3* and *ABI3*) (Wang et al., 2007). These observations suggested that *CHR5* might play a role in late embryo development and seed maturation.

2.3.2 *chr5* mutants characterization

To study *CHR5* function in gene expression and plant development, we characterized *Arabidopsis* T-DNA insertion lines of the gene in Columbia-0 (Col-0) and Wassilewskija (Ws) backgrounds (Figure S2). The insertions interrupted the production of the full length transcript of the gene (Figure S2). The mutants did not display any visible morphological defect, except a weak long hypocotyl phenotype in different light conditions (Figure S3).

Because *CHR5* expression was activated during embryo development, we examined whether the *chr5* mutations affected the expression of the embryo regulatory genes in developing siliques (at stage 6) of by RT-qPCR. Two mutant alleles, *chr5-1* and *chr5-6*, were selected for the analysis. In the mutants, the mRNA levels of *LEC1*, *FUS3* and *ABI3* were reduced compared to wild type, while that of *LEC2* was not clearly affected (Figure 2A). In addition, the expression of seed storage protein genes (i.e., *2S2*, *7S1*, *OLE1* and *CRA1*) which are downstream targets of *LEC1* and *AFL* (Kagaya et al., 2005), was also reduced (Figure 2A). By contrast, the expression of *PIL5*, a *PHYTOCHROME-INTERACTING FACTOR3-LIKE* gene that inhibits seed germination (Oh et al., 2004), was not changed in the mutants (Figure 2A). To confirm the data, we analyzed the seed storage protein accumulations by SDS-PAGE according to previous description (Finkelstein and Somerville, 1990). Fifty seeds per genotype were used for protein extraction; one fifth of the extraction was loaded for the analysis. The analysis revealed a decrease of 2S albumin and 12S globulin proteins in mature seeds of the mutants compared to wild type (Col-0) (Figure 2B). More pronounced decrease was also detected in *abi3* and *fus3* loss-of-function mutants (Figure 2B).

To confirm the effect of *chr5* mutation on seed gene expression and storage protein accumulation, we made complementation tests by transforming *chr5-6* plants with the *pCHR5-CHR5-HA* fusion construct under the control of the 2.0 kb promoter region of *CHR5*. The production of the fusion protein in the complementation plants was detected by western blotting methods using the anti-HA antibody (Figure S4). The levels of seed storage proteins in *chr5-6* were restored to wild type levels in the complementation plants (Figure 2B), indicating that the decrease of storage proteins was caused by the mutation of *CHR5*. These data suggested that *CHR5* might be involved in the gene expression program of embryo development and seed maturation in *Arabidopsis*.

2.3.3 Antagonistic function between *CHR5* and *PKL* in seed maturation gene expression

The *pickle* (*pkl*) mutation derepresses embryo regulatory genes in young seedlings with the production of embryonic traits in the primary root of seedlings, accumulation

of seed storage reserves, and formation of somatic embryos (Ogas et al., 1997). However, this *pickle* root phenotype only occurs with a low penetrance (Ogas et al., 1997). To study functional interaction between *CHR5* and *PKL*, we generated *chr5-6 pkl* double mutants and examined the seedling root phenotype. The penetrance of the embryonic root in *pkl* (*pkl1* allele) (about 15%), revealed by Fat red dye staining of fatty acids present in embryonic roots, was decreased to about 5% in the double mutants (Figure 3A, 3B). Similar results were obtained in the *chr5-2 pkl1* double mutant (Figure S5). Ectopic expression of *LEC1*, *ABI3*, and *FUS3* in *pkl* seedlings or seedling roots was suppressed in the double mutants (Figure 3C, 3D), suggesting antagonistic actions of the two CHD proteins on the expression of *LEC1*, *ABI3* and *FUS3*. No clear decrease of *LEC2* ectopic expression in the double mutant siliques (Figure 3E), corroborated the observation that *CHR5* was not or weakly involved in the activation of the gene (Figure 2A).

In *pkl* siliques, the expression levels of *LEC1*, *ABI3* and *FUS3* were higher than in wild type (Figure 3E), indicating that *PKL* may also repress these genes during embryo and seed development. This was in agreement of gradual activation of the *PKL* gene during seed maturation (Fig. 1B). Consistently, a higher accumulation of seed storage proteins was detected in *pkl* compared to wild type (Figure 2B). In the siliques of the *chr5-6 pkl* double mutants, the expression of *LEC1*, *ABI3* and *FUS3* was lower than that in *pkl* (Figure 3E). In addition, the increased accumulation of seed storage proteins in *pkl* was restored to wild type levels in the double mutants (Figure 2B), further supporting the idea that *CHR5* and *PKL* have an opposite function to regulate the seed maturation gene expression program.

2.3.4 *CHR5* acted on the promoter of *ABI3* and *FUS3*

We next studied whether *chr5* and *pkl* mutations affected the expression pattern of the embryo regulatory genes during embryo development. Because of the similar expression pattern of *CHR5* with *ABI3* and *FUS3*, we chose the promoter region of *ABI3* and *FUS3* (with 5'-UTR) to control *GUS* expression (translational fusion) in transgenic wild type, *chr5-6* and *pkl* single and double mutant plants. A 4 kb promoter region of *ABI3* and a 2.2 kb promoter region of *FUS3* were used. Several (6-11) independent transgenic lines for each construct in each genotype were characterized.

GUS staining revealed that *ABI3* and *FUS3* promoters started to be active in the embryo during the late globular or early heart stages in the wild type (Figure 4A), which corresponded to the expression profile of the genes (Wang et al., 2007) indicating that the used promoter regions contained necessary elements for the expression patterns. We also noticed that the *FUS3* promoter was active in the suspensor in the early stages of embryogenesis. The *chr5* and *pkl* single and double mutations did not clearly alter the expression pattern during embryo development (Figure 4A). Quantification of the *GUS* transcripts in siliques (at stage 6) of several independent transgenic plants indicated that the promoter activities of both genes were reduced in *chr5-6*, but increased in *pkl* background compared to wild type (Figure 4B). In the double mutants, the promoter activities were lower than that in *pkl* (Figure 4A, 4B). These results confirmed the above data and revealed that *CHR5* and *PKL* act antagonistically to regulate the promoter activity of *ABI3* and *FUS3* in developing embryo.

2.3.5 CHR5 binds directly to the promoter region of *ABI3* and *FUS3*

To examine whether *CHR5* was directly associated with the embryo regulatory genes, we isolated chromatin from *chr5-6* plants complemented by *pCHR5-CHR5-HA* (Figure S4). The chromatin fragments were immuno-precipitated with anti-HA and analyzed by qPCR using primer sets corresponding to several regions of *AFL* and *LEC1* loci and to *ACTIN2* gene and Ta3 transposon as controls (Figure 5). The analysis revealed that only the regions upstream to or near the transcription start site (TSS), not the gene body regions, of *ABI3* and *FUS3* were clearly enriched in the precipitated fractions (Figure 5), indicating that *CHR5* was associated with the promoter and TSS of *ABI3* and *FUS3*. No association with *LEC1* or *LEC2* was detected, suggesting that *CHR5* was not directly involved in their expression. The effect of *chr5* mutations on *LEC1* may be due to an indirect effect.

2.3.6 Chromatin modifications of *LEC1* and *AFL* loci in *chr5* and *pkl* mutants

To study whether *chr5* mutations affected histone modification on *LEC1* and *AFL* genes during seed development, chromatin fragments isolated from wild type and *chr5-6* developing seeds (from siliques at stage 6) were immunoprecipitated by

antibodies of H3K4me3 and H3K27me3 and analyzed by qPCR using primer sets corresponding to the 5'-UTR region of *FUS3*, *ABI3*, *LEC1*, *LEC2* and, as a control, *PIL5*. The analysis revealed that *LEC2* and *PIL5* displayed relatively higher levels of H3K4me3 compared to the other genes (Figure 6A). However, there was no clear difference observed between the mutant and wild type. By contrast, relatively higher levels of H3K27me3 were detected on *FUS3*, *ABI3* and *LEC2* and *LEC1* compared to the control genes *RBCS* and *PIL5* (Figure 6B). The *chr5* mutation led to about 2 fold increases of H3K27me3 on *LEC1* and *AFL* (including *LEC2*) genes, but not on the control genes (Figure 6B). Similar assays with chromatin fragments directly isolated from siliques (stage 6) of the two *chr5* mutant alleles confirmed the results (Figure 6C). These data suggested that CHR5 was not involved in modulating H3K4me3 for *LEC1* and *AFL* activation during seed development. The increased H3K27me3 on *LEC1* and the late *AFL* genes may be associated with their repression in *chr5* mutants.

We also analyzed histone methylation in *chr5* and *pkl* seedlings. The results revealed that the amount of H3K4me3 on *LEC1*, *LEC2*, *FUS3* and *ABI3* was not clearly altered in *chr5-6*, *pkl* and *chr5 pkl* compared to wild type, except some increase on *LEC2* was observed in *pkl* plants (Figure S6). This corroborated the data obtained in developing seeds/siliques (Figure 6). In *pkl* seedlings that ectopically express *LEC1* and *AFL* genes, H3K27me3 was reduced on *LEC1* and *LEC2*, but not on *ABI3* and *FUS3* (Figure S6), confirming previous observations (Aichinger et al., 2009; Zhang et al., 2012). Unlike in developing seeds, the *chr5* mutation did not lead to any clear change of H3K27me3 on the seed maturation genes in seedlings. In addition, the decrease of H3K27me3 on *LEC1* and *LEC2* in *pkl* was maintained in the *chr5 pkl* double mutants, suggesting that the antagonistic function of *CHR5* and *PKL* on these genes expression in seedlings may not rely on the regulation of H3K27me3 level.

2.3.7 CHR5 may modulate nucleosome occupancy on *FUS3* promoter

Recent results have shown that CHD1 protein is involved in nucleosome positioning and turnover and thus regulates transcription rate (Zentner et al., 2013). To study whether CHR5 was involved in chromatin structure of target genes, we next examined nucleosome positioning and occupancy at the *FUS3* TSS region in chromatin isolated

from siliques (stage 6) using high-resolution micrococcal nuclease (MNase) mapping (Chodavarapu et al., 2010; Han et al., 2012). We identified one nucleosome upstream of the likely nucleosome-free region (NFR) between -182 and -17 relative to TSS and two nucleosomes downstream of the NFR of *FUS3*. NFR that is located just upstream of the TSS is commonly found in eukaryotic promoters. A typical nucleosome protects about 140-150 bp of genomic DNA from MNase digestion (Yen et al., 2012), as was the case for the three nucleosomes near the TSS of *FUS3*. The upstream one was roughly located between -465 and -265, the two downstream ones protected from -40 to +97 and from 122 to 259 relative to the TSS (Figure 7). In *chr5* mutants, we observed reproducibly a moderate increase in nucleosome occupancy at the three positions compared to wild type (Figure 7). This was consistent with the lower expression of *FUS3* in the mutants. No alteration in positioning of the three nucleosomes was observed. Thus, CHR5 was likely to be required to reduce nucleosome occupancy near the NFR of *FUS3*, which might contribute to increased activation of the gene in developing seeds.

2.4 Discussion

2.4.1 CHD1 (CHR5) and CHD3 (PKL) function in embryo/seed gene expression

In this work, we have shown that the *Arabidopsis* CHD1 gene *CHR5* is involved in embryo regulatory gene expression and seed storage protein accumulation. The observations that *chr5* mutations reduced the levels, but not patterns of *LEC1* and *AFL* expression in developing embryo suggest that *CHR5* may be mostly involved in modulating gene expression levels instead of defining expression pattern of the key regulatory genes. The *CHR5* expression pattern is similar to that of *ABI3* and *FUS3* during embryo development (Figure 1) (Wang et al., 2007). However, expression of *ABI3* and *FUS3* has been detected in earlier stages (Le et al., 2010; Raissig et al., 2013). In addition *FUS3* expression was first detected in the suspensor when the embryo was at octant stage and was not stained (Figure 4A.). Therefore, *CHR5* expression in the seed partially overlaps with that of *ABI3* and *FUS3*, suggesting that *CHR5* may be involved in establishing a favorable chromatin environment to increase the expression levels of the seed maturation genes instead of initiating the transcriptional activation process. This is consistent with normal embryo development in the mutants. The high accumulation of *CHR5* transcripts in mature embryo/seed and the effects of the mutation of the gene on seed storage protein accumulation suggest that, in addition to *LEC1*, *AFL* and the tested seed storage genes, *CHR5* may regulate many other seed maturation genes. Nevertheless, unlike *lec1*, *abi3* or *fus3* loss-of-function mutants that display a wrinkled seed phenotype with accumulation of anthocyanin in *lec1* and *fus3* cotyledons or chlorophyll in *lec1* and *abi3*, the *chr5* mutant seeds germinated normally and did not display any visible abnormality. Possibly, the reduction (about 50%) of *ABI3*, *FUS3*, *LEC1* expression and storage protein accumulation in *chr5* mutants was not sufficient to lead to a seed phenotype.

In addition to developing seeds, *CHR5* is also expressed in many rapidly growing organs/tissues, suggesting that the gene may be involved in other plant developmental processes. However, loss-of-function mutations of the gene did not produce any severe developmental defects. This is consistent with the observations that *D. melanogaster chd1* mutant zygotes are viable and display only a mild notched-wing phenotype (McDaniel et al., 2008). In yeast, the phenotype associated with *Chd1*

deletion was also minor, whereas deletion of *Chd1* together with two additional chromatin remodeling factor genes *ISWI* and *ISW2* results in synthetic phenotypes (Tsukiyama et al., 1999). This indicates the existence of functional redundancy between *CHD1* and other chromatin remodeling factors, which may explain partly the mild developmental phenotype observed in *chr5* mutants.

Previous studies have shown that the *Arabidopsis* *CHD3* protein *PKL* represses embryo regulatory genes after seed germination. The present data revealed that *PKL* also represses these genes during embryo development and seed maturation (Figure 2-4). Although *PKL* transcripts started to be detected after stage 4, its repressive function may act mostly during the maturation stages. Since the expression of *PKL* was sharply increased after stage 7 (Figure 1B), the repressive role of *PKL* may be effective during late embryogenesis as well. This is consistent with previous results showing that *PKL*-dependent repression of embryonic gene expression extends to late-embryogenesis genes during germination under stress or ABA (Perruc et al., 2007). The increase of *pABI3-GUS* and *pFUS3-GUS* expression in *pkl* developing seeds suggests that *PKL* may act on the promoter of the genes. However, it was shown that *PKL* is not directly involved in the repression of *ABI3* (Aichinger et al., 2009), while recent data indicated that *PKL* is present at *LEC1*, *LEC2* and *FUS3* loci during germination to repress these master regulators of embryonic identity (Zhang et al., 2012). Increased *ABI3* and *FUS3* promoter activities in *pkl* may be mediated by *LEC1* that was induced in *pkl* developing seeds (Figure 3E), and was previously shown to activate expression of *ABI3* and *FUS3* (Kagaya et al., 2005). As *PKL* is associated to the *FUS3* locus (Zhang et al., 2012), the derepression of *FUS3* promoter activity in *pkl* mutants suggests that the repressive function of *PKL* may act on the promoter of the gene, but it is not excluded that *PKL* may repress *FUS3* promoter through an indirect effect.

The opposite function of *CHR5* and *PKL* on *LEC1* and *AFL* expression suggests that chromatin state and/or chromatin remodeling controlled by the *CHD* proteins plays an important role for the gene expression program of embryo and seed development. *PKL* is suggested to repress genes by promoting H3K27me3. The mild increases of H3K27me3 on *LEC1* and *AFL* genes in *chr5* mutant siliques/seeds might be related to antagonist actions of *CHR5* and *PKL* during embryo development.

However, the suppression of ectopic expression of *LEC1* and *AFL* in *chr5 pkl* seedlings was not accompanied by an increase of H3K27me3 (Figure S6). It is possible that increased H3K27me3 on *LEC1* and *AFL* genes in *chr5* developing seeds might be a consequence of the repression of the genes, as H3K27me3 can be deposited to mark down-regulated genes and to memorize the repressive state (Bonasio et al., 2010). Although CHD1 is reported to binds to H3K4me3 (Flanagan et al., 2005; Sims et al., 2005; Vermeulen et al., 2010), our analysis indicates that CHR5 is not involved in modulating H3K4me3 for *LEC1* and *AFL* activation during seed development (Figure 6). Therefore, CHR5-mediated gene activation may act at different levels of chromatin modification or remodeling.

2.4.2 Mechanism of CHR5-mediated gene activation

CHD1 was initially thought to be integral to transcriptional activity. The relatively higher enrichment of the *Arabidopsis* CHD1 protein, CHR5, near the transcriptional start sites (TSS) of *FUS3* and *ABI3* is consistent with the recent data showing that *S. cerevisiae* Chd1 is enriched within the nucleosome free regions in the genome (Zentner et al., 2013). The enrichment of CHD1 proteins at TSS may be mediated through the interaction of their tandem chromodomain with H3K4me3, a hallmark of actively transcribed chromatin which is enriched at the TSS (Flanagan et al., 2005; Sims et al., 2005). However, we have shown that CHR5 targets only to the promoters of *ABI3* and *FUS3*, but not that of *LEC1*, *LEC2* or *PIL5* (Figure 5). The underlying mechanism of the locus-specific targeting of CHR5 is not known. It is unlikely that the locus-specific targeting of CHR5 is only through interaction with H3K4me3 at TSS, as the two relatively highly H3K4me3-marked genes (i.e., *LEC2* and *PIL5*) were not targeted by CHR5 (Figure 5; Figure 6).

It is shown that the binding of human CHD1 at TSS mediates subsequent recruitment of post-transcriptional initiation and pre-messenger-RNA splicing factors (Sims et al., 2005; Sims et al., 2007). More recent data indicated that CHD1 is involved in H2B monoubiquitination (Lee et al., 2012), which may facilitate transcriptional elongation and prevent cryptic transcriptional initiation from the gene bodies (Hennig et al., 2012). The deletion of *S. cerevisiae* Chd1 results in a clear decrease of the overall H2Bub level but not that of H3K4me3 (Lee et al., 2012). Consistently, *chr5* mutations did not affect H3K4me3 on the targeted loci (Figure 6).

However, our analysis of *chr5* mutants did not reveal a clear change of the overall level of H2Bub (Figure S7), suggesting that this function of maintaining H2Bub may not be conserved in CHR5.

Nucleosomes are organized into uniformly spaced arrays at the 5' end of the genes, which starts with the +1 nucleosome at around the TSS (Jiang and Pugh, 2009). A “-1” nucleosome located on the upstream in the promoter is positioned to potentially control access to gene regulatory sequences. It is suggested that CHD1 enrichment within the NFR is to position nucleosomes within the promoter or around the TSS to facilitate gene transcription and also to position nucleosomes within the coding regions of genes, which are aligned with respect to TSS to prevent cryptic transcription within gene bodies by suppressing histone turnover (Gkikopoulos et al., 2011; Hennig et al., 2012; Smolle et al., 2012; Zentner et al., 2013). Our data showing that *chr5* mutations also led to a relatively higher occupancy of the nucleosomes located near the NFR of *FUS3* are in agreement with the finding that in *S. cerevisiae* with the deletion of Chd1 nucleosomes near the NFR display a relatively higher occupancy genome-wide (Gkikopoulos et al., 2011). Possibly, the reduction of nucleosome occupancy in the promoter and near the TSS by the binding of CHD1 within NFR may facilitate the functioning of regulatory sequence such as transcription factor binding and transcription initiation complex recruitment to stimulate gene transcription. It remains to know whether CHR5 regulated nucleosome occupancy near the TSS of *FUS3* affects the binding of transcription factors including LEC1 and AFL proteins to the gene.

2.5 Methods

Plant Growth

The *Arabidopsis thaliana* genetic resources used in this study were mostly in the Columbia ecotype. Mutant lines *chr5-1* (Sail_504_D01), *chr5-2* (Salk_020296), *chr5-3* (Flag_130A16), *chr5-4* (GABI_773A12), *chr5-5* (Sail_1259_B05) and *chr5-6* (Salk_046838) were obtained from the Nottingham *Arabidopsis* Stock Center and ABRC collections. The *pkl* mutant used in this study was the *pkl-1* allele as described (Ogas et al., 1997). The *abi3* mutant (Salk_023411) and *fus3* mutant (GABI_612E06) were used. The T-DNA insertion was confirmed by PCR using the primers described in supplemental Table on line. Double mutants *chr5-6 pkl* and *chr5-2 pkl* and were obtained by genetic crossed between *chr5-6* and *chr5-2* with *pkl*. Seeds were surface-sterilized and plated on 0.5 x Murashige and Skoog medium. After stratification at 4°C for 2 d, plants were grown in a growth room under a long day photoperiod (16h light/8h dark) at 22°C.

Constructs and Transformation

For the histochemical GUS assay, the 2 kb promoter of *CHR5* was amplified from WT DNA using proCHR5-F and proCHR5-R and then cloned into the pPR97 vector. For the complementation experiment, the full length cDNA without a stop codon was amplified from total cDNA of Col-0 using primers comCHR5-F and comCHR5-R. *CHR5* promoter and cDNA were inserted into the binary vector pFA1300, which was modified based on pCAMBIA1300 (CAMBIA) and contained 2 x HA tag. The *pCHR5-GUS* and *pCHR5-CHR5-HA* constructs were introduced into Col-0 and *chr5* mutants via *Agrobacterium*-mediated transformation by the floral dip method. The *pABI3:GUS* and *pFUS3:GUS* were made using a PCR-based Gateway system. The promoter and 5' UTR of 4 kb for *ABI3* and 2.2 kb for *FUS3* were inserted as translational fusion with the *uidA* gene into the vector pGWB553.

Gene Expression Analysis

Definition of stages for seed and silique development were according to previous description (Kleindt et al., 2010). Total RNA of siliques was extracted as described (Meng and Feldman, 2010) with minor modification. The crude RNA was further

purified via the clean-up protocol of the RNeasy Plant RNA isolation kit (Qiagen) according to the manufacturer's protocol instead of Trizol reagent. Two micrograms of total RNA were treated with DNase I (Promega) and transcribed into cDNA by ImPromII reverse transcriptase (Promega). Real-time PCR was performed with the LightCycler[®] 480 SYBR Green I Master (Roche) following the manufacturer's instructions. Primers are listed in Supplemental Table 1. Three biological replicates were performed for each sample and the expression level was normalized with that of *ACTIN2*.

ChIP Assay

The ChIP experiment was performed as previously described (Benhamed et al., 2006). Chromatin was isolated from siliques and dissected seeds after cross-linking proteins and DNA with 1% formaldehyde for 1 hr under vacuum and termination of the reaction with glycine. Chromatin was fragmented to 200–1000 bp by sonication, and ChIP was performed using the following antibodies: anti-H3H4me3 (Millipore, 07–473), anti-H3K27me3 (Millipore, 07–449), anti-H3 (Abcam, ab1791), anti-H3ac (Millipore, 06–599), and anti-HA antibody (Sigma, H6908).

Histochemical GUS and Lipid Staining

GUS staining was performed as previously described (Bertrand et al., 2003). Briefly, plant siliques were fixed with 90% acetone on ice for 30 min and were washed with staining buffer (0.2% Triton X-100, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 100 mM NaH₂PO₄ and 100 mM Na₂HPO₄ pH 7.2). The dissected seeds from the fixed siliques were immersed in GUS staining solution with 1 mM X-Gluc and placed under vacuum for 1 h. After incubation at 37°C overnight, the staining solution was removed and samples were cleared by sequential changes of 75 and 95% (v/v) ethanol. Lipid staining with Fat red 7B was carried out as described previously (Ogas et al., 1997). Whole seedlings were incubated for 1 h in filtered Fat red solution (0.5% Fat red 7B in 60% isopropanol), washed three times with water and analyzed under a dissecting microscope.

Seed Protein Analysis

Seed protein was extracted from 50 seeds/ genotype by grinding mature seeds in an ice-cold mortar with 20 $\mu\text{L}/\text{mg}$ seed of extraction buffer (100mM Tris-HCl, PH 8.0, 0.5% SDS, 10% glycerol and 2% β -mercaptoethanol). Extracts were boiled for 3 min and centrifuged. The gel was loaded on a seed number basis and the proteins were resolved by 12% SDS-PAGE. Proteins were visualized by Coomassie blue staining.

MNase Assay

Siliques were harvested in liquid nitrogen after cross-linking in 1% formaldehyde. Nuclei and chromatin were isolated as described (Chodavarapu et al., 2010) with the following changes. The isolated chromatin was digested with 0.1 units/ μL of Micrococcal Nuclease (Takara) for 10 min in digestion buffer at 37°C. Mononucleosomes were excised from 1.5% agarose gels and purified using a gel purification kit (MACHEREY-NAGEL). The purified DNA was quantified using a NanoDrop-1000 spectrophotometer. Two nanograms of purified DNA were used for qPCR to monitor nucleosome occupancy. The fraction of input was calculated as $2^{-\Delta\text{Ct}}$ ($2^{-[\text{Ct}(\text{mono})-\text{Ct}(\text{gDNA})]}$) using undigested genomic DNA followed by normalization over that of gypsy-like retrotransposon -73 loci for each sample according to previous description (Gevry et al., 2009). The tiled primer sets used for realtime PCR are listed in Supplemental Table online.

Accession Numbers

Sequence data for the genes in this articles can be found in the *Arabidopsis* Genome Initiative under the following accession numbers: *CHR5* (AT2G13370), *PKL* (AT2G25170), *ABI3* (AT3G24650), *FUS3* (AT3G26790), *LEC1* (AT1G21970), *LEC2* (AT1G28300), *2S2* (AT4G27150), *7S1* (AT4G36700), *CRA1* (AT5G44120), *OLE1*(AT4G25170), *PIL5* (AT2G20180), *RBCS1A* (AT1G67090), *ACTIN2* (AT3G18780), and gypsy-like retrotransposon (AT4G07700). Mutants investigated in this study are listed in Methods.

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2.7 Figures

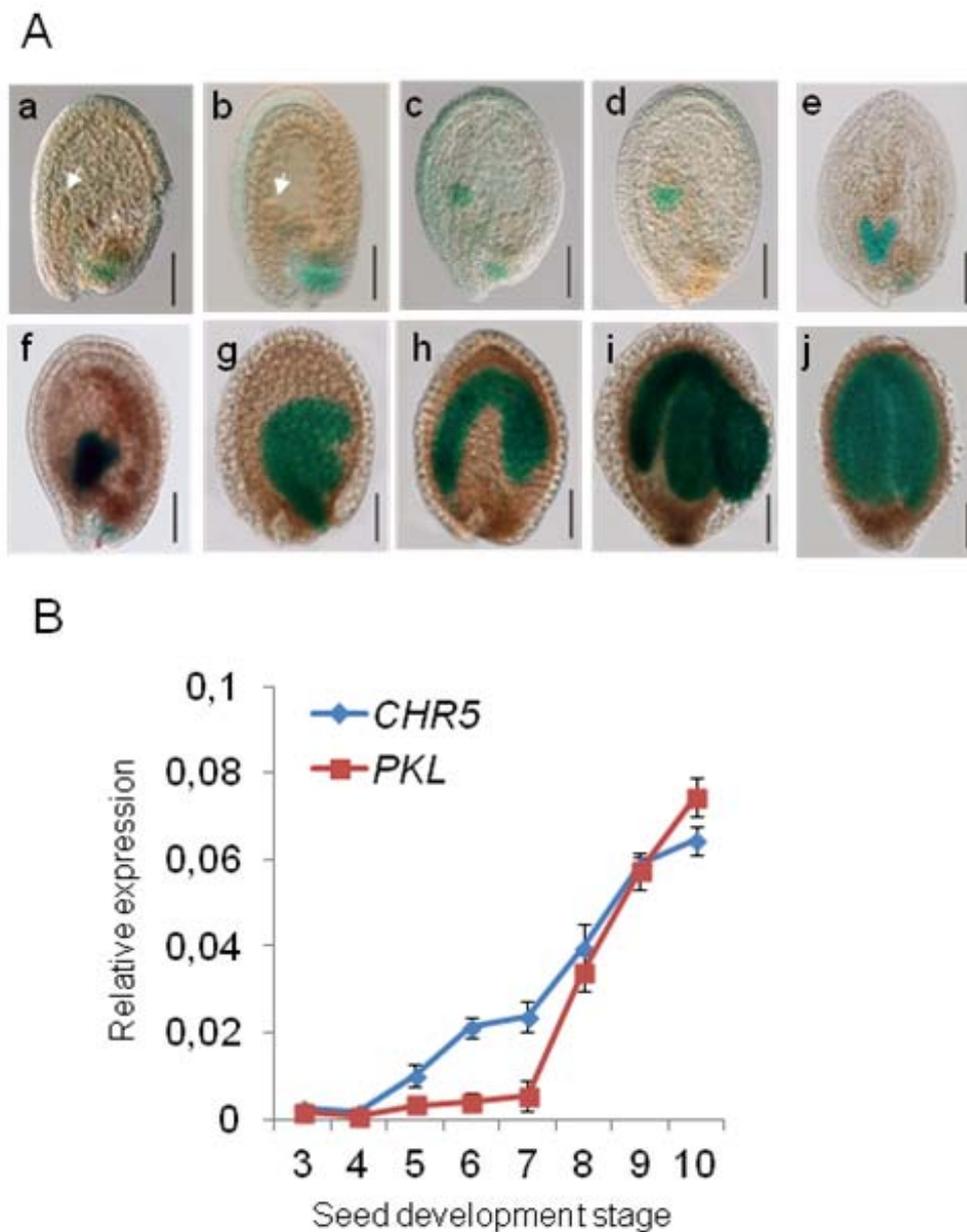


Figure 1. Expression pattern of CHR5 in developing embryo. **A.** Developing seeds of *Arabidopsis* plants transformed by pCHR5-GUS were stained for GUS activity detection. Photographs of representative seeds at different stages of embryo development (a - j) are shown. White arrow heads indicate unstained embryo at the early stages. Scale bars= 0.1 mm. **B.** qRT-PCR detection of *CHR5* and *PKL* transcripts during the different stages of silique development as defined previously (<https://www.genomforschung.uni-bielefeld.de/GF-research/AtGenExpress-SeedsSiliques.html>). Relative expression levels (with *ACTIN2* mRNA set as 1) are shown.

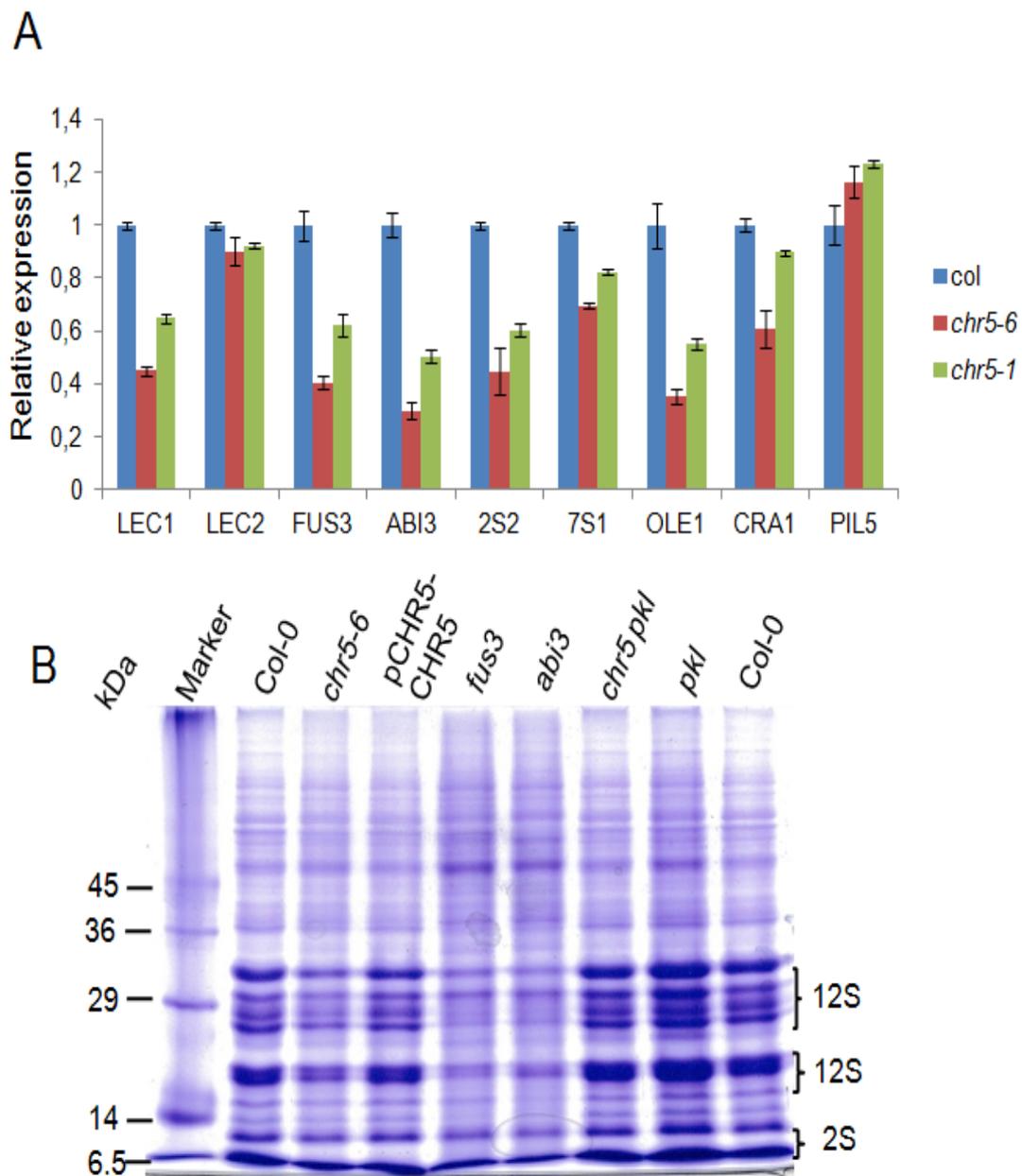


Figure 2. *CHR5* mutations reduced the mRNA levels of embryo and seed maturation genes and seed storage protein accumulation. **A.** transcript levels of *LEC1*, *AFL* and the indicated seed storage genes in siliques harvested at stage 6. Bars = means values \pm SD from three biological repeats. **B.** SDS-PAGE analysis of mature seed proteins from the wild type (*Col-0*), mutants (*chr5-6*, *fus3*, *abi3*, *pkl* and *chr5-6 pkl*) and *chr5-6* complementation (*pCHR5-CHR5-HA*) plants. Peptides corresponding to 2S albumin and 12S globulin are indicated.

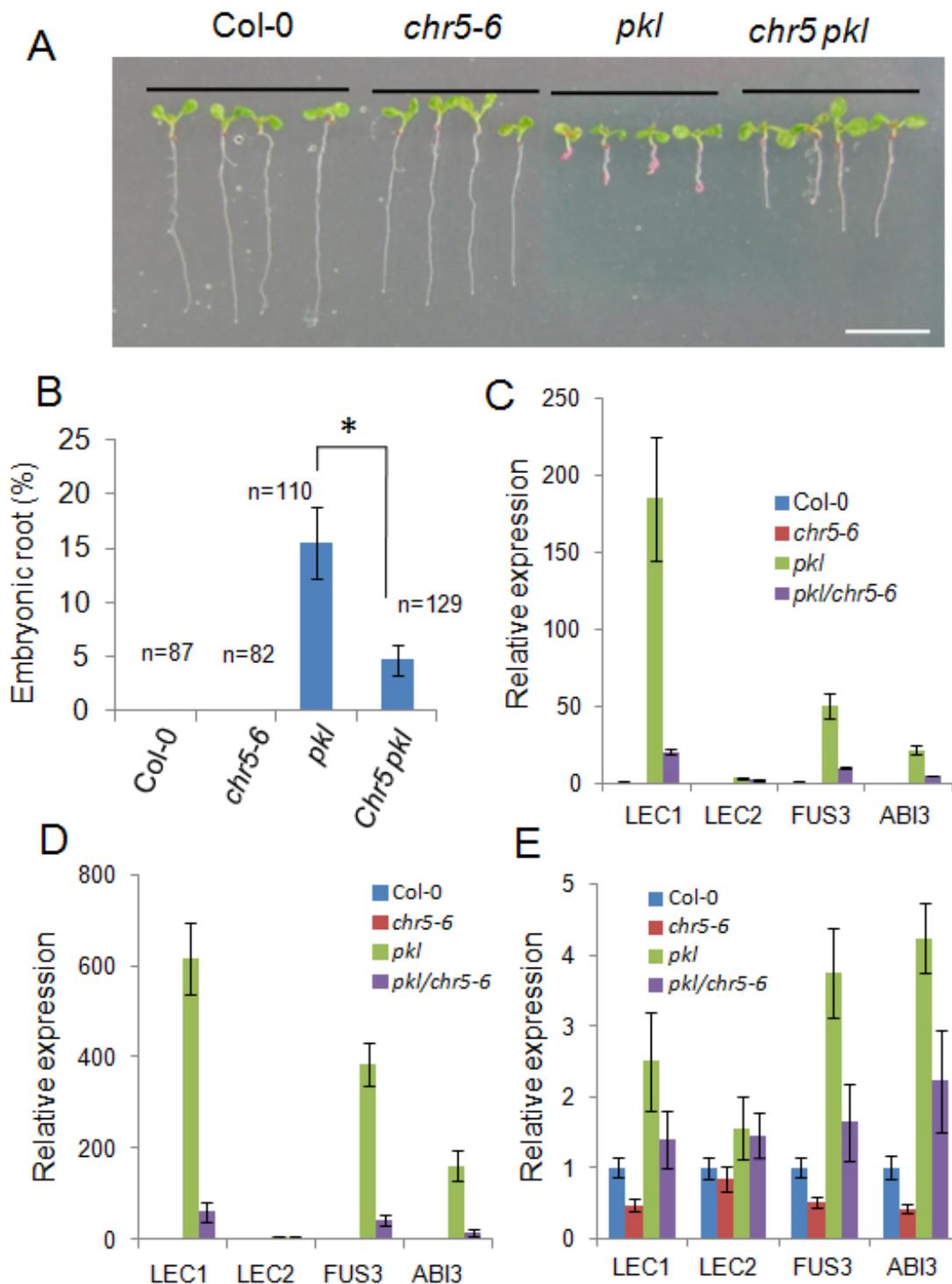


Figure 3. CHR5 and PKL have antagonistic function in the expression of embryo regulatory genes. **A.** Seedling phenotype of wild type (Col-0), *chr5-6*, *pk1* and *chr5 pk1* double mutants stained by Fat red dye. Bar= 5mm. **B.** Embryonic root penetrance of the 4 genotypes calculated from 82 – 129 plants. Asterisks indicate the significance of difference by Student's t-test. **C-E.** Relative expression of *LEC1* and *AFL* genes in 5 day-old seedlings (**C**), 5 day-old seedling roots (**D**) and Siliques (**E**), with the levels in wild type set as 1. Bars = means values +/-SD from three biological repeats.

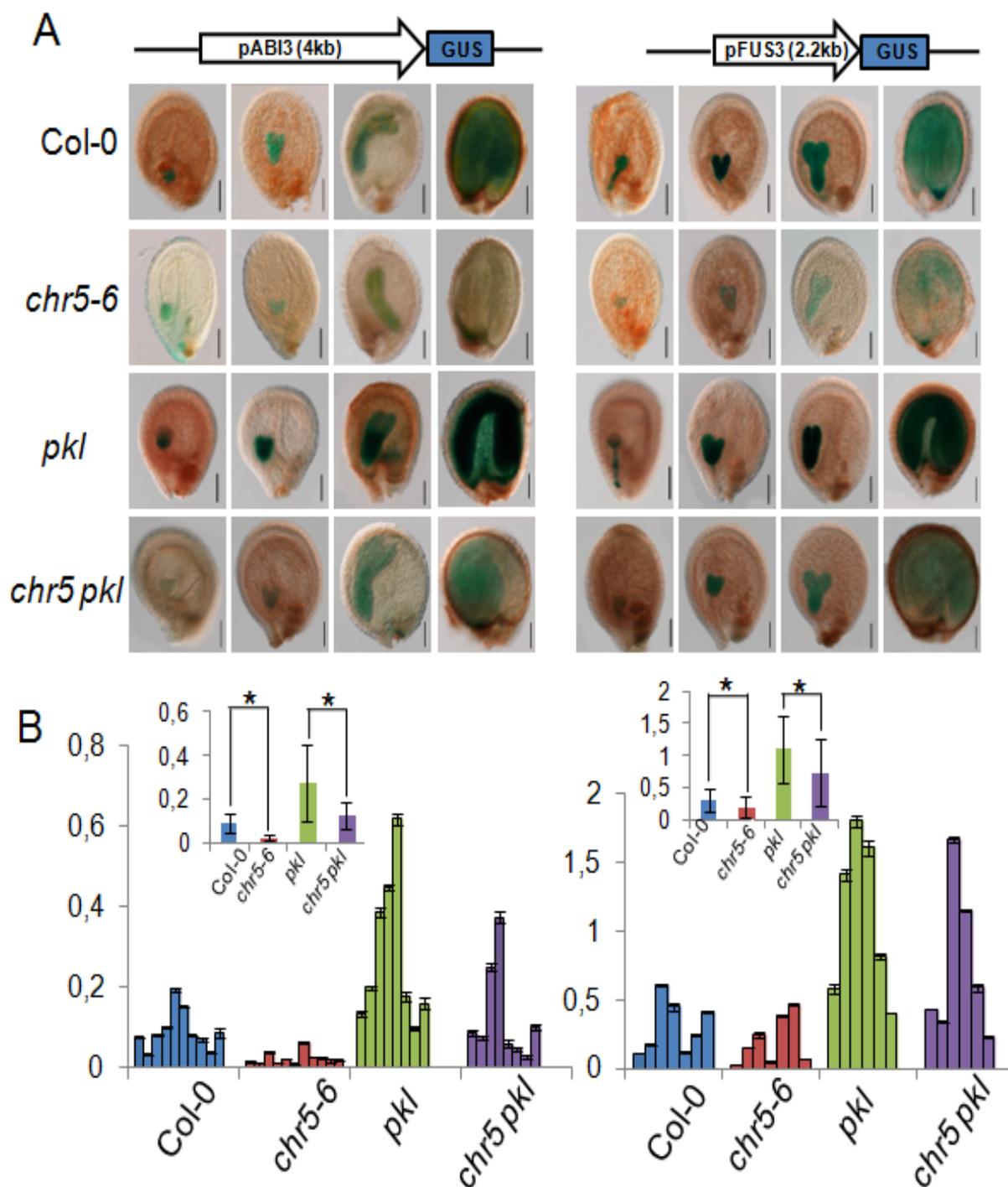


Figure 4. Promoter activity of ABI3 and FUS3 in wild type (Col-0), *chr5-6*, *pk1* and *chr5-6 pk1* double mutants. **A.** GUS staining of developing seeds of plants transformed by *pABI3-GUS* (left panels) and *pFUS3-GUS* right panels. **B.** Quantification of GUS transcripts (relative to *ACTIN2* mRNA) from 6-10 independent transgenic lines per genotype for each construct. Insets: means values \pm SD are shown. Significance of differences by Student's t-test is indicated by asterisks.

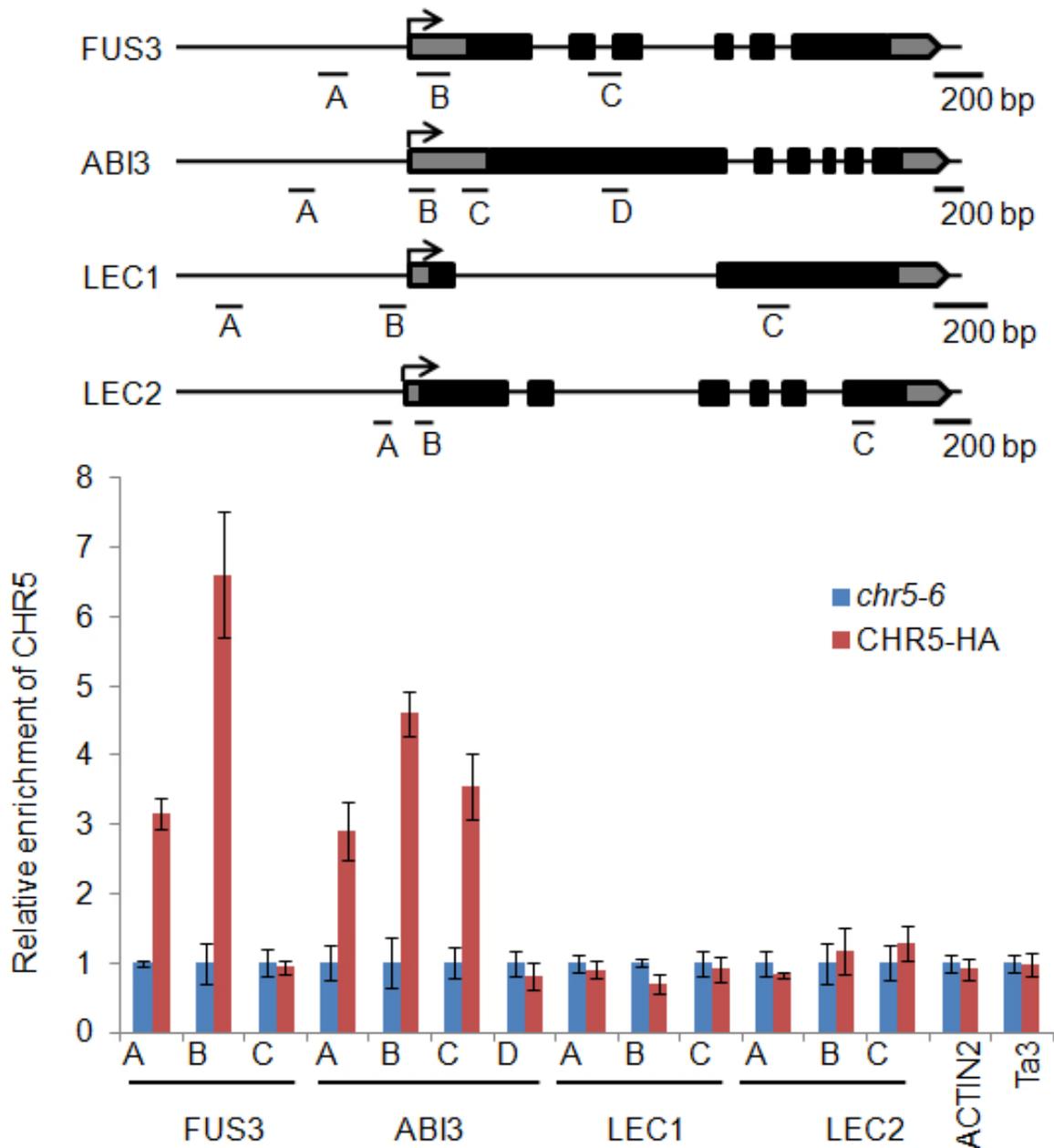


Figure 5. CHR5 is enriched near the transcriptional start site of *ABI3* and *FUS3*. ChIP-qPCR analyses for the binding of CHR5 to *LEC1* and *AFL* genes. Chromatin isolated from siliques of *chr5-6* complementation plants by *pCHR5-CHR5-HA* (CHR5-HA) was immunoprecipitated with anti-HA antibody, and the amount of precipitated DNA was measured by quantitative PCR using 3-4 primer sets (A-D) as indicated on the 4 genes (top). Transcriptional start sites of the four genes are indicated by arrows. Levels in non-complemented mutant (*chr5-6*) were set to 1 after normalization to the levels of input DNA. *ACTIN2* and *Ta3* DNA were tested as controls.

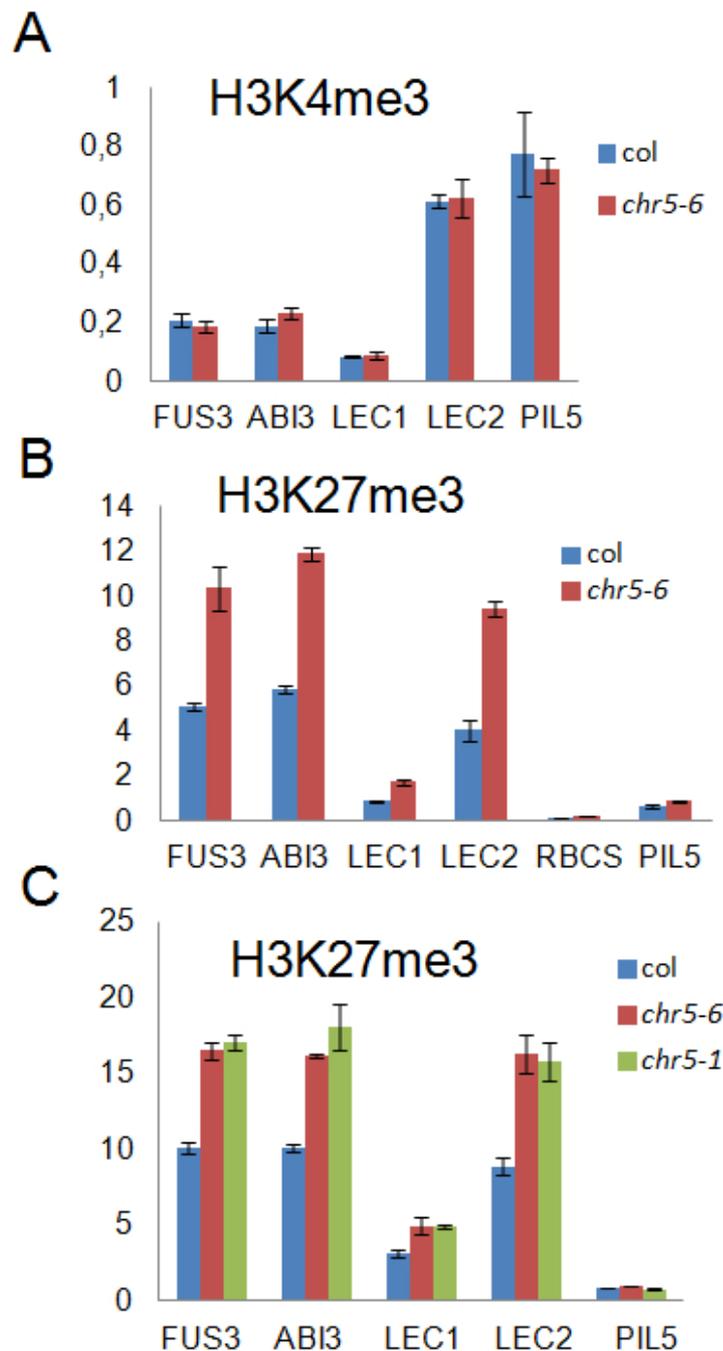


Figure 6. Histone methylation on *LEC1*, *AFL* and *PIL5* genes in wild type and *chr5* mutants. **A.** H3K4me3 on the 5 genes in seeds harvested from developing siliques (at stage 6) of wild type and *chr5-6*. **B.** H3K27me3 on the 5 genes and *RBCS* (as a control) in seeds harvested from developing siliques (at stage 6) of wild type and *chr5-6*. **C.** H3K27me3 on the 5 genes in developing siliques (at stage 6) of wild type, *chr5-1* and *chr5-6*. Levels are relative to H3K4me3 or H3K27me3 levels on the *ACTIN2* locus.

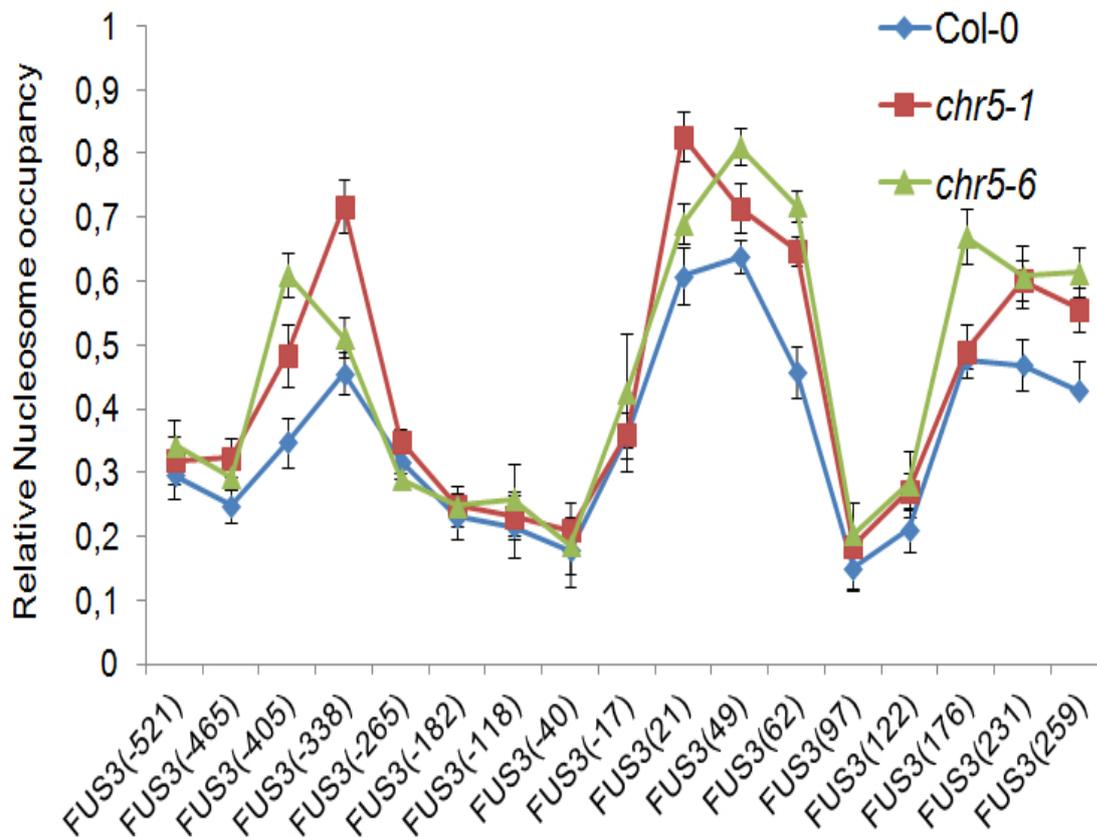


Figure 7. *CHR5* mutations lead to increased nucleosome occupancy near the transcriptional start site of *FUS3*. Chromatin isolated from wild type (Col-0), *chr5-1* and *chr5-6* was digested by micrococcal nuclease, then analyzed tiled primer qPCR to monitor nucleosome positioning and occupancy at the 5'-end of the *FUS3* locus. The fractions of input were calculated as $2^{-\Delta Ct}$ ($2^{-[Ct(\text{mono})-Ct(\text{gDNA})]}$) using undigested genomic DNA followed by normalization over that of gypsy-like retrotransposon -73 loci for each sample. NFR, nucleosome free region.

2.8 Supplemental data

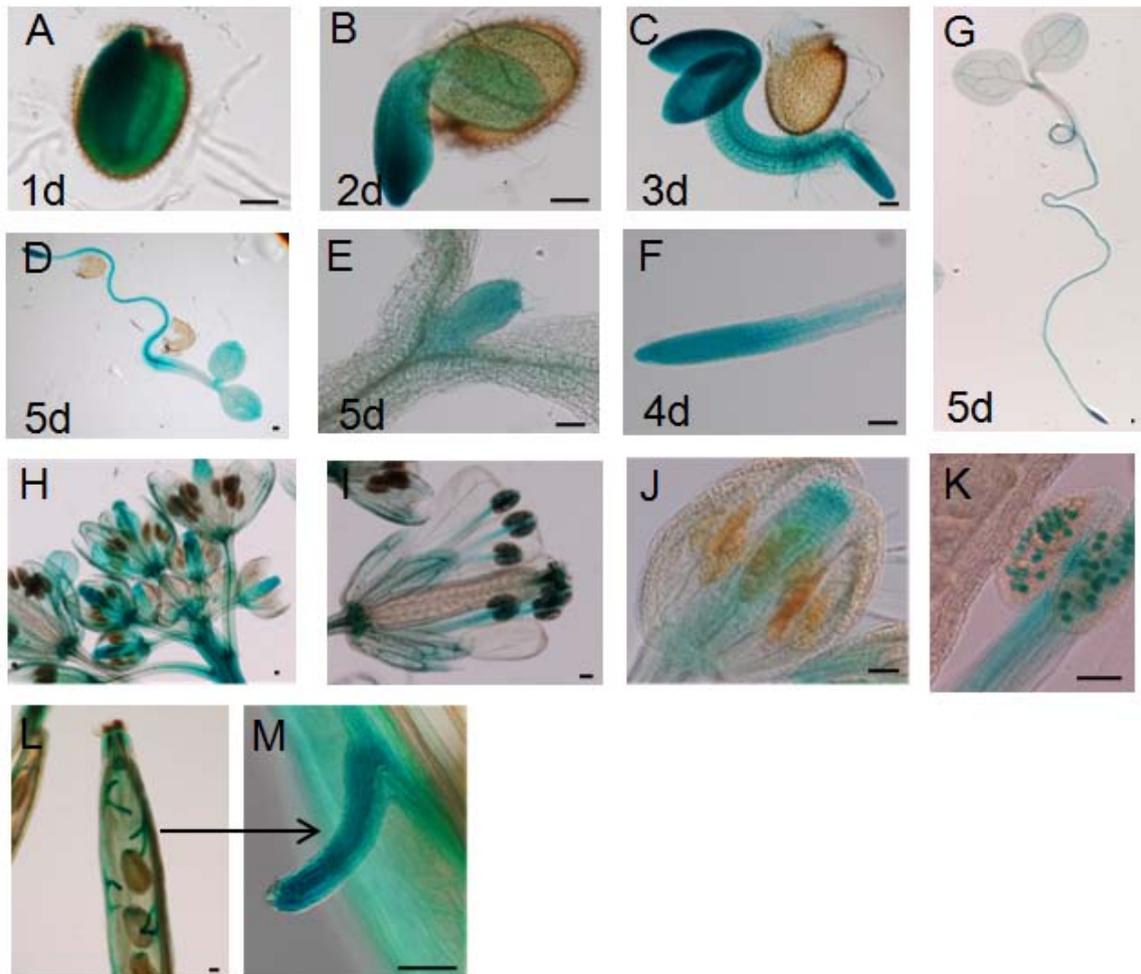


Figure S1. The CHR5 2 kb promoter activity in transgenic plants. GUS staining of germinating seeds (1-5 days, A-D), young seedlings (G), flowers and floral organs (H-K) of transgenic plants with the pCHR5-GUS construct. GUS activity in a 5 day-old apex (E), in a 4 day-old primary root (F) and in funiculus (L, M) are shown. Bars=0.1 mm.

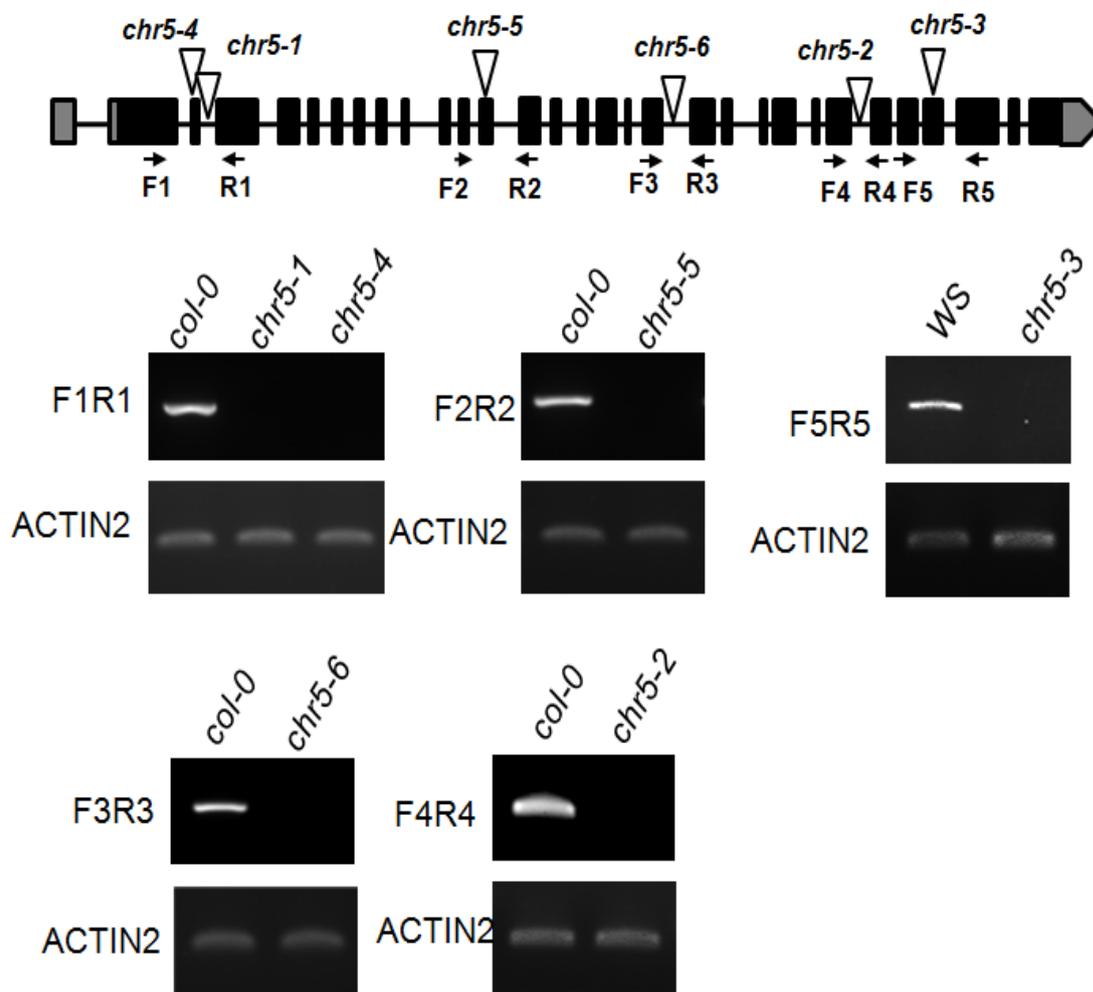


Figure S2. Characterization of *chr5* T-DNA insertion mutants. Upper part: six T-DNA insertions in the locus are indicated by arrows. The positions of the primer sets used for genotyping and RT-PCR analysis are indicated. Lower parts: RT-PCR analysis of *CHR5* transcripts in wild type (Col-0 or Ws) and the 6 mutant alleles by using the indicated primer sets.

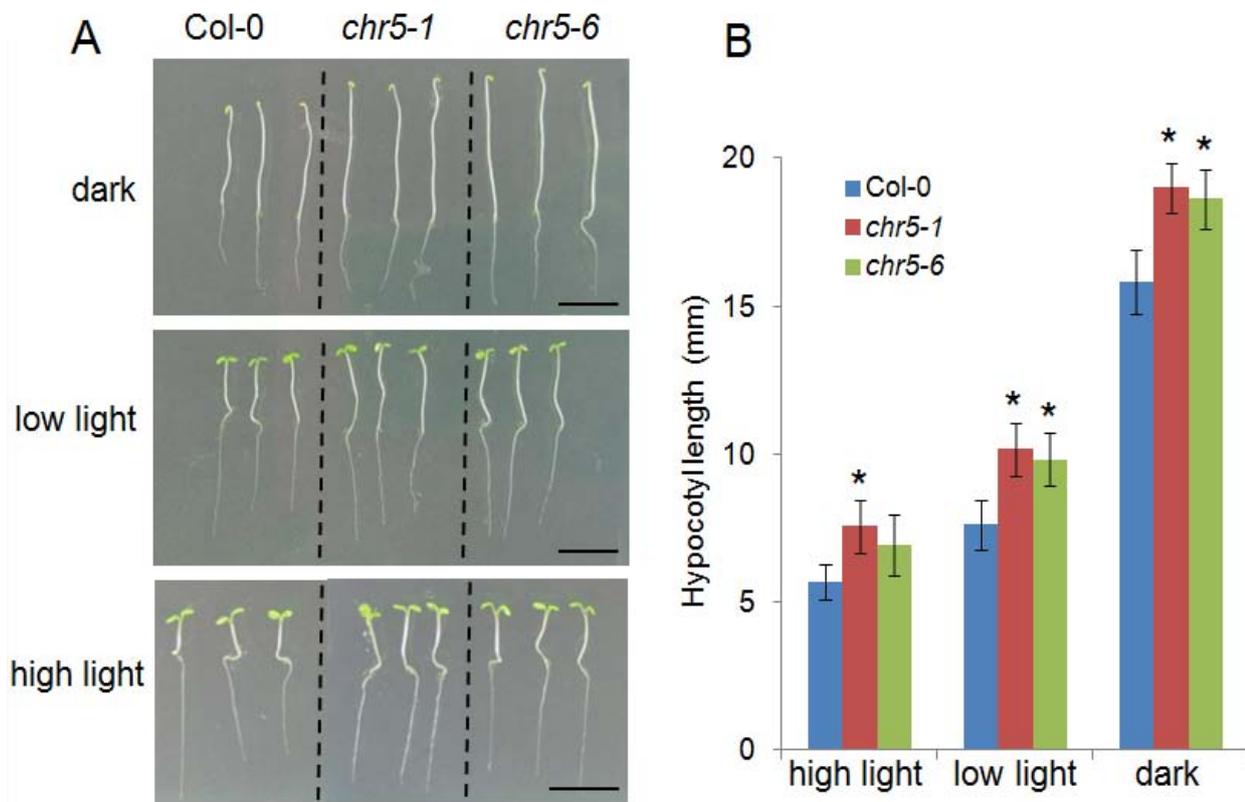


Figure S3. CHR5 mutations display a slight long hypocotyl phenotype. **A.** Hypocotyl length of *chr5* mutants grown in the dark, low white light ($12 \mu\text{mol m}^{-2}\text{s}^{-1}$) and high white light ($35 \mu\text{mol m}^{-2}\text{s}^{-1}$) with 8h light/16h dark cycles for 5 days. Bar = 1 cm. **B.** Quantification of hypocotyl lengths of the seedlings shown in A. Data represent the mean \pm SD of 50 seedlings. The asterisks indicate that the difference is significant at $P < 0.01$ between mutants and Col-0 according to Student's t test.

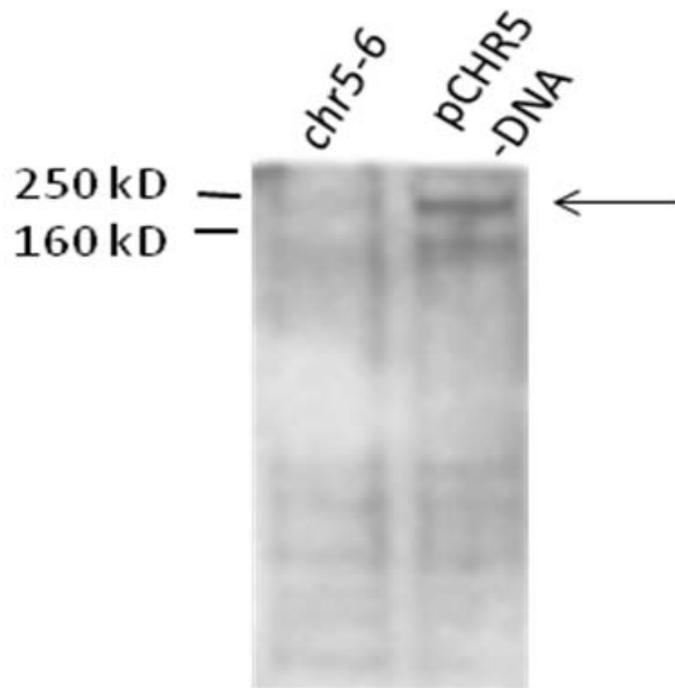


Figure S4. Characterization of a complementation line of the *chr5-6* allele. Western blot analysis of nuclear proteins extracted from *chr5-6* and *chr5-6* transformed with pCHR5-CHR5-HA construct using anti HA antibody.

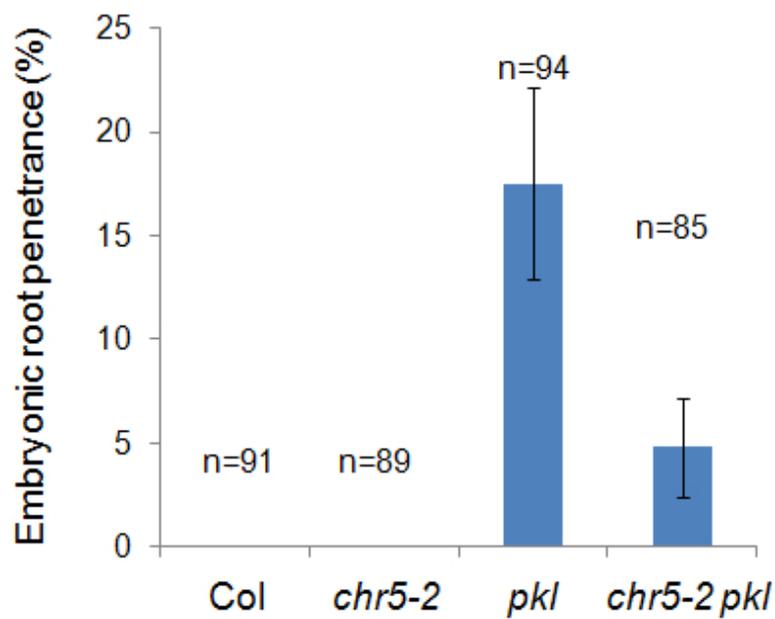
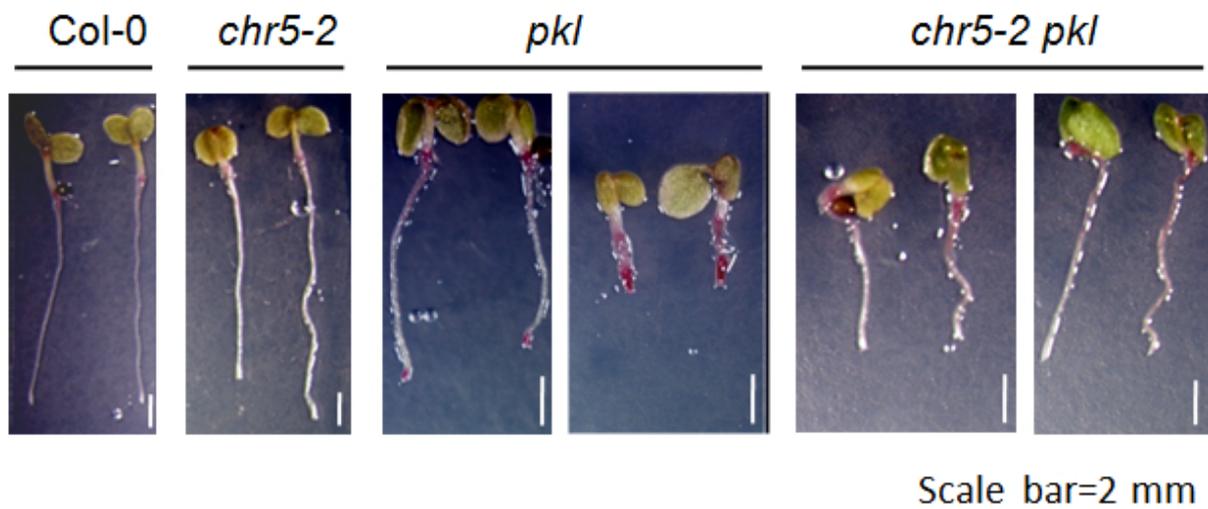


Figure S5. Comparison of Fat red dye stained seedling phenotypes of wild type, *chr5-2*, *pkl* and *chr5-2 pkl* double mutants. Percentages of embryonic roots (stained red) were calculated from 85-94 seedlings.

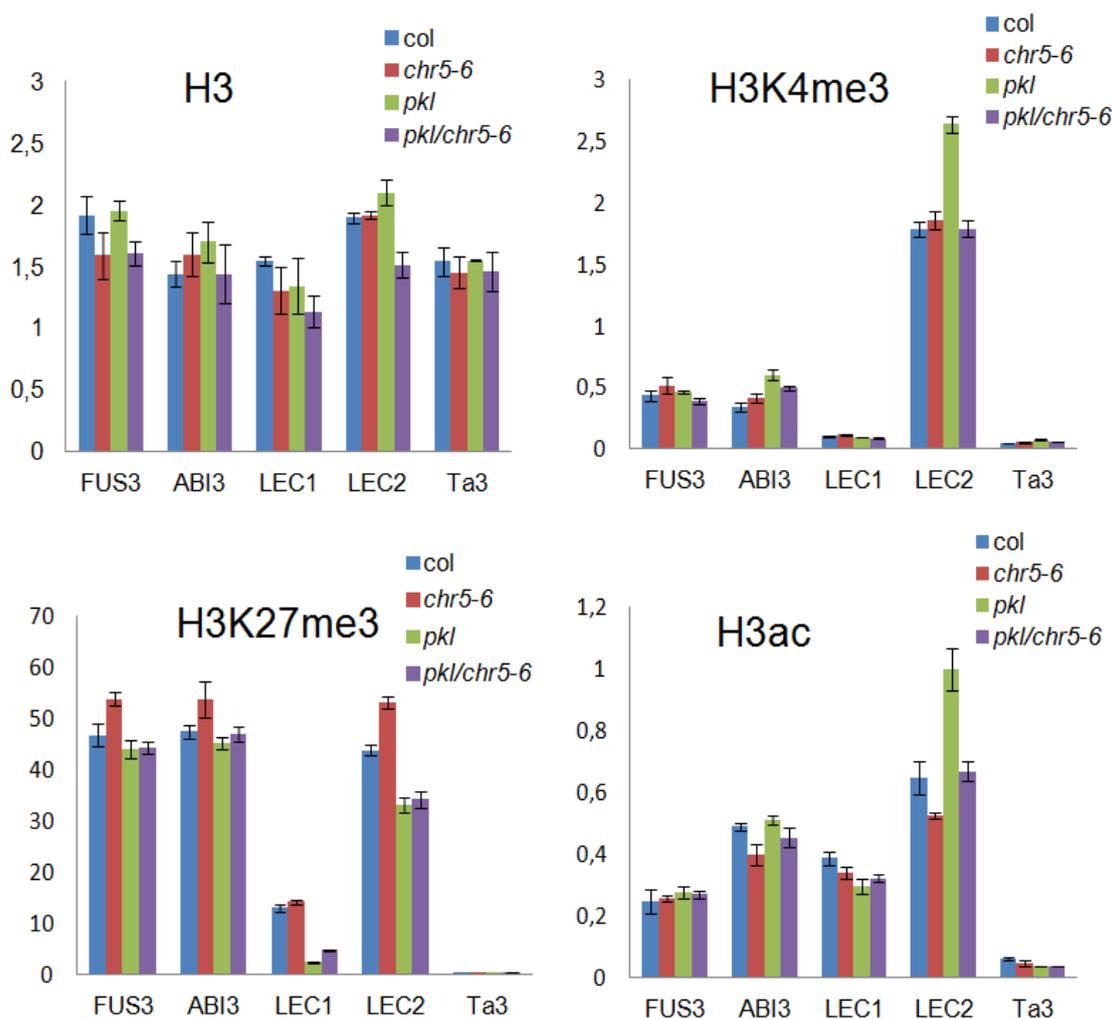


Figure S6. Histone modifications on *LEC1* and *AFL* loci in seedlings of wild type, *chr5-6*, *pkl*, and *chr5-6 pkl* double mutants. Chromatin fragments isolated from 5 day-old seedlings were immunoprecipitated with antibodies of histone H3, H3K4me3, H3K27me3 and acetylated H3 (H3ac), and quantified by qPCR using primer sets corresponding to the 4 genes and, as a control, to the *Ta3* locus. Y-axis: relative levels to histone modifications on *ACTIN2*.

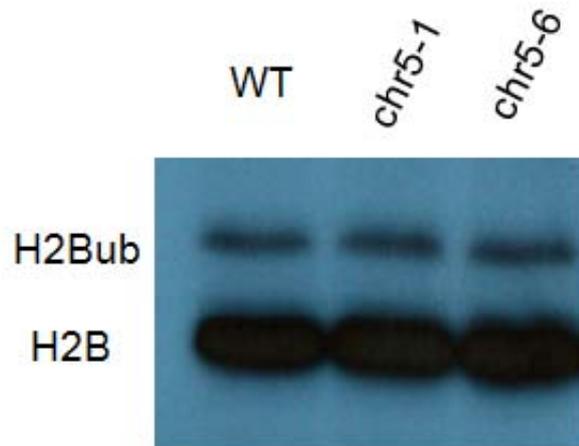


Figure S7. CHR5 mutations do not affect the overall levels of histone H2B monoubiquitination (H2Bub). Histones isolated from wild type and chr5-1 and chr5-6 were analyzed by western blotting with anti H2B antibody.

Supplemental Table 1. Sequences of primers used in this study.

Primers used for genotyping

Loci	Forward (5'→3')	Reverse (5'→3')
CHR5-F1,R1	TCCATAGCAACTCAAGGACT	GCTGCGAACTTCACTACTCT
CHR5-F2,R2	GATGAAATGGGTCTTGGTAA	GCTGTGCCTCACTATTTTTTC
CHR5-F3,R3	CTGCGTGAGACTAAGCATCG	CGTCACTAGCAGGTGCGTTA
CHR5-F4,R4	TCTTCCTAAGAGAGATGCGC	CGTTTGCTTTAACTGGCACA
CHR5-F5,R5	TGTGCCAGTTAAAGCAAACG	GCTCGTAAACTTCCCCATCA

Primers used for RT-qPCR and ChIP-qPCR

Gene	Forward (5'→3')	Reverse (5'→3')
LEC1(RT)	AAATCCATCTCTGAATTGAACTT	CACGATACCATTGTTCTTGT
LEC2(RT)	CGCTCGCACTTCAACAAGTCC	TCATCACCGCCGCCATCTGC
FUS3(RT)	CGATGGGCTCATGGTATTCT	CAACACACCTTCATGACTCACA
ABI3(RT)	ATGTATCTCCTCGAGAACAC	CCCTCGTATCAAATATTTGCC
PIL5(RT)	TGTTAGCCGCTCAAAGACCT	GCGGAACTGCTGATATGGAT
2S2(RT)	AACTTTCGCCCTCTGCTTC	TCTTAGGTGCTGTGATTGCT
7S1(RT)	GCAGTTCACGGTTACATCC	CTCCAACAATCCATCAATC
CRA1(RT)	CTCCGTTGCTCAGGTGTCT	ACCGCTCCTAATGTGCTCC
OLE1(RT)	ACCTCTGCTCGTTATCTTC	GTTCCCAACTTCATCC
ACTIN2(RT)	CGCTGACCGTATGAGCAAAGA	GCAAGAATGGAACCACCGATC
CHR5(RT)	AGGAACTTCCAGAGGCAACA	TGTTATCGTTGGTCGCTCAG
PKL(RT)	GAGCGAATTGATGGAAAGGT	TTCCTAAGCCACCAGCTCTT
GUS(RT)	GCCGATGCAGATATTCGTAATTATGC GGGCAAC	TTCAAATGGCGTATAGCCGCCCTGATGC TCCAT
LEC1(ChIP)-A	GGACTGGGCCGTAAACTCTA	CCCTAGTAATGGAATGGTGGA
LEC1(ChIP)-B	AGCAGTTTGACTGGCCACATAG	CAAAGGAGGCGGTTCTTACG
LEC1(ChIP)-C	CGTCTCACGCCAAAATCTCT	TCTTACGTTGCTCACGTTGG
LEC2(ChIP)-A	CCCCATAAGCTGCAAAACATTG	CAGATGTTGGAAAAACACAAAAGAA
LEC2(ChIP)-B	GCTCGCACTTCAACAAGTC	GTAACCGGCTGAGGATTCA
LEC2(ChIP)-C	ACGAAGAGGCAATGCTTGAT	GGTGAAGTCCATGAGATCGT
FUS3(ChIP)-A	AAGACGCCATTGAATTCTGG	CAAAGGGACGAGTGGATTGT
FUS3(ChIP)-B	GCGTCCATTTTAAGCCTCTG	TGGTGGATCTCTTTGGGTTT
FUS3(ChIP)-C	CTCCCGAAGGTGATCTTTGA	AGGAATCCCTTCTTGCATT
ABI3(ChIP)-A	AAAACCGACCGAGGAAACTT	AGACCAAGCGGTGGTTCTTA
ABI3(ChIP)-B	CTGGAACACATGGGCTCTCT	CGTTTGGGTTTACTCCAACAA
ABI3(ChIP)-C	TTCCTTGCCTCTTACTCACA	GCTTTTCATCGTTGAAGTGA
ABI3(ChIP)-D	AGGCAAGAAAGAAACGGATG	ATTTGGGTTTGGTTCTGCTG
ACINT2(ChIP)	CGCTGACCGTATGAGCAAAGA	GCAAGAATGGAACCACCGATC
Ta3(ChIP)	CTGCGTGGAAGTCTGTCAA	CTATGCCACAGGGCAGTTT
RBCS(ChIP)	TGGTCGCTCCTTTCAACGGACTTA	CGCCGTTGCTTGTGATGGAAGTAA

Primers used for cloning

Name	Forward (5'→3')	Reverse (5'→3')
ProCHR5	<u>AAGCTTCGCTAAATACAAATGTCTCG</u> added HindIII site underlined	<u>GGTACCGTAAAGAAAGCCATCTCGTCT</u> added KpnI site underlined
CHR5(cDNA)	<u>GGTACCAGACGAGATGGCTTTCTTTAG</u> GA added KpnI site underlined	<u>ACTAGTTCTTGGGGAAATCCGGT</u> added SpeI site underlined

Primers used for FUS3 nucleosome mapping

Center of amplicon	Forward (5'→3')	Reverse (5'→3')
FUS3(-521)	TGAGACAAATGTGCTATTTCAACC	TGATTTGGAAGAGAAGTGAAGGA
FUS3(-465)	TCCTTCACTTCTCTTCCAATCA	GAACATTTTTGCGACCAAGA
FUS3(-405)	TTCTTGGTCGCAAAAATGTTT	CTTACTGGTCAACATGTGATTTG
FUS3(-338)	CAAATCACATGTTGACCAGTAAG	CCAGAATTCAATGGCGTCTT
FUS3(-265)	AAGACGCCATTGAATTCTGG	CAAAGGGACGAGTGGATTGT
FUS3(-182)	CTTGGTTCTAAACTTTAACCTAAC	CGCAACAAGATCTAATGCCACT
FUS3(-118)	GGCATTAGATCTTGTGCGTTA	TTCATGAATCTAGGGTTTTGGAA
FUS3(-40)	AATCTTCCAAAACCCTAGATTCA	ACGCTGGGCAATCAGAGAT
FUS3(-17)	CAAATGTCACCTTCTTACCATAAA	ACGCTGGGCAATCAGAGAT
FUS3(21)	CCAATGGTAAACCCAAATCTC	TGTGTGTTGTGCAGATCGAA
FUS3(49)	GCGTCCATTTTAAGCCTCTG	CTGTGGTTTGTGTTGTGTTGTG
FUS3(62)	AAGCCTCTGTTTCGATCTGC	GAGAGAAAGAGAGACTGTGGTTTG
FUS3(97)	TTCGATCTGCACAACACACA	TGGTGGATCTCTTTGGGTTT
FUS3(122)	CACAACACAACACAAACCACAG	GAAAACAGTTCTCTGTGTGAAGGA
FUS3(176)	AAACCCAAAGAGATCCACCA	TGCCTAGTAAAAGGAAGTGTGG
FUS3(231)	ACTGTTTTCTCCACACTTCT	AGTAGAGGCCTTGGTTTCCA
FUS3(259)	GCAAGTGAACCAATTGAGAGAGAA	ACCCATGATCAACACTTGCCA
gypsy(-73)	TACACATTCCTCAGCCGTTG	CCTATCTCCAGCTAGCAACC

CHAPTER 3

Functional characterization of a histone demethylase JMJ15 in *Arabidopsis*

Introduction

JMJ15 belongs to the KDM5/JARID1 group of JmjC-domain-containing proteins, which was previously shown to be a H3K4 demethylase (Liu et al., 2010; Yang et al., 2012a). However, little is known concerning to its function in stress-responsive gene regulation and plant growth. In this work, I analyzed the tissue expression pattern of *JMJ15* during different plant development stages using *proJMJ15-GUS* construct. To study the function of JMJ15 in gene expression and plant development, I obtained 3 alleles of T-DNA insertion mutants: *jmj15-1* and *jmj15-2* were gain-of-function lines, and *jmj15-3* was loss-of-function line. The *jmj15-3* mutant did not show obvious phenotypes, whereas gain-of-function mutations reduced the length of hypocotyls and inflorescence stems with higher accumulation of lignin. Furthermore, the gain-of-function mutants showed enhanced salt tolerance whereas the loss-of-function mutant was more sensitive to salt compared to the wild type. To study the effect of JMJ15 over-expression on gene expression, we analyzed the transcriptome of the *jmj15-1* and *jmj15-2* mutant alleles using Affymetrix ATH1 microarrays. The transcriptomic analysis revealed that there were much more down-regulated genes compared to up-regulated ones in the over-expression plants. Most of the down-regulated genes are related to stress, among which most encode transcription factors, suggesting that JMJ15 may be a higher hierarchical regulator involved in stress-responsive gene expression. On the other hand, the H3K4 methylation level of down-regulated genes was analyzed using publicly epigenomic datasets. The result showed that most down-regulated genes were highly enriched for H3K4me3 and H3K4me2. In general, our results indicate that JMJ15, as a H3K4 demethylase, plays important roles in plant responses to stress. This part of the results is written in an article that will be submitted for publication.

Over-expression of Histone H3K4 Demethylase Gene *JMJ15* Enhances Stress Tolerance in *Arabidopsis*

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Running title: Function of H3K4me3 demethylation in stress tolerance

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3.1 Abstract

Histone H3 lysine 4 trimethylation (H3K4me3) has been shown to be involved in stress-responsive gene expression and gene priming in plants. However, the role of H3K4me3 resetting in stress tolerance is not clear. In this work we studied the expression and function of *Arabidopsis* H3K4 demethylase gene *JMJ15*. We show that the expression of *JMJ15* was restricted to a few tissues during vegetative growth but relatively highly expressed in young floral organs. Over-expression of the gene in gain-of-function mutations reduced the length of seedling hypocotyls and inflorescence stems with higher accumulation of lignin in the stem, while the loss-of-function mutants did not show any visible phenotype. The gain-of-function mutants showed enhanced salt tolerance, whereas the loss-of-function mutants were more sensitive to salt compared to the wild type. Transcriptomic analysis revealed a much higher number of genes down-regulated in *JMJ15* over-expression plants, which are highly enriched for H3K4me3 and H3K4me2. Among the down-regulated genes, many encode transcription regulators of stress responsive genes. The data suggest that increased *JMJ15* levels may regulate the gene expression program that enhances stress tolerance.

3.2 Introduction

Histone lysine methylation is an important epigenetic modification for gene expression in eukaryotic cells (Martin and Zhang, 2005; Kouzarides, 2007). Histone lysines can be mono-, di-, and tri- methylated. Genome-wide analysis has indicated that about two-thirds of *Arabidopsis* genes are marked by mono-, di- or trimethylation on histone H3 at residue lysine 4 (H3K4me_{1/2/3}) (Zhang et al., 2009). H3K4me₃ is predominantly found at the promoter and 5' regions of genes and is strongly associated with transcriptional activation in plants. H3K4me₃ was found to be increased on responsive genes upon stress treatment, but the increase was found to be lagged behind gene activation in a few experiments (Kim et al., 2008; Hu et al., 2011), suggesting that H3K4me₃ may have a function to mark the active gene state. In addition, H3K4me₃ in gene bodies has been suggested to play a role in transcriptional memory of stress-responsive genes in *Arabidopsis* (Alvarez-Venegas et al., 2007; Jaskiewicz et al., 2011). Recent results indicated that the enriched H3K4me₃ in gene bodies decreased after stress recovery but remained higher than basal state, suggesting that a regulated resetting mechanism is involved for partial removal of H3K4me₃ and that remaining H3K4me₃ may contribute for the transcriptional memory in *Arabidopsis* (Ding et al., 2012; Kim et al., 2012).

Histone methylation marks are established by evolutionarily conserved SET-domain proteins (named after 3 *Drosophila* genes: Su(var)3-9, Enhancer of zeste and Trithorax). H3K4 methylation is mediated by the Trithorax group proteins (TRX). *Arabidopsis* Trithorax ATX1 and ATX2 respectively trimethylates and dimethylates H3K4 (Saleh et al., 2008). ATX1 is found to be necessary for stress-induced gene expression (Alvarez-Venegas and Avramova, 2005; Alvarez-Venegas et al., 2007; Ding et al., 2011). Other SET-domain genes (SDG) such as SDG4 and SDG2 also methylate H3K4 and are involved in the control of many aspects of plant development (Cartagena et al., 2008; Berr et al., 2010; Guo et al., 2010).

Histone methylation is reversed by histone demethylases. Lysine Specific Demethylase 1 (LSD1) is the first identified histone demethylase to remove mono-

and di-methyl groups from H3K4 (Shi et al., 2004). In *Arabidopsis* there are 4 LSD1-like genes including *FLOWERING LOCUS D (FLD)*, *LSD1-LIKE 1 (LDL1)* and *LSD1-LIKE 2 (LDL2)* that are shown to be involved in flowering time control (Jiang et al., 2007). The second class of histone demethylases containing the jumonji C (JmjC) domain catalyze histone lysine demethylation through a ferrous ion (Fe(II)) and α -ketoglutaric acid (α -KG)-dependent oxidative reaction (Tsukada et al., 2006). Multiple JmjC domain-containing histone demethylases are identified in animal cells, which are divided into distinct groups including JARID/KDM5, JMJD1/JHDM2/KDM3, JMJD2/KDM4, JMJD3/UTX/KDM6, JHDM1/FBX/KDM2 and the “JmjC domain-only” group. Members of each group target to specific histone lysine residues at different methylation states (Klose et al., 2006). About 20 JmjC domain-containing protein genes are found in *Arabidopsis* (Lu et al., 2008; Sun and Zhou, 2008; Chen et al., 2011). Most animal and plant JmjC proteins are conserved, while some animal proteins, such as JMJD3/UTX/KDM6 group that has the H3K27 demethylase activity, is not found in plants. Recent data have shown that plant JMJD2/KDM4 homologues can demethylate H3K27 (Lu et al., 2011; Li et al., 2013).

The JARID/KDM5 group catalyzes H3K4me_{2/3} demethylation in mammalian cells. *Arabidopsis* genome has one JARID/KDM5-like gene (*JMJ17*), whose function is presently unknown. There is a specific group in plants which includes *Arabidopsis* JMJ14, JMJ15, JMJ16, JMJ18 and JMJ19. The JmjC domains of this group are more closely related to that of the JARID, but structurally similar to that of JMJD2/KDM4 (Chen et al., 2013). JMJ14, JMJ15 and JMJ18 have been reported to have the H3K4me_{2/3} demethylase activity and to regulate diverse aspects of chromatin function and plant development (Deleris et al., 2010; Lu et al., 2010; Searle et al., 2010; Le Masson et al., 2012; Yang et al., 2012a; Yang et al., 2012b; Cui et al., 2013). However, the function of these H3K4 demethylases in plant stress tolerance has not been evaluated. In this work we provide evidence that increased expression of *JMJ15* preferentially down-regulates H3K4me_{2/3}-marked stress-related genes and enhance salt stress tolerance.

3.3 Materials and methods

Plant growth

The *Arabidopsis thaliana* ecotype Columbia (Col-0) was used throughout this study. T-DNA mutant lines *jmj15-1* (GABI_257F10), *jmj15-2* (GABI_876B01) and *jmj15-3* (GABI_663C11) were obtained from the Nottingham *Arabidopsis* Stock Center (NASC) and confirmed by PCR. Seeds were surface-sterilized and plants were grown on 0.5 x Murashige and Skoog (MS) medium after stratification at 4°C for 2 days. Plants were analyzed on plates under long-day (LD, 16h light/8h dark) or short-day (SD, 8h light/16h dark) photoperiods at 20°C. Ten days after germination, plants were transferred to soil and kept in growth rooms under LD condition.

To test *JmjC* gene expression in response to salt, experiments were carried out with 8 day-old plants, treated with 0.5 x MS supplemented with or without 100 mM NaCl for 5h. For germination tests, seeds of WT and *jmj15* mutants were sown on medium containing indicated concentration NaCl. Images of the Petri dishes were taken 10 days after germination.

Constructs and Transformation

For the histochemical GUS assay, the 2 kb promoter of *JMJ15* was amplified from wild type genomic DNA using the following primers: 5'-GGATCCAGAGCTTGGCCATTTCTTGA-3' (forward) and 5'-GGTACCGC ACTGAAAGGCTCCATTG-3' (reverse). *Bam*HI and *Kpn*I (underlined) were used for digestions. The *JMJ15* promoter fragment was inserted as translational fusion with the *uidA* gene into the pPR97 vector. To generate the *35S-JMJ15-HA* construct, the full length cDNA without a stop codon was amplified from total cDNA of Col-0 using primers: TCTAGACCTTTGGGTTTTGTGGAGTG (forward) and TCTAGACCAATTCAAATCAACCCCAA (reverse). Using *Xba*I site, *JMJ15* cDNA was inserted into the binary vector pFA121, which was modified based on pBI121 and contained 2 x FLAG-HA tag. The pMJ15-GUS and

35S-JMJ15-HA constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 and then transformed the plants using floral dip method.

Microarray analysis

Total RNA was extracted from 12 day-old seedling using Trizol (Invitrogen) and cleaned using the RNeasy isolation kit (Qiagen). Hybridization with Affymetrix GeneChip *Arabidopsis* ATH1 Genome Array was performed at CapitalBio Corporation. Wild type and both *jmj15* overexpression alleles were performed in two biological repeats. Gene expression changes between the samples were analyzed by the AffyLmGUI package from R software. GO annotation was carried out with the GO terms of the TAIR database (<http://arabidopsis.org/tools/bulk/go/index.jsp>). The percent of significantly changed genes in each TAIR annotated category was calculated as follows: percent = the number of significantly changed genes divided by N x 100, where N represents the total number of genes annotated in each ontology. Significantly changed genes were subsequently analyzed for their H3K4 methylation levels at epigenomics database (<http://epigenomics.mcdb.ucla.edu/H3K4m1m2m3/>). The microarray data are deposited to the NCBI databases (accession number pending).

Real-Time PCR

For gene expression analysis, two micrograms of total RNA were reversed transcribed into cDNA by ImPromII reverse transcriptase (Promega). Real-time PCR was performed with the LightCycler® 480 SYBR Green I Master (Roche) on a LightCycler 480 (Roche). At least two biological replicates and two technical repeats for every biological replicate were tested. The primers used in this study are listed in supplementary Table 1.

Histochemical GUS and Lignin Staining

GUS staining was performed as previously described (Bertrand et al., 2003). Briefly, plant samples were fixed with 90% acetone on ice for 20 min and were washed with staining buffer (0.2% Triton X-100, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 100 mM NaH₂PO₄ and 100 mM Na₂HPO₄ pH 7.2). Then the samples were immersed in GUS staining solution with 1 mM X-Gluc and placed under vacuum for 20 minutes. After incubation at 37°C overnight, the staining solution was removed and samples were cleared by sequential changes of 70% (v/v) ethanol and stored at 4°C.

The histological comparative analysis of inflorescence stems between Col-0 and *jmj15* mutants was done at the stage of newly formed green siliques, about 2 weeks after bolting, when the inflorescence stems of wild type reach 20 cm in height. Cross-sections of the inflorescence stems at the basal end were stained for 3 min in phloroglucinol-HCl reagent (Prolabo, VMR International, France) and then observed in ethanol 100 % : HCl 37 % (9/1, v/v) using a light microscope (Nikon, MULTIZOOM AZ 100).

3.4 Results

3.4.1 Expression levels of H3K4 demethylase genes

To investigate whether H3K4 demethylase genes are involved in plant stress responses, we analyzed the mRNA levels of *JMJ14*, *JMJ15*, *JMJ16*, *JMJ17*, *JMJ18* and *JMJ19* genes in 8 day-old seedlings grown in ½ MS media under continuous light, then transferred to 100 mM NaCl or to ½ MS solution for 5 hours. In untreated (½ MS) seedlings, the expression levels of the 6 genes varied considerably. The relative expression levels of *JMJ17*, *JMJ18* and *JMJ19* were much higher ($>10^2$) than that of *JMJ14*, *JMJ15* and *JMJ16* (Supplementary Figure 1). NaCl treatment did not dramatically affect the expression of these genes, although some decrease of *JMJ14* and *JMJ18* and some increase of *JMJ15* transcript levels were detected.

3.4.2 *JMJ15* displayed a highly tissue-specific expression pattern

The relatively low expression level of *JMJ15* was in agreement with previous data showing that the 1.5 kb promoter region of *JMJ15* is weak in vegetative tissues (Hong et al., 2009). To study the temporal and spatial expression pattern of *JMJ15*, we used a larger promoter region of *JMJ15* (-2051 bp to +14 bp relative to ATG) to make a *GUS* reporter translational fusion construct and transformed *Arabidopsis* plants. Three independent *GUS* reporter lines were characterized. All showed the same pattern of *GUS* expression. In seedlings, *GUS* activity was detected only at the base of rosette leaves and root vascular tissues, but not in the root tip (Figure 1). Interestingly, a higher accumulation of *GUS* activity was detected in pericycle cells that initiated to lateral root meristem. The *GUS* activity remained to be detected at the base of the growing lateral roots (Figure 1). In the inflorescence, *GUS* activity was strong in the young anthers and detected in carpels, but the activities become weaker in the mature flower (Figure 1). This temporal and tissue-specific expression pattern suggested that *JMJ15* may have a function in plant development.

3.4.3 JMJ15 gain-of-function mutations showed a reduced plant height phenotype

The *JMJ15* gene contains 10 exons and encodes a polypeptide of 806 amino acids with distinct domains, including the JmjN domain, JmjC domain, a C5HC2 zinc finger and the FY-rich N-terminus (FYRN)/FY-rich C-terminus (FYRC) domains, which are conserved in JMJ14, JMJ16, and JMJ18 (Supplementary Figure 2) (Lu et al., 2008). To study the function of JMJ15 in gene expression and plant development, we characterized 3 T-DNA insertion mutants: *jmj15-1*(GK-257F10), *jmj15-2* (GK-876B01) and *jmj15-3* (GK-663C11). In *jmj15-1* and *jmj15-2*, the T-DNA was inserted in the 5' end, and in *jmj15-3* the T-DNA was inserted in the seventh exon of the gene (Figure 2A). RT-PCR analysis with 4 pairs of primers that covered the whole coding region of the gene, revealed that the transcript level of *JMJ15* was dramatically increased in *jmj15-1* and *jmj15-2*, but the transcript was interrupted in *jmj15-3* compared to wild-type (Figure 2B). The insertion in *jmj15-1* and *jmj15-2* did not alter the 5' end of the coding region, as the primer set F1 (that cover the 5' end of the coding region) and R1 successfully amplify the transcripts from the mutants. The data suggested that *jmj15-1* and *jmj15-2* were gain-of-function mutants that overexpressed the gene and that *jmj15-3* was a loss-of-function mutant.

The *jmj15-3* loss-of-function mutation did not display any visible phenotype in normal growth conditions, confirming previous results (Yang et al., 2012a). However, in short day (8h light/16h dark) grown seedlings, *jmj15-1* and *jmj15-2* mutants produced slightly shorter hypocotyls compared to wild type (Figure 3A). At the mature stage, the plant height of *jmj15-1* and *jmj15-2* were also reduced compared to wild type (Figure 3B). The stronger phenotypes in *jmj15-1* were correlated with the higher expression of the gene in the allele compared to in *jmj15-2*. To study whether the plant height phenotype of *jmj15-1* and *jmj15-2* was due to increased expression of the gene, we made *35S-JMJ15-HA* construct and obtained *JMJ15* over-expression transgenic plants. The transgenic plants also displayed the reduced plant height phenotype at mature stage (Figure 3B).

The plant height phenotype of the *jmj15* gain-of-function mutants prompted us to further investigate the stem cell structure by using histochemical method. Sections of the basal part of the inflorescence stem of 5 week-old plants (grown in long day) were stained with phloroglucinol and examined by light microscopy. Phloroglucinol reacts with coniferaldehyde groups in lignin, and the color intensity reflects the total lignin content. The analysis revealed that *jmj15-1* and *jmj15-2* exhibited a significantly deeper red staining in the stem vascular system and interfascicular fibers compared to that in wild type and *jmj15-3* (Figure 4). This observation suggested that over-expression of *JMJ15* resulted in an increase of the total lignin content in the stems concurrently with stem growth reduction.

3.4.4 Over-expression of *JMJ15* preferentially repressed genes marked by H3K4 methylation

To determine the effect of *JMJ15* over-expression on gene expression, the transcriptomes of *jmj15-1*, *jmj15-2* and wild type seedlings (12 day-old) were analyzed by using the Affymetrix *Arabidopsis* ATH1 Genome Array. Two biological replicates for each sample were analyzed. Pair-wise plots of the microarray data revealed a good correlation of the hybridization signals between the biological replicates of each sample and between the 2 mutant alleles (Figure 5A). The average hybridization signals of the replicates of both mutants were normalized and compared with the wild type signals. Up- and down-regulated genes in both *jmj15-1* and *jmj15-2* were filtrated with the threshold >2 fold changes compared to wild type. The analysis revealed 23 up-regulated and 194 down-regulated genes in the *jmj15* mutant lines (Supplementary Dataset 1). The analysis revealed a much high expression level of *JMJ15* itself (>7-8 folds) in the mutants compared to wild type (Supplementary Dataset 1), confirming the over-expression of the gene in the mutants. The higher number of down-regulated genes compared to up-regulated ones suggested that elevated *JMJ15* expression mainly repressed genes and that *JMJ15* acted as a transcriptional repressor, consistent with its H3K4 demethylase activity (Liu et al., 2010; Yang et al., 2012a). To validate the microarray data, we checked 4

down-regulated genes by RT-qPCR. The relative transcript level changes in the mutants compared to wild type detected by RT-qPCR were in agreement with that from the microarray analysis (Figure 5B).

To evaluate whether there was any enrichment of the deregulated genes for H3K4me2/3, we compared the deregulated genes with the genome-wide H3K4me2/3/1 data obtained from wild type seedlings (Zhang et al., 2009). The analysis revealed that about 85% of the down-regulated genes were marked by the H3K4 methylation (mostly by H3K4me2, H3K4me3, or both) in the gene bodies, compared to about 50% of up-regulated genes (Figure 6, Supplementary Dataset 1). About 60% of the down-regulated genes were marked by H3K4me3, H3K4me2 or H3K4me2/3 in the promoter region (in the -500 bp region relative to TSS), compared to about 30% of up-regulated genes. Compared to up-regulated genes, the down-regulated ones were clearly enriched for the H3K4me2/3 double methylation marks. This analysis suggested that JMJ15-mediated gene repression might be achieved by demethylating H3K4 and indicated that JMJ15 preferentially repressed genes that have the H3K4me2/3 double methylation marks.

3.4.5 Over-expression of JMJ15 preferentially repressed stress regulatory genes

Gene ontology (GO) analysis of the deregulated genes using the GO Slim Classification (http://www.arabidopsis.org/help/helppages/go_slim_help.jsp) revealed that a large proportion of the deregulated genes (23.73%) in the *jmj15* mutants had a function in the nucleus (Supplementary Figure 3A). Interestingly, 37 out of the 194 down-regulated genes were transcriptional regulators (Supplementary Dataset 1). Remarkably, more than 32% of the down-regulated genes belonged to the stress-responsive categories (Supplementary Figure 3B), suggesting that JMJ15 may have a function in stress-responsive gene expression. We noticed that among the greater than 5 fold down-regulated genes, more than 50% were transcription factors or stress-responsive signalling protein genes (Supplementary Dataset 1). These included the stress-responsive zinc finger protein STZ/Zat10 (At1g27730) (Sakamoto et al., 2000), the stress-responsive WRKY proteins WRKY40 (At1g80840) and WRKY33

(At2g38470) (Jiang and Deyholos, 2009), the cold-responsive factor CBF2 (At4g25470) (Vogel et al., 2005), the ethylene-responsive-element binding proteins ATERF6 (At4g17490) and ATERF11 (At1g28370) (Li et al., 2011; Dubois et al., 2013), the stress-responsive F-box protein ATFBS1 (At1g61340) and the cytochrome P450 CYP707A3 (At5g45340) genes (Umezawa et al., 2006; Maldonado-Calderon et al., 2012) (Supplementary Dataset 1). The decreased expression of *WRKY33* and *CYP707A3* genes was validated by RT-qPCR (Figure 5B).

3.4.6 JMJ15 gain-of-function mutations enhanced salt stress tolerance

To study whether JMJ15 mutations affected plant tolerance to stress, we germinated seeds of wild type, *jmj15-1*, *jmj15-2* and *jmj15-3* mutants on ½ MS media containing 130 mM or 150 mM NaCl. The seedling growth phenotype shown in Figure 6 indicated that the gain-of-function mutations (*jmj15-1* and *jmj15-2*) enhanced plant tolerance to salt stress, whereas the loss-of-function mutation (*jmj15-3*) reduced the stress resistance.

3.5 Discussion

3.5.1 Function of JMJ15 in stress tolerance

In this work we have shown that *JMJ15* displayed a high tissue-specific expression pattern. Constitutive or over-expression of the gene, as a result of T-DNA insertions in the 5' region, preferentially repressed genes marked by H3K4me_{2/3}, suggesting that JMJ15-mediated gene repression may be mainly mediated through H3K4me_{2/3} demethylation. The observation that about a third of the down-regulated genes are related to stress implies that H3K4me_{2/3} levels are important for the expression of this category of genes. This is consistent with numerous observations that H3K4me₃ is associated with the induction of biotic and abiotic stress-responsive genes (van Dijk et al., 2010; Hu et al., 2011; Jaskiewicz et al., 2011; Zong et al., 2013; To and Kim, 2014), and ATX1 that trimethylates H3K4 in the genic region is required for stress-responsive gene expression (Ding et al., 2009; Ding et al., 2011). Microarray analysis of *atx1* mutant seedlings revealed that 424 genes were up-regulated and 328 genes were down-regulated more than 2 folds compared to the wild type (Alvarez-Venegas et al., 2006). Similarly, 271 genes were found to be up-regulated and 321 genes down-regulated in mutant seedlings of another H3K4 methyltransferase gene *SDG2* (Guo et al., 2010). However there was no clear correlation between transcription changes in *sdg2* and *atx1* (Guo et al., 2010), suggesting that the two enzymes may regulate different targets. Comparison of the deregulated genes in *jmj15* and *atx1* or *sdg2* did not reveal a clear overlap, although there were a relatively higher number of overlapped genes between *jmj15* and *atx1* than between *jmj15* and *sdg2* (Supplementary Figure 4). These proteins may targets to different loci. In addition, the data showing that the mostly down-regulated genes were those encoding transcription factors and signaling proteins involved in stress responses raise the hypothesis that *JMJ15* might be a higher hierarchical regulator primarily to regulate stress-responsive gene transcription programs in *Arabidopsis*. Since JMJ15 is closely related to JMJ14, JMJ16 and JMJ18 (Lu et al., 2008; Sun and

Zhou, 2008), the ectopically expressed JMJ15 may also regulate the targets of the other related demethylases.

The observations that the *jmj15* gain-of-function mutants showed enhanced salt tolerance at seedling stage and that the loss-of-function mutant was more sensitive to salt stress than the wild type, suggest that JMJ15 is required for stress tolerance. The data showing that the gain-of-function mutants displayed reduced growth and increased stem lignification, which are suggested to be associated with stress responses (Moura et al., 2010; Golldack et al., 2013), support the hypothesis that increased JMJ15 levels may regulate the gene expression program that integrates plant growth to stress tolerance. Among the mostly repressed genes in *jmj15* gain-of-function mutants was *STZ/Zat10* that encodes a C2H2-zinc finger protein associated with the ERF amphiphilic repression (EAR) domain (Supplementary Dataset 1). *STZ/Zat10* has been shown to be a transcriptional suppressor of stress-responsive genes (Sakamoto et al., 2004). Knockout and RNAi of the gene could enhance plant tolerance to abiotic stress (Mittler et al., 2006). As the *STZ* locus displays a high level of H3K4me3 (Supplementary Figure 5), it is possible that the repression of *STZ/Zat10* by JMJ15 through H3K4me2/3 demethylation is associated with the enhanced salt tolerance phenotype of the gain-of-function mutants. *STZ/Zat10* may be a major player in JMJ15-mediated regulatory network of stress tolerance.

3.5.2 Developmental function of JMJ15

Consistent with previous results (Yang et al., 2012a), the loss-of-function mutation identified in this study (*jmj15-3*) did not produce any visible phenotype. *JMJ15* was first identified as *Maternal Effect Embryo Arrest 27 (MEE27)* in a genetic screen for mutants defective in female gametophyte development (Pagnussat et al., 2005). However, no embryonic defect was observed in *jmj15* loss-of-function mutants (Yang et al., 2012a). Either the mutation was compensated by highly expressed homologues (e.g., JMJ18, Hong et al, 2009) or JMJ15-dependent H3K4 demethylation is not sufficient to lead to any morphological change. In addition, another study has identified *JMJ15* as a maternally imprinted gene (Hsieh et al., 2011), however, our

data showing the high promoter activity of *JMJ15* in anthers do not support that observation.

It is reported that JMJ14 demethylates H3K4me_{2/3} at the *Flowering Locus T* (*FT*) locus and represses expression of the gene and that *jmj14* loss-of-function mutants display an early flowering phenotype (Jeong et al., 2009; Lu et al., 2010). Conversely, JMJ18 directly binds to and represses the flowering repressor gene, *Flowering Locus C* (*FLC*), through H3K4me_{2/3} demethylation. Consequently, loss-of-function mutations of *JMJ18* result in a weak late-flowering phenotype, while *JMJ18* overexpressors exhibit an early flowering phenotype (Yang et al., 2012b). These observations support the notion that members of this H3K4 demethylase group target to different loci and have distinct functions in plant development control. However, Yang et al have shown that, like JMJ18, JMJ15 over-expression plants showed repressed *FLC* expression and produced an early flower phenotype (Yang et al., 2012a). But unlike *jmj18* mutants (Yang et al., 2012a), the *jmj15-3* loss-of-function mutation did not alter the flowering phenotype. Possibly, JMJ15 at elevated levels may demethylate and repress genes that normally targeted by JMJ18 in wild type plants. However, the *jmj15-1* and *jmj15-2* gain-of-mutation mutants did not show any clear flowering phenotype. This discrepancy may be due to difference in expression levels of JMJ15 in the overexpression plants and the mutant alleles.

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3.7 Figures

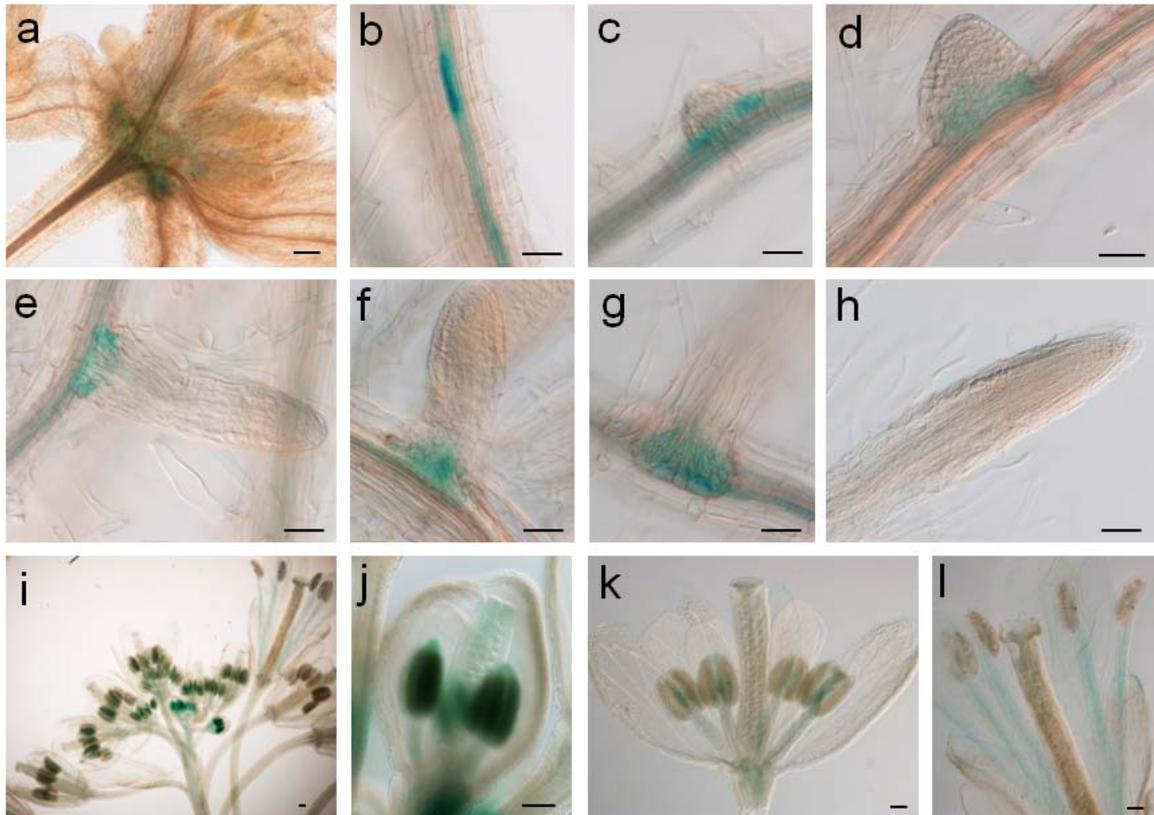


Figure 1. The 2.0 kb promoter activity of *JMJ15* in transgenic plants. In seedlings, the GUS activity was detected in the base of rosette leaves (A), root vascular tissues (B), and bases of growing lateral roots (C-G), but not in root tip (H). In flower buds, the GUS activity was detected in anthers and in carpels (I, J), which become weak in opened flowers, but remained in stamen filaments (K, I). Bar = 0.1mm.

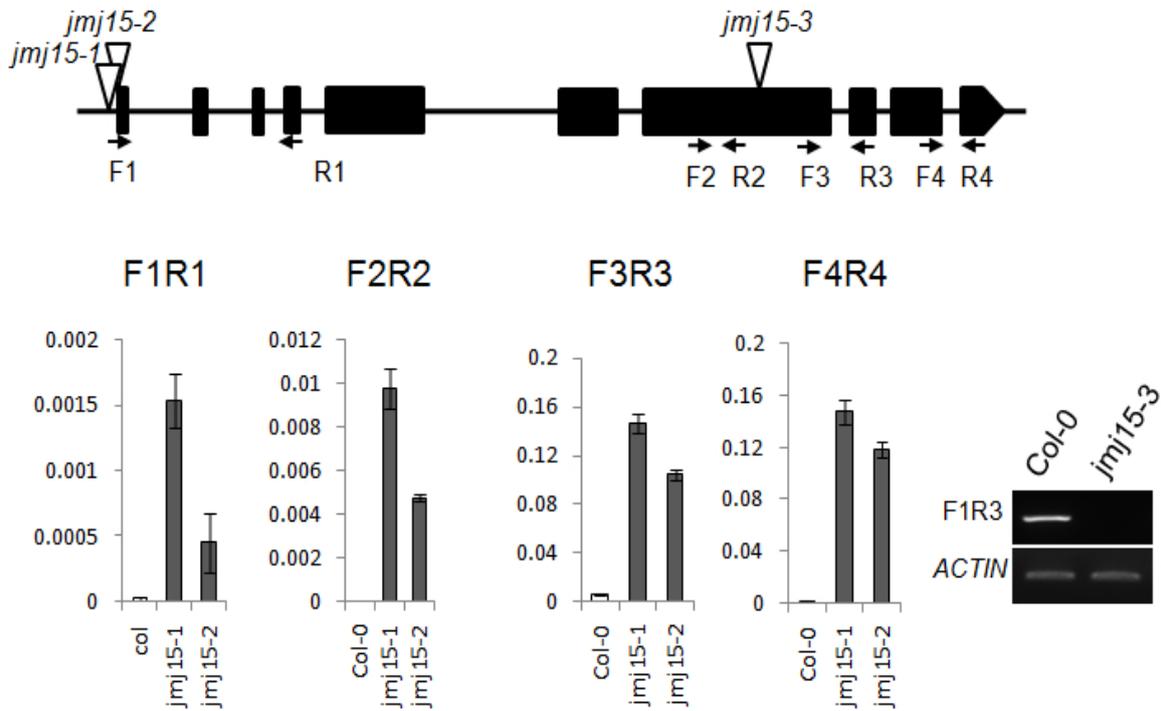


Figure 2. Caution of *JMJ15* T-DNA insertion mutants. The insertion positions of the 3 alleles are indicated by open arrows. The exons are represented by black boxes. The positions of forward (F) and reverse (R) primers are indicated by arrows. The expression levels (relative to ACTIN2 mRNA) in *jmj15-1* and *jmj15-2* compared to wild type were tested by qRT-PCR using the 4 indicated primer sets. The transcript in *jmj15-3* compared to wild type was analyzed by RT-PCR using the indicated primers.

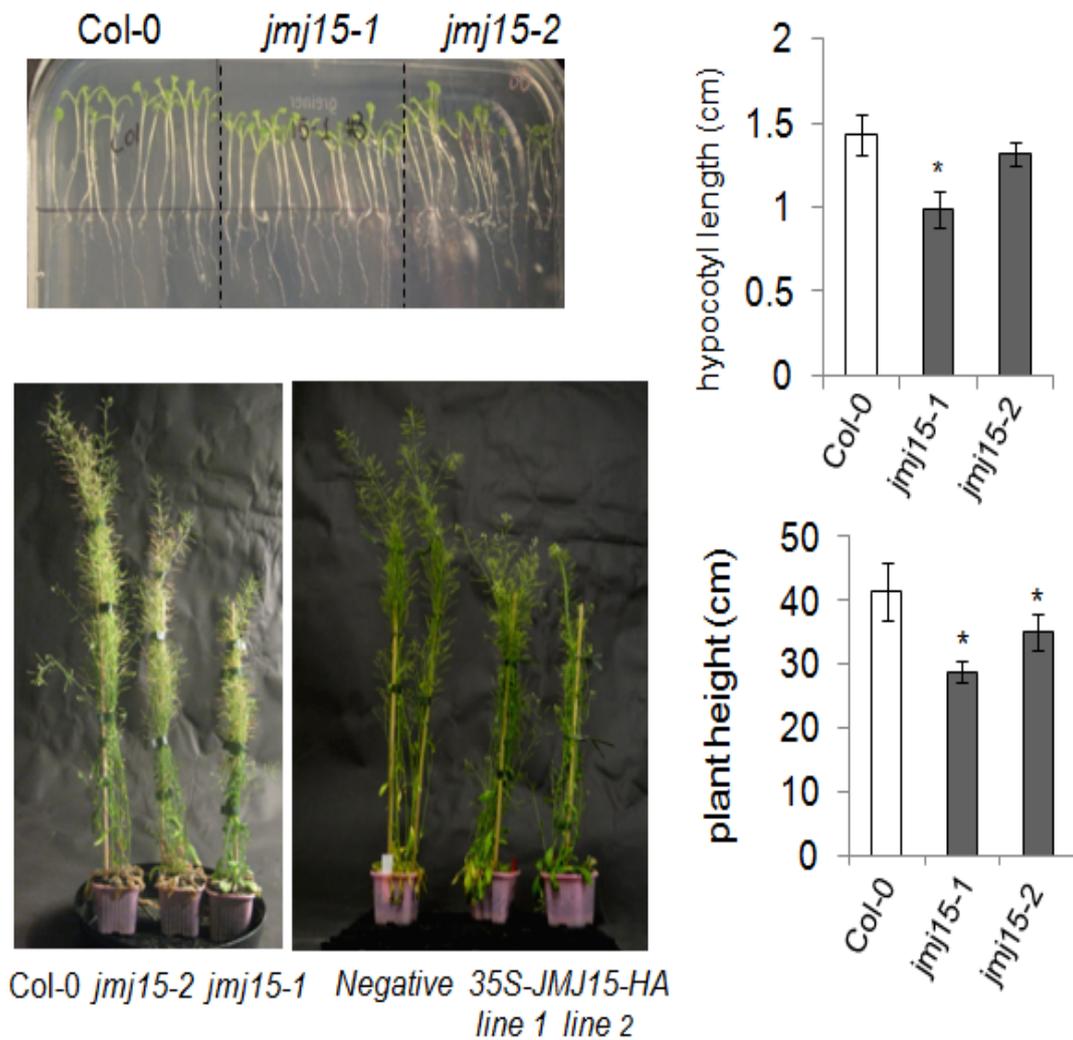


Figure 3. *jmj15* gain-of-function mutations reduced plant height. A. Phenotype of hypocotyls of short day-grown seedlings of *jmj15-1* and *jmj15-2* compared to wild type (left). Lengths of hypocotyls were measured from 30 wild type, *jmj15-1* and *jmj15-2* plants (right). B. Plant height at mature stage of *jmj15-1* and *jmj15-2* compared to wild type (left) and of 35S-JMJ15-HA overexpression plants compared to negative transgenic plants (middle). Plant height was measured from 10 wild type, *jmj15-1* and *jmj15-2* plants (right). Bar = means \pm SD. Asterisks indicate the significance of difference from wild type by student t-tests ($P < 0.01$).

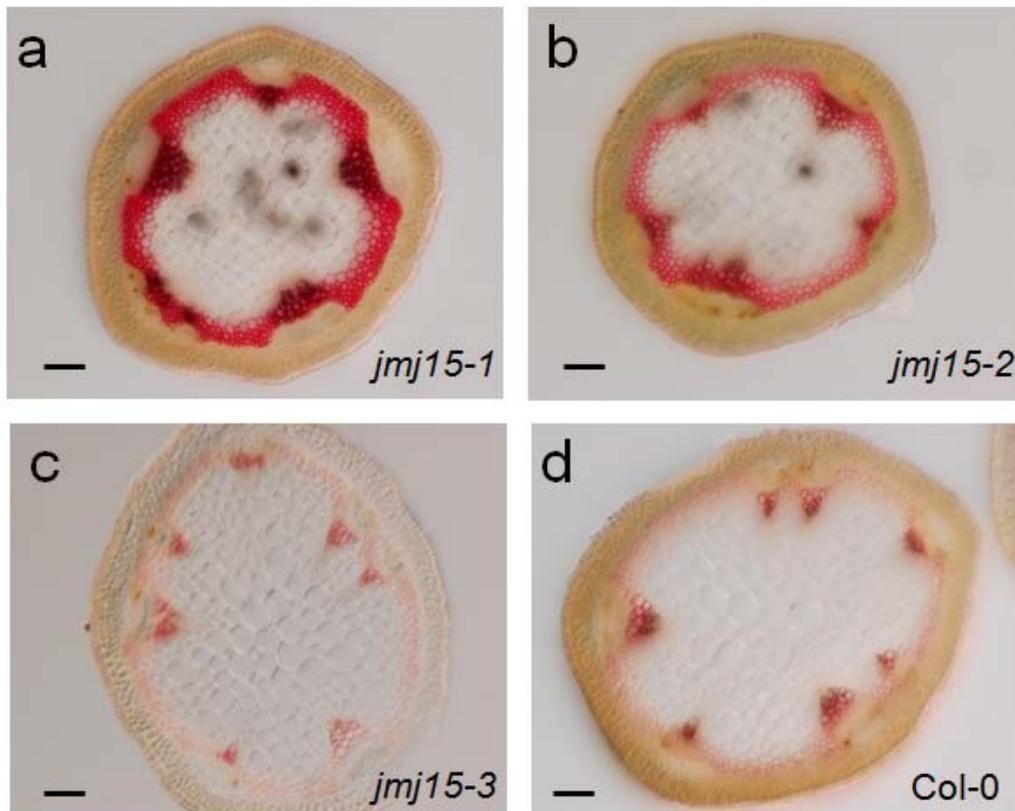


Figure 4. Lignin accumulation in *j mj15-1*, *j mj15-2*, and *j mj15-3* mutants compared to wild type. Inflorescence sections at the base were stained by Phloroglucinol stain. Bar = 0.1 mm

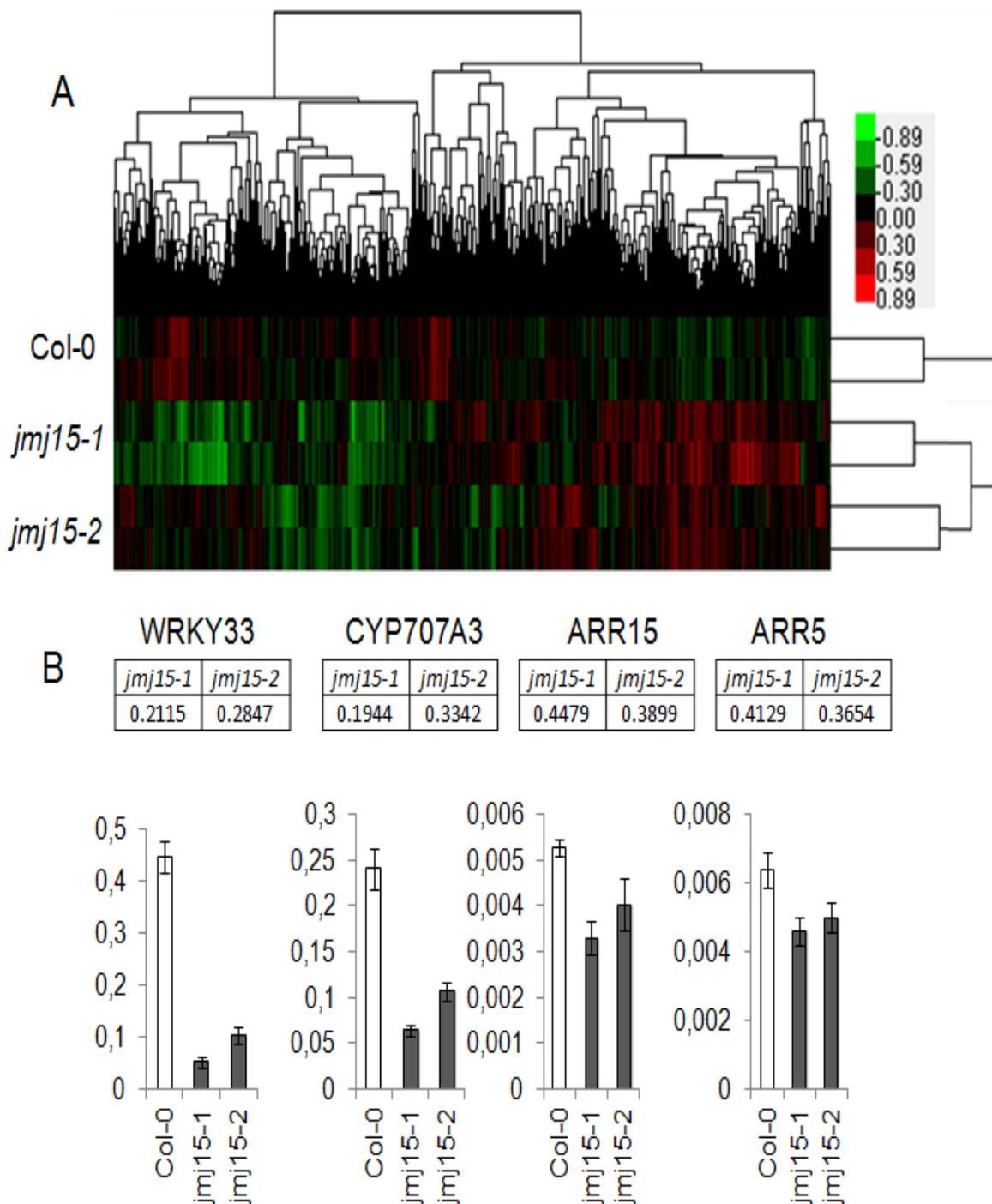


Figure 5. Transcriptomic analysis of *jmj15-1* and *jmj15-2* (12 day-old) seedlings compared to wild type. A. Pair-wise plots of the microarray hybridization signals between the biological replicates of each sample and the between wild type and two mutant alleles. B. Four down-regulated genes in the two mutants (microarray signals relative to wild type are indicated below the respective genes) were validated by qRT-PCR. Bar = means \pm SD from 3 replicates.

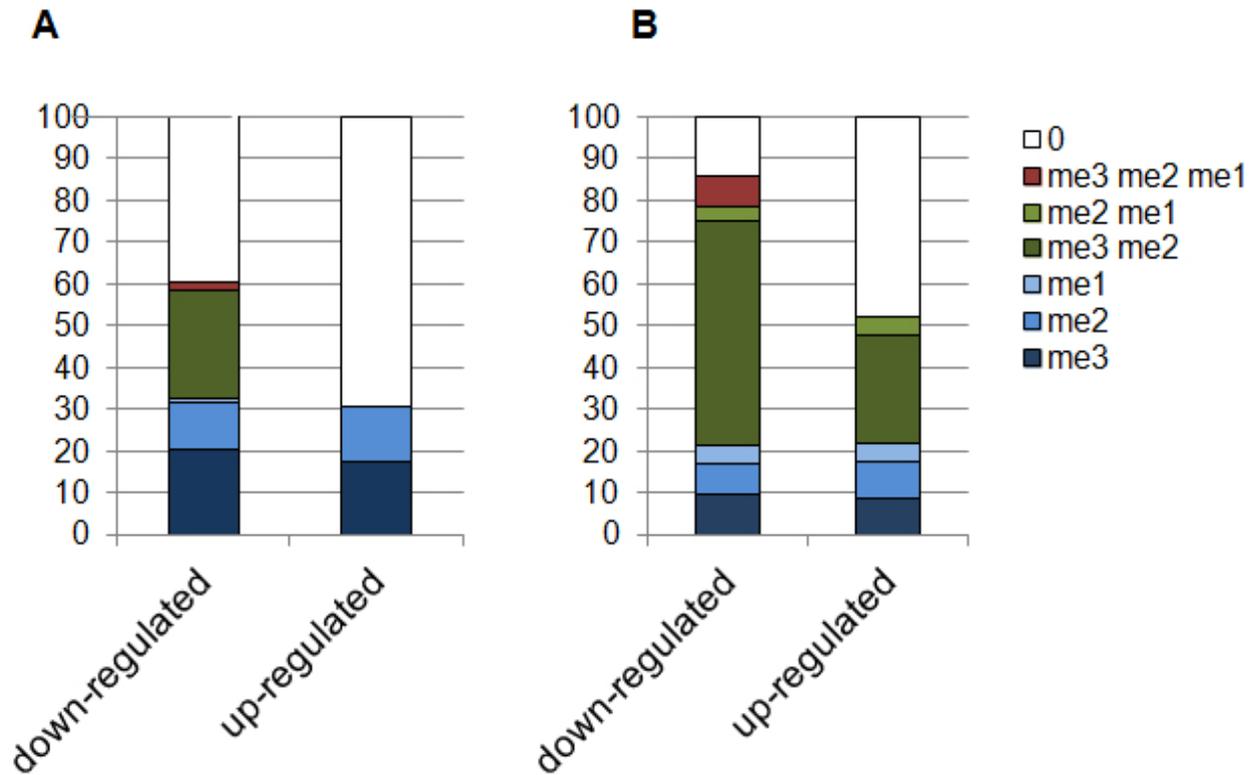


Figure 6. Down-regulated genes are highly enriched for H3K4me2/3. Up-regulated (23) and down-regulated (194) genes in *jmj15-1* or *jmj15-2* mutants (changes >2 folds) were compared with genome-wide H3K4 methylation data. Percentages of H3K4me3, H3K4me2, H3K4me1 and their combinations on the promoter (A) and the gene body (B) regions are presented.

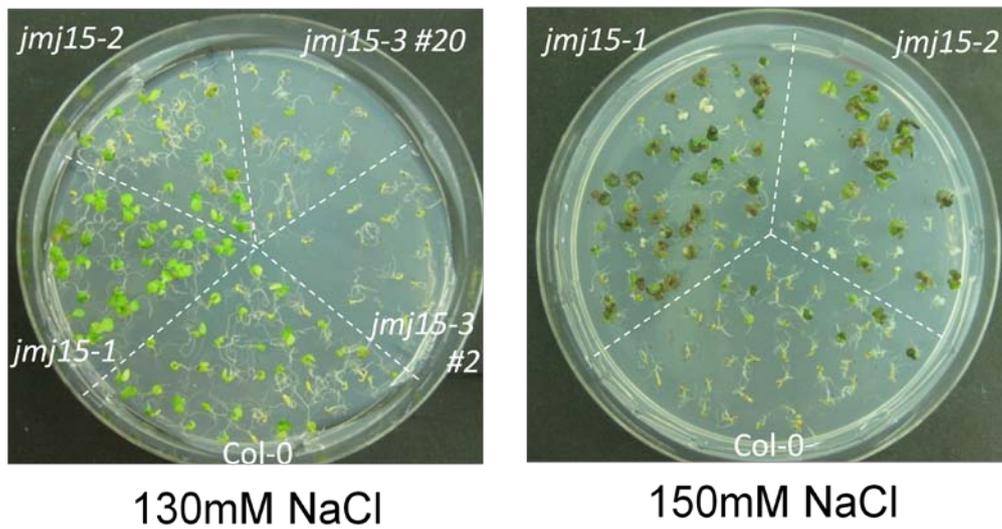


Figure 7. Comparison of sensitivity to NaCl of *jmj15* mutants with wild type during seed germination. Wild type, *jmj15-1*, *jmj15-2*, and *jmj15-3* seeds were germinated for 12 days on $\frac{1}{2}$ MS supplemented with indicated concentrations of NaCl.

3.8 Supplementary Material

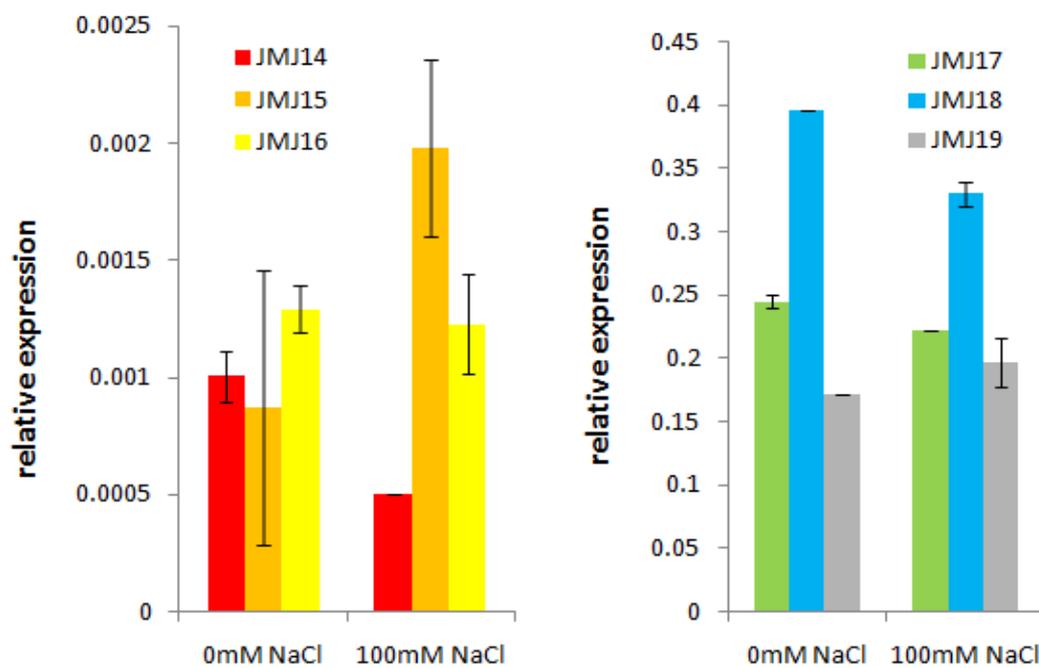


Figure S1. Expression levels of 6 potential H3K4 demethylase genes in *Arabidopsis* seedlings treated with or without 100 mM NaCl for 5 hours. The transcript levels detected by RT-qPCR are presented as relative to that of the reference gene *At4g34270*.

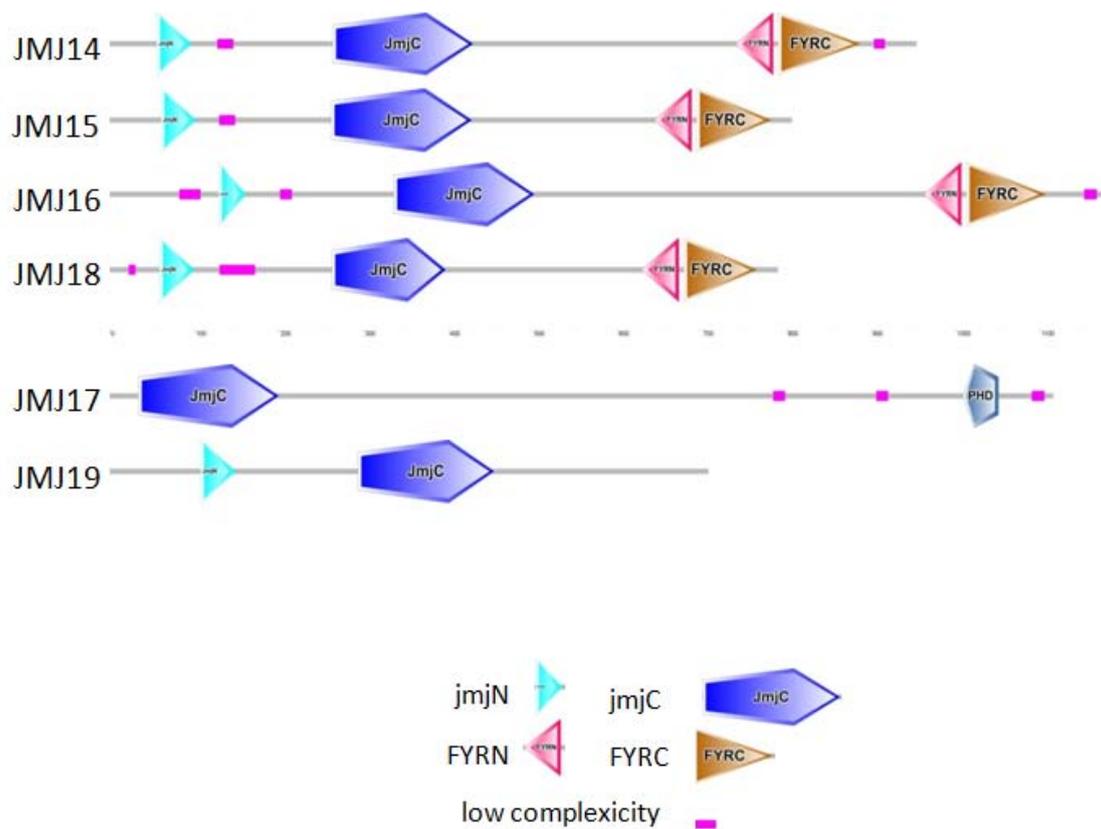


Figure S2. Comparison of protein structures of the 6 JmjC genes specific to plants.

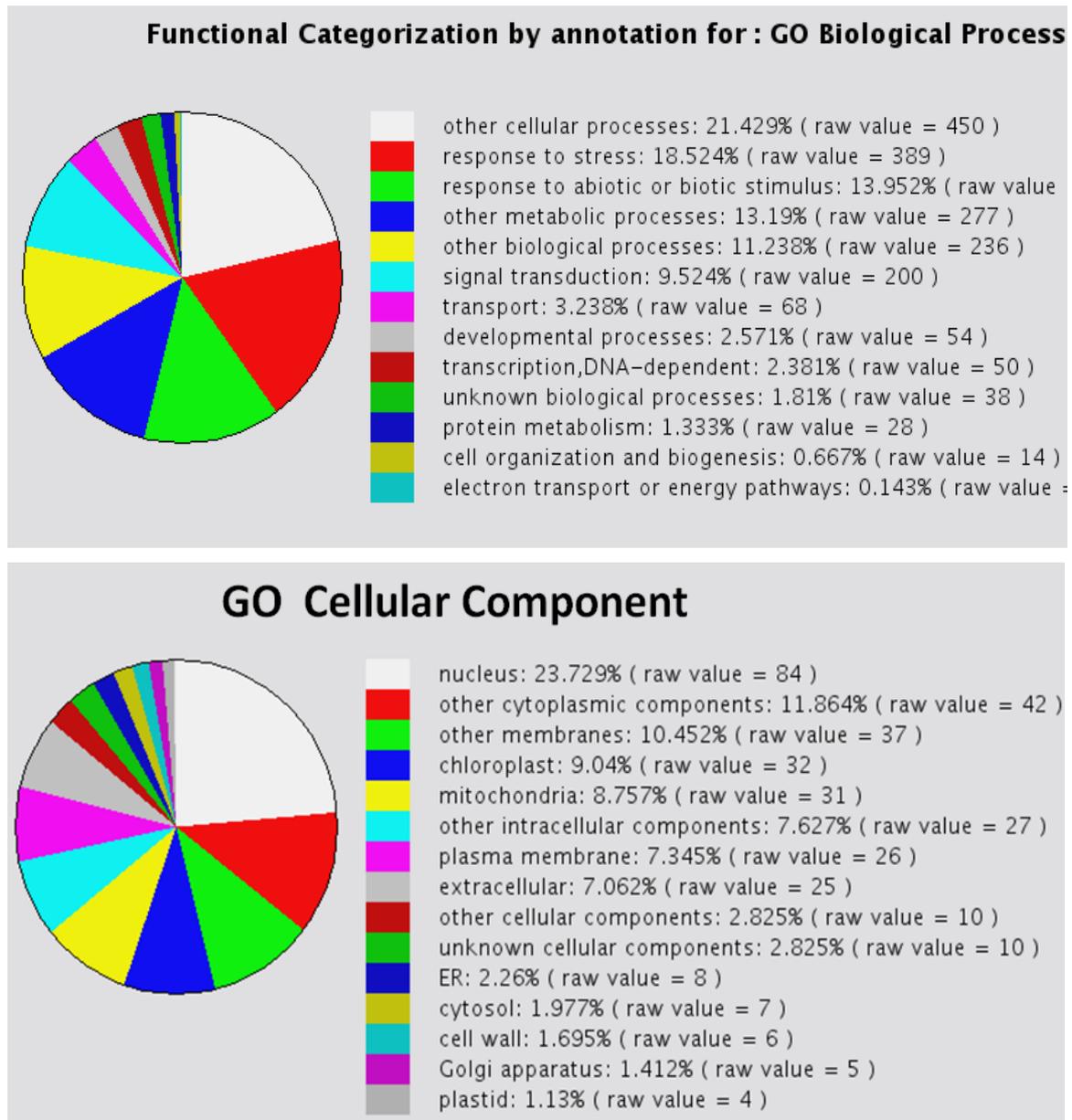


Figure S3. Gene ontology analysis of down-regulated genes in *jmj15* gain-of-function mutants.

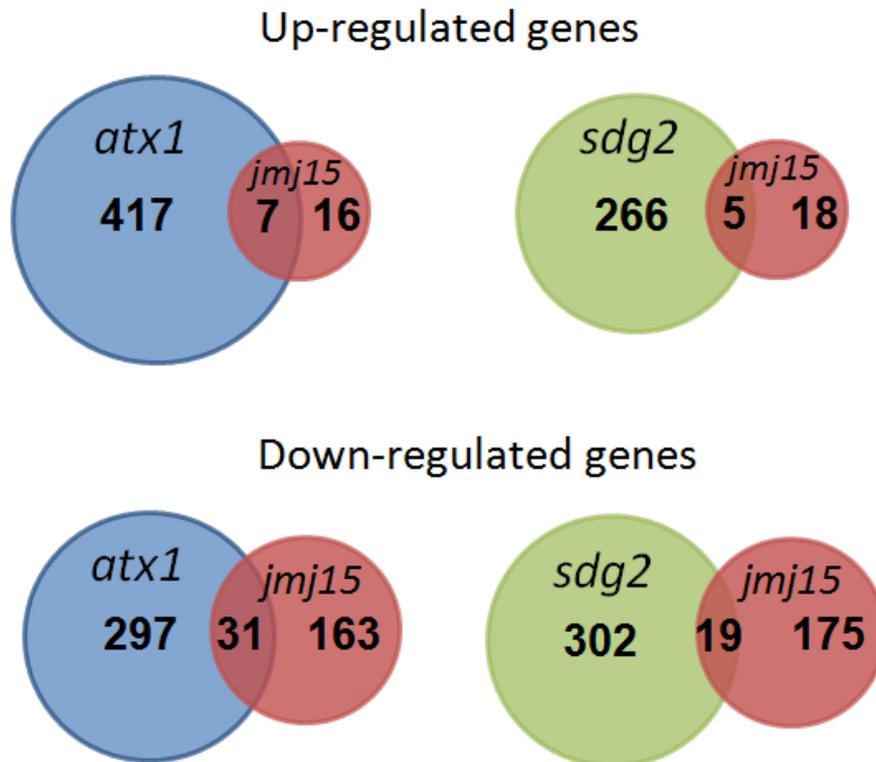


Figure S4. Overlaps of de-regulated genes between *jmj15* gain-of-function mutants and *atx1* or *sdg2* mutants.

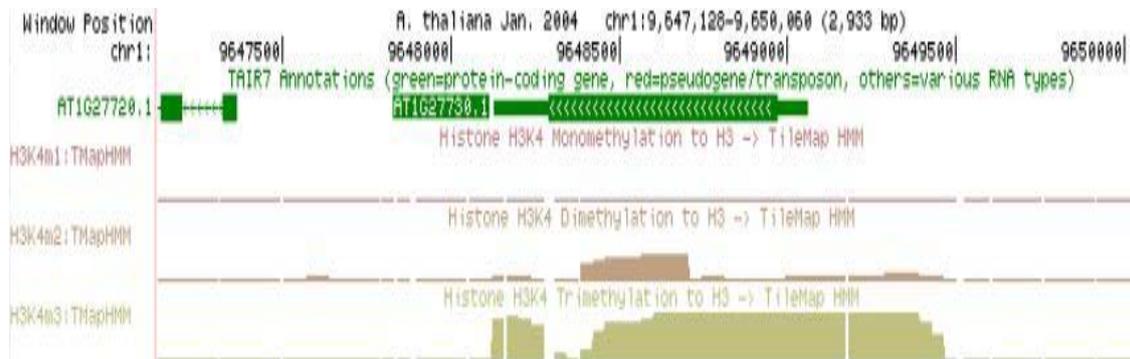


Figure S5. H3K4 methylation on the STZ/Zat10 locus.

Supplementary Dataset 1. Down- and up-regulated genes in *jmj15-1* and *jmj15-2* mutants compared to wild type. Genes encoding transcription factors or signaling regulators are indicated. Genes that are marked H3K4me3/2/1 are also indicated.

ProbeSetID	AGI ID	Target Description	Jm15-1 vs Col-C Jm15-2 vs Col-C		transcription factors	stress-responsive genes	H3K4 methylation (Zhang et al., 2009)	
			Ratio (Jm15-1/Col)	Ratio (Jm15-2/Col)			promoter (500bp)	gene body
261648_at	At1g27730	salt-tolerance zinc finger protein identical to salt-tolerance zinc fi	0.0650	0.2516			unstream the	me3 me2
261892_at	At1g98040	transcription factor, putative similar to WRKY transcription factor	0.0703	0.1741				me3 me2
254120_at	At4g24570	putative mitochondrial uncoupling protein mitochondrial uncoupl	0.0993	0.3023			me3	me3 me2
248448_at	At5g51190	putative protein contains similarity to ethylene responsive elem	0.1045	0.3087			me3 me2 me1	me3 me2 me1
256442_at	At3g10930	hypothetical protein predicted by genefinder; supported by full-len	0.1068	0.2049				
253643_at	At4g29780	hypothetical protein ; supported by full-length cDNA: Ceres: 2497	0.1203	0.2133				me3 me2
253832_at	At4g27654	Expressed protein ; supported by full-length cDNA: Ceres: 8827	0.1313	0.2052				me3
254075_at	At4g25470	DRE CRT-binding protein DREB1C: involved in low-temperature-	0.1360	0.3177			me3 me2	me3 me2
251745_at	At3g55980	putative protein zinc finger transcription factor (PE1), Arabidops	0.1457	0.2371			me3	me3 me2 me1
245250_at	At4g17490	ethylene responsive element binding factor-like protein (ATERF6)	0.1546	0.191			me3 me2	me3 me2
245041_at	At2g26530	AR781, similar to yeast pheromone receptor identical to GB:D88	0.1575	0.314			me3 me2	me3 me2
264758_at	At1g61340	late embryogenesis abundant protein, putative similar to late em	0.1619	0.2225			me3 me2	me3 me2
254926_at	At4g11280	ACC synthase (ATACS-6) ; supported by cDNA: gi_16226285_g	0.1658	0.3886				me3 me2
250098_at	At5g17350	putative protein ; supported by full-length cDNA: Ceres: 1198	0.1816	0.3182				me3
252679_at	At3g44260	CCR4-associated factor 1-like protein CAF1_MOUSE CCR4-AS	0.1842	0.4041			me3	me3
247543_at	At5g61600	DNA binding protein - like DNA binding protein EREBP-4, Nicoti	0.1848	0.3791			me3 me2	me3 me2
253259_at	At4g34410	putative protein ethylene-responsive element binding protein ho	0.1913	0.2266				me2
248964_at	At5g45340	cytochrome P450	0.1944	0.3342			me3 me2	me3 me2
261470_at	At1g28370	ethylene-responsive element binding factor, putative similar to e	0.1961	0.2817			me3 me2	me3 me2
251336_at	At3g61190	putative protein hypothetical protein F418.26 - Arabidopsis thali	0.1967	0.2765				me3 me2
253830_at	At4g27652	Expressed protein ; supported by full-length cDNA: Ceres: 4250	0.1967	0.3579			me3	me3
259879_at	At1g76650	putative calmodulin similar to calmodulin GB:CAA56517 [Leishm	0.2007	0.2114			me2	me3 me2
252131_at	At3g50930	BCS1 protein-like protein Homo sapiens h-bcs1 (BCS1) mRNA,	0.2057	0.3227				me3 me2
263379_at	At2g40140	putative CCH-type zinc finger protein also an ankyrin-repeat pr	0.2107	0.3246			me3	me3 me2 me1
267028_at	At2g38470	putative WRKY-type DNA binding protein	0.2115	0.2847			me3	me3 me2
261033_at	At1g17380	unknown protein ; supported by full-length cDNA: Ceres: 37370	0.2143	0.2295				me3 me2 me1
249928_at	At5g22250	CCR4-associated factor-like protein	0.2179	0.346			me3 me2	me3 me2
262382_at	At1g72920	virus resistance protein, putative similar to virus resistance prote	0.2207	0.4153			me3	me3 me2
266834_s_at	At2g30020	putative protein phosphatase 2C	0.2279	0.324			me3	me3
260744_at	At1g15010	hypothetical protein predicted by genemark hmm; supported by f	0.2285	0.3726			me3 me2	me3 me2
256356_s_at	At1g65500	hypothetical protein	0.2292	0.4414			me3 me2	me3
253859_at	At4g27657	Expressed protein ; supported by full-length cDNA: Ceres: 12938	0.2294	0.3765			me3	me3
247655_at	At5g59820	zinc finger protein Zat12 ; supported by full-length cDNA: Ceres:	0.2327	0.4255			me3 me2	me3 me2
249205_at	At5g42600	cytochrome P450	0.2444	0.5183				
256017_at	At1g19180	unknown protein ; supported by full-length cDNA: Ceres: 38751	0.2475	0.3806			me3	me3 me2
263182_at	At1g05575	Expressed protein ; supported by full-length cDNA: Ceres: 2708	0.2529	0.2702			me3 me2	me3 me2
266800_at	At2g22880	hypothetical protein predicted by genefinder	0.2585	0.3323			me2	
248799_at	At5g47230	ethylene responsive element binding factor 5 (ATERF5) (sp)080	0.2640	0.3768				me3 me2
265327_at	At2g18210	unknown protein	0.2704	0.3862				
264000_at	At2g22500	putative mitochondrial dicarboxylate carrier protein ; supported b	0.2711	0.4091			me3 me2	me3 me2
245711_at	At5g04340	putative c2h2 zinc finger transcription factor	0.2729	0.3542			me3 me2	me3 me2
263935_at	At2g35930	unknown protein	0.2733	0.3299			me3 me2	me3 me2
257644_at	At3g25780	unknown protein ; supported by full-length cDNA: Ceres: 3457	0.2867	0.3956			me2	me3 me2
267623_at	At2g39650	unknown protein	0.2887	0.4037			me3 me2	me3 me2
258792_at	At3g04640	hypothetical protein predicted by genefinder; supported by full-len	0.2908	0.4212			me3 me2	me3 me2
267357_at	At2g40000	putative nematode-resistance protein ; supported by full-length c	0.2921	0.3338			me3	me3
266316_at	At2g27080	unknown protein ; supported by cDNA: gi_15450380_gb_AY052	0.2969	0.3996				me3 me2
253915_at	At4g27280	putative protein centrin, Marsilea vestita supported by full-length	0.2986	0.5356			me3 me2 me1	me3 me2 me1
252474_at	At3g46620	putative protein several hypothetical proteins - Arabidopsis thalia	0.3024	0.5066			me3 me2 me1	me3 me2
257919_at	At3g23250	myb-related transcription factor, putative similar to myb-related t	0.3059	0.3107				
247047_at	At5g66650	putative protein contains similarity to unknown protein (gb AAC1	0.3117	0.4941			me3 me2	me3 me2
254231_at	At4g23810	putative protein AR411 - Arabidopsis thaliana (thale cress), PID	0.3150	0.3105			me3 me2	me3 me2
249202_at	At5g42580	cytochrome P450	0.3214	0.5135				
261713_at	At1g32640	protein kinase, putative identical to bHLH protein GB:CAA67885	0.3278	0.4318			me3	me3 me2
249203_at	At5g42590	cytochrome P450	0.3301	0.5482				
249719_at	At5g35735	Expressed protein ; supported by full-length cDNA: Ceres: 32456	0.3324	0.5556			me3 me2	me3 me2
266098_at	At2g37870	hypothetical protein predicted by genescan; supported by cDNA: (0.3327	0.8934			me2	
245119_at	At2g41640	unknown protein ; supported by cDNA: gi_16930450_gb_AY419	0.3351	0.3944			me3 me2	me3 me2
245777_at	At1g73540	unknown protein contains similarity to diphosphoinositol polypho	0.3356	0.4845			me3	me3
247177_at	At5g65300	unknown protein ; supported by cDNA: gi_13877834_gb_AF370	0.3370	0.4175			me3	me3 me2
247215_at	At5g64905	Expressed protein ; supported by full-length cDNA: Ceres: 3657	0.3378	0.4234			me3	me2
248794_at	At5g47220	ethylene responsive element binding factor 2 (ATERF2) (sp)080	0.3419	0.516			me3	me3 me2
262211_at	At1g74930	AP2 domain containing protein, putative similar to AP2 domain c	0.3490	0.3526			me3	me3 me2
250676_at	At5g06320	harpin-induced protein-like ; supported by cDNA: gi_9502175_g	0.3516	0.5263			me3	me3 me2
256633_at	At3g28340	unknown protein	0.3600	0.4934			me3 me2	me3 me2
256306_at	At1g30370	lipase, putative contains Pfam profile: PF01764 Lipase	0.3653	0.4303				
265725_at	At2g32030	putative alanine acetyl transferase	0.3664	0.4255				me3 me2
247708_at	At5g59550	putative protein COP1-interacting protein CIP8, Arabidopsis thali	0.3666	0.4955			me3	me3 me2
246099_at	At5g20230	blue copper binding protein ; supported by full-length cDNA: Ceri	0.3712	0.3806				me3 me2
248164_at	At5g54490	putative protein similar to unknown protein (pir T05752); support	0.3732	0.4466				me3 me2
245840_at	At1g58420	hypothetical protein predicted by genemark hmm; supported by f	0.3740	0.4451			me2	me3 me2
252193_at	At3g50060	R2R3-MYB transcription factor ; supported by cDNA: gi_159834	0.3760	0.4183			me3 me2	me3 me2
249197_at	At5g42380	putative protein contains similarity to calmodulin supported by fu	0.3769	0.3684			me2	me2
248327_at	At5g52750	putative protein similar to unknown protein (emb CAA71173.1).st	0.3782	0.4598				me3
247240_at	At5g64660	putative protein strong similarity to unknown protein (emb CAB88	0.3807	0.4778			me3 me2	me3 me2
248727_at	At5g47990	cytochrome P450	0.3809	0.5915				
249417_at	At5g39670	calcium-binding protein - like cbp1 calcium-binding protein, Lotu	0.3814	0.4887				me2 me1
256526_at	At1g66090	disease resistance protein, putative similar to disease resistance	0.3841	0.3981			me3 me2	me3 me2
258436_at	At3g16720	putative RING zinc finger protein similar to RING-H2 zinc finger p	0.3842	0.4884			me3	me3 me2
245731_at	At1g73500	MAP kinase, putative similar to MAP kinase kinase 5 GI: 321927	0.3846	0.5915			me3	me3
257022_at	At3g19580	zinc finger protein, putative similar to Cys2/His2-type zinc finger	0.3889	0.3644			me3 me2	me3 me2
249683_at	At5g37770	CALMODULIN-RELATED PROTEIN 2, TOUCH-INDUCED (TCH	0.3907	0.5152			me3	me3
256093_at	At1g20823	predicted protein ; supported by cDNA: gi_15027984_gb_AY405	0.3924	0.485				me3
246289_at	At3g56880	putative protein predicted protein At2g41010 - Arabidopsis thalia	0.3934	0.5572			me3	me3 me2 me1
256522_at	At1g66160	unknown protein ; supported by full-length cDNA: Ceres: 35218	0.3955	0.4303			me2	me3 me2
259850_at	At1g72240	hypothetical protein predicted by genemark hmm	0.3979	0.4662				me2
245252_at	At4g17500	ethylene responsive element binding factor 1 (frameshift !); supp	0.3997	0.4898			me3	me3 me2
262128_at	At1g52690	late embryogenesis-abundant protein, putative similar to late em	0.4022	0.7624				me1
254158_at	At4g24380	putative protein dihydrofolate reductase - Schizosaccharomyces	0.4046	0.5108			me3	me3 me2
255733_at	At1g25400	unknown protein similar to unknown protein GI: 6714347 from [Ar	0.4068	0.4467			me3 me2	me3 me2
247431_at	At5g62520	putative protein similar to unknown protein (pir T07711); support	0.4104	0.3249			me2	me3 me2
252374_at	At3g48100	response reactor 2 (ATRR2) ; supported by cDNA: gi_3273197	0.4129	0.3654				me3 me2
247208_at	At5g64870	nodulin-like ; supported by full-length cDNA: Ceres: 142026	0.4133	0.4861				me2 me1
262381_at	At1g72900	virus resistance protein, putative similar to virus resistance prote	0.4143	0.4875			me3 me2 me1	me3 me2
265630_at	At2g06050	12-oxophytodienoate-10,11-reductase ; supported by cDNA: gi_	0.4148	0.6358			me3	me3 me2
248392_at	At5g52050	integral membrane protein-like	0.4151	0.5489			me2	me2

ProbeSetID	AGI ID	Target Description	Jmj15-1 vs Col-0mj15-2 vs Col-0		H3K4 methylation (Zhang et al., 2009)	
			Ratio (mj15-1/Col)	Ratio (mj15-2/Col)	500bp upstream the gene body	gene body
262383_at	At1g72940	disease resistance protein, putative similar to disease resistance	0.4188	0.5448	me3 me2	me3 me2 me1
264213_at	At1g65400	hypothetical protein contains similarity to lectin polypeptide Gl.410	0.4218	0.4083		me1
267069_at	At2g41010	unknown protein	0.4227	0.5461	me3 me2	
252368_at	At3g48520	cytochrome P450-like protein cytochrome P450 CYP9A41 - Vicia	0.4251	0.5377	me3 me2	me3 me2
263783_at	At2g46400	putative WRKY-type DNA binding protein ; supported by cDNA: g	0.4251	0.5618	me3 me2	me3 me2
262105_at	At1g02810	pectin esterase, putative similar to pectin esterase Gl.1213628 fr	0.4272	0.5898	me2	me3 me2
261476_at	At1g14480	hypothetical protein contains similarity to alpha-latroinsectotoxin p	0.4275	0.4919	me1	me2 me1
250472_at	At5g10210	putative protein	0.4296	0.5654		
262369_at	At1g73010	hypothetical protein predicted by genemark.hmm,supported by full	0.4325	0.3722	me2	
266645_at	At2g35290	hypothetical protein predicted by genefinder	0.4341	0.5005		me3 me2
253113_at	At4g35985	putative protein physical impedance induced protein, Zea mays, g	0.4397	0.6055	me3 me2	me3 me2
252278_at	At3g49530	NAC2-like protein NAC2 - Arabidopsis thaliana, EMBL_AF201456	0.4410	0.4782	me2	me3
259479_at	At1g19020	Expressed protein ; supported by full-length cDNA: Ceres: 31015.	0.4423	0.5058		me3 me2
245275_at	At4g15210	beta-amylase ; supported by cDNA: gi_166601_gb_M73467.1_AT	0.4447	0.4927		me2
255844_at	At2g33580	putative protein kinase contains a protein kinase domain profile (P	0.4458	0.5561	me3 me2	me3 me2
249918_at	At5g19240	putative protein predicted protein, Arabidopsis thaliana	0.4459	0.5383	me3 me2	me3 me2
262212_at	At1g74890	response regulator 7, putative similar to response regulator 7 Gl.	0.4479	0.3899	me2	me2
265665_at	At2g27420	cysteine proteinase contains similarity to cysteine protease SPCP	0.4480	0.3524		me2
260135_at	At1g66400	calmodulin-related protein similar to GB P25070 from [Arabidopsi	0.4483	0.5149	me3 me2	me3 me2
247925_at	At5g57560	TCH 4 protein (gb AAA92363.1) ; supported by cDNA: gi_141941	0.4488	0.4946	me2	me3 me2
245422_at	At4g17470	putative protein	0.4491	0.7861		me1
249042_at	At5g44350	ethylene-regulated nuclear protein ERT2-like protein	0.4508	0.6014		me3 me2
259979_at	At1g76600	unknown protein ; supported by full-length cDNA: Ceres: 29624.	0.4510	0.6163		me3 me2
250292_at	At5g13220	putative protein predicted proteins, Arabidopsis thaliana, supporte	0.4512	0.4933	me3 me2	me3 me2
261443_at	At1g28480	glutaredoxin, putative similar to glutaredoxin Gl.2244924 from [Ar	0.4547	0.5486	me2	
253485_at	At4g31800	Expressed protein ; supported by full-length cDNA: Ceres: 40692.	0.4561	0.3536		me3 me2
250239_at	At5g13580	ABC transporter-like protein	0.4578	0.5873		me2 me1
251423_at	At3g60550	regulatory protein - like regulatory protein preg, Neurospora crass	0.4600	0.5098		me2
251705_at	At3g56400	DNA-binding protein-like DNA-binding protein 4 WRKY4 - Nicotia	0.4627	0.4719	me3 me2	me3 me2 me1
258322_at	At3g22740	putative selenocysteine methyltransferase similar to selenocystey	0.4647	0.5912		me2
263584_at	At2g17040	NAM (no apical menstem)-like protein similar to petunia NAM (X9	0.4656	0.4743		me3 me2
253483_at	At4g31910	putative protein anthranilate N-hydroxycinnamoyl/benzoyltransfer	0.4665	0.941		me2 me1
257824_at	At3g25290	unknown protein ; supported by cDNA: gi_16323199_gb_AY0577	0.4669	0.772		me3 me2
258941_at	At3g09940	putative monodehydroascorbate reductase (NADH) similar to mor	0.4682	0.5201	me3	me3
266658_at	At2g25735	Expressed protein ; supported by full-length cDNA: Ceres: 7152.	0.4695	0.3723		
245329_at	At4g14365	Expressed protein ; supported by full-length cDNA: Ceres: 37809.	0.4698	0.5025		me3 me2 me1
256763_at	At3g16860	unknown protein	0.4700	0.5882	me3 me2	me3 me2 me1
266017_at	At2g18690	unknown protein ; supported by cDNA: gi_14517479_gb_AY0395	0.4700	0.6369		
246228_at	At4g36430	peroxidase like protein	0.4706	0.631		me1
252045_at	At3g52450	putative protein arm repeat containing protein ARC1 -Brassica nap	0.4717	0.4941	me3 me2 me1	me3 me2 me1
264404_at	At2g25160	putative cytochrome P450	0.4717	0.5681		
264261_at	At1g09240	putative nicotianamine synthase similar to GB:BAA74589;support	0.4718	0.5307		me1
265846_at	At2g35770	putative serine carboxypeptidase II	0.4726	0.5934		
251507_at	At3g59080	putative protein CND41, chloroplast nucleoid DNA binding protein	0.4736	0.5298	me3	me3 me2 me1
252053_at	At3g52400	syntaxin-like protein synt4 ; supported by full-length cDNA: Ceres	0.4752	0.5173	me3	me3 me2
255877_at	At2g40460	putative PTR2 family peptide transporter	0.4763	0.6729	me3 me2	me3 me2
265184_at	At1g23710	unknown protein ; supported by full-length cDNA: Ceres: 36437.	0.4768	0.5297	me3	me3 me2
246270_at	At4g36500	putative protein	0.4772	0.4782		me3 me2
266418_at	At2g38750	putative annexin ; supported by full-length cDNA: Ceres: 32721.	0.4781	0.634		
261405_at	At1g18740	unknown protein ; supported by full-length cDNA: Ceres: 40753.	0.4810	0.5668	me3	me3 me2
259518_at	At1g20510	hypothetical protein ; supported by cDNA: gi_13430675_gb_AF36	0.4827	0.6064	me3 me2	me3 me2
265188_at	At1g23800	putative aldehyde dehydrogenase ; supported by cDNA: gi_14334	0.4830	0.702		me1
261193_at	At1g32920	unknown protein ; supported by cDNA: gi_15450636_gb_AY0526	0.4831	0.5082	me3	me3 me2
248790_at	At5g47450	membrane channel protein-like, aquaporin (tonoplast intrinsic prot	0.4835	0.5019		me3 me2
247755_at	At5g59090	subtilisin-like serine protease contains similarity to prepro-cucumis	0.4839	0.5866		me1
252200_at	At3g50280	anthranilate N-hydroxycinnamoyl/benzoyltransferase - like protein	0.4847	0.5473	me2	me2
247393_at	At5g63130	unknown protein	0.4848	0.6296	me3 me2	me3 me2
264434_at	At1g10340	hypothetical protein predicted by genscan; supported by cDNA: gi	0.4849	0.7225		me3 me2 me1
245412_at	At4g17280	hypothetical protein	0.4863	0.5233		
251668_at	At3g57010	putative protein stricotosidine synthase (EC 4.3.3.2) - Rauvolfia ma	0.4882	0.6488	me2	me2
255753_at	At1g18570	myb factor, putative similar to myb factor Gl.1946266 from [Oryza	0.4900	0.5819	me3	me3 me2
257925_at	At2g23170	unknown protein ; supported by full-length cDNA: Ceres: 92314.	0.4906	0.5665	me3	me3
263005_at	At1g54540	hypothetical protein predicted by genemark.hmm	0.4907	0.4603		
249097_at	At5g43520	putative protein strong similarity to unknown protein (pir J T02393)	0.4908	0.5739		
255668_at	At4g01250	putative DNA-binding protein ; supported by cDNA: gi_15028172	0.4937	0.4402	me3 me2	me3 me2
265841_at	At2g35710	putative glycogenin	0.4940	0.67	me2	me3 me2 me1
248750_at	At5g47530	putative protein similar to unknown protein (pir J H71441)	0.4953	0.4593		
252594_at	At3g45680	putative transporter protein peptide transport protein - Hordeum v	0.4957	0.6836		me1
245755_at	At1g35210	hypothetical protein predicted by genemark.hmm,supported by full	0.4966	0.5759	me3 me2	me3 me2
246777_at	At5g27420	RING-H2 zinc finger protein-like RING-H2 zinc finger protein ATL	0.4968	0.4714	me3	me3 me2
260662_at	At1g19540	2-hydroxyisoflavone reductase, putative similar to PIR T08106 fr	0.4977	0.6085		me2 me1
249715_at	At5g35760	putative protein similar to unknown n protein (emb CAB69836.1)	0.4984	0.5941		
250230_at	At5g13900	putative protein similar to unknown n protein (pir J T14827)	0.4984	0.4984		me3 me2
262751_at	At1g16310	hypothetical protein predicted by genscan+	0.5000	0.6862		me3
254574_at	At4g19430	expressed protein ; supported by cDNA: Ceres: 13917	0.9354	0.2955		
258133_at	At3g24500	ethylene-responsive transcriptional coactivator, putative similar to	0.7948	0.3411	me3 me2	me3
252367_at	At3g48360	BTB and TAZ domain protein 2, protein binding / transcription regu	0.7612	0.384		me3 me2
261450_s_at	At1g21110	O-methyltransferase, putative similar to GB:AAF28353	0.5165	0.3536		
249337_at	At5g41080	putative protein contains similarity to unknown protein (gb AAF148	0.5609	0.3887	me2	me3 me2 me1
245757_at	At1g35140	phosphate-induced (phi-1) protein, putative similar to phi-1	0.5634	0.3977		me3 me2
261567_at	At1g33055	Expressed protein	0.8762	0.3996	me3	me3 me2
259399_at	At1g17710	hypothetical protein similar to putative phosphatase Gl:3218467	0.7019	0.4142		
248434_at	At5g51440	mitochondrial heat shock 22 kd protein-like	0.6903	0.416	me3	me3 me2
247293_at	At5g64510	putative protein similar to unknown n protein (pir T03813)	0.7474	0.4202	me2	me2 me1
258158_at	At3g17790	acid phosphatase 5/ protein serine/threonine phosphatase	0.5483	0.4202	me2	me3 me2
264153_at	At1g65390	transmembrane receptor, disease resistance protein RPS4	0.5811	0.4265	me2	me2
265387_at	At2g20670	unknown protein	0.9323	0.4277	me3 me2	me3 me2
263216_s_at	At1g30720	putative reticuline oxidase-like protein similar to GB P30986	0.5601	0.4319		me3 me2
251356_at	At3g61060	putative protein hypothetical proteins	0.6445	0.463		me3
258487_at	At3g02550	unknown protein ; supported by full-length cDNA: Ceres: 20907.	0.8134	0.4642	me3 me2	me3 me2
253814_at	At4g28290	putative protein ; supported by full-length cDNA: Ceres: 10077.	0.6229	0.4661		me3 me2
250541_at	At5g09520	putative proline-rich protein surface protein C PSpC, Streptococcu	0.5162	0.4688	me3 me2	me2
253061_at	At4g37610	BTB and TAZ domain protein 5; protein binding / transcription regu	0.5319	0.4702	me3	me3 me2
247406_at	At5g62920	response regulator 6 (ARR6), transcription regulator	0.593	0.4802	me3	me3 me2
263168_at	At1g03020	putative glutaredoxin Strong similarity to gb Z49699 glutaredoxin	0.7675	0.4807		
258468_at	At3g06070	unknown protein predicted by genscan	0.7713	0.4853	me3 me2	me3 me2

ProbeSetID	Representative Public ID	Target Description	data Jacobsen			
			Ratio	Ratio	promoter	gene body
267407_at	At2g34880	JMJ15	8.3689	7.2483		
251677_at	At3g56980	putative protein hypothetical protein At2g41240 - Arabidopsis thaliana, EMBL:AC005662	8.5218	2.3644		
254650_at	At4g19690	Fe(II) transport protein ; supported by full-length cDNA. Ceres:3164.	5.7238	3.2572		
253301_at	At4g33720	pathogenesis-related protein 1 precursor, 19.3k ; supported by full-length cDNA. Ceres:5743.	5.4322	2.0545	me2	me2
261684_at	At1g47400	hypothetical protein predicted by genemark hmh	4.8653	1.9126		
253502_at	At4g31940	Cytochrome P450-like protein cytochrome P450 monooxygenase, Pisum sativum, PATCHX.G	4.7761	2.0154		
248048_at	At5g56080	nicotianamine synthase	3.8378	2.0558		me3
246273_at	At4g36700	globulin-like protein	3.7989	1.149	me2	me3 me2
248270_at	At5g53450	putative protein similar to unknown protein (sp P29618).supported by full-length cDNA. Ceres:	3.2196	1.6649		
251293_at	At3g61930	hypothetical protein	2.6078	1.3479		me2
250828_at	At5g05250	unknown protein ; supported by cDNA: gi_15810044_gb_AY054291.1_	2.597	1.5287		me3me2
262446_at	At1g49310	hypothetical protein predicted by genscan1	2.3311	1.6505		
264433_at	At1g61810	beta-glucosidase, putative similar to beta-glucosidase GI:804655 from (Hordeum vulgare)	2.226	1.0228		
264751_at	At1g23020	putative superoxide-generating NADPH oxidase flavocytochrome highly similar to GB_CAA707	2.1952	1.1255	me3	me3 me2
247540_at	At5g61590	ethylene responsive element binding factor - like ethylene responsive element binding factor 5	2.0825	1.2048	me3	me3 me2
252510_at	At3g46270	putative protein serine/threonine-specific receptor protein kinase (EC:2.7.1.-), Arabidopsis thali	2.0823	1.8489		
263364_at	At2g41230	unknown protein	2.0683	1.2025	me2	me3 me2
263593_at	At2g01860	hypothetical protein predicted by genscan and genefinder	2.059	1.0228	me3	me3 me2
252882_at	At4g39675	Expressed protein ; supported by full-length cDNA: Ceres: 14423.	2.0215	1.0184		
245076_at	At2g23170	unknown protein	1.7126	2.1658		
259813_at	At1g49860	glutathione S-transferase, putative similar to GI:860955 from [Hyoscyamus muticus] (Plant Ph	1.7111	2.1157		me2 me1
256940_at	At3g30720	unknown protein	1.6412	2.564		me3 me1
260152_at	At1g52830	putative IAA6 protein similar to IAA6 protein GB:S5849 from [Arabidopsis thaliana].supported b	1.4391	2.0293		
247718_at	At5g59310	nonspecific lipid-transfer protein precursor - like nonspecific lipid-transfer protein precursor, Bri	1.0384	2.712		me3

Table S1. Sequences of the primers used in this study.

Gene	Forward (5'→3')	Reverse (5'→3')
JMJ15-F1, R1	CCTTTGGGTTTTGTGGAGTG	CACCAATGTCTGGCCTCTTT
JMJ15-F2, R2	CCTTGGAGCAGCTTTTGAAG	AACCATTTCCAAGAGCCTCA
JMJ15-F3, R3	ATCTTGGGTTCTTGGTCGTG	GAAGAGTGGACCCAGAAGTCC
JMJ15-F4, R4	GAGATCATGAGACGCAGCAA	TCATTTCCAGCGAATCCTTC
JMJ14	TGGTTGTTTAGGAGGGAAGG	TGGTTAGGTTTGTGGGTCA
JMJ16	GGAGATTCGCTGCGTGATAC	CGTGGCTGTTAGTTGCCATT
JMJ17	CATGCAGAGCAGGAACTTCA	GTGGAGTGGTTTCTGCCAGT
JMJ18	GAAATGGTGATGCGGAGAGT	AGTCGATGGTTTGGGTCAAG
JMJ19	AATCGCAAAGGATGGACAAG	CCTCATGTGGTTCAGACACG
WRKY33	AAGGGAAACCCAAATCCAAG	AAGACGAATCCTGTGGTGCT
CYP707A3	AAAGCAGGATTAACCGACGA	TTGCCATTTGCTCTTCAGTG
ARR15	GAAGGAGCAGAGGAGTTTTTG	GTCGTCATCAAGGGAGGAA
ARR5	TGTCCTGATTCTTTCGGCTTAC	AAAAACCCATCTTTGTCACTCTT
	A	GA
ACTIN2	CGCTGACCGTATGAGCAAAGA	GCAAGAATGGAACCACCGATC
At4g34270	GCACCAACTGTTCTTCGTGA	GGTCGCTCCAGACTGCTAAG

CHAPTER 4

GENERAL DISCUSSION & PERSPECTIVES

4.1 Function of CHR5 in plant gene expression

Several CHD3 proteins have been reported in plants. *Arabidopsis* CHD3 protein, PKL, represses *LEC1/AFL* gene expression via promoting H3K27me3 on these loci (Aichinger et al., 2009; Zhang et al., 2012). Rice CHD3 protein, CHR729, recognizes and modulates H3K4 and H3K27 methylation over repressed tissue-specific genes (Hu et al., 2012). Unlike the CHD3 family, only one CHD1 gene (CHR5) is found in the genome of *Arabidopsis*. CHR5 is mostly expressed in growing or developmental tissues/organs, suggesting that this chromatin remodeler may play an important role in facilitating gene expression reprogramming during plant development. Our data on the function of CHR5 to modulate seed maturation gene expression during embryogenesis support this hypothesis. The role of CHR5 in other developmental aspects could be exploited to further understand the function and mode of action of CHR5.

The CHD1 proteins in animal and yeast cells have shown to play various roles in regulating chromatin dynamics during transcription at different levels. For instance, the chromodomain of human CHD1 was found to specially bind to H3K4me3, a mark for gene activation, and to be associated with the pre-initiation transcription complex (PIC) through interacting with Mediator (Lin et al., 2011). In fly and yeast, CHD1 is localized to transcriptionally active genes and physically interacts with elongation factors and RNA Polymerase II (Simic et al., 2003; Srinivasan et al., 2005). In *S. cerevisiae*, Chd1 was shown to positively regulate gene expression through promoting nucleosome reassembly coupled with H2B monoubiquitination (Lee et al., 2012). In yeast, Chd1 was also reported to maintain chromatin structure at active genes via modulating nucleosome spacing integrated with the prevention of cryptic transcription and histone exchange within gene bodies (Hennig et al., 2012; Shim et al., 2012; Smolle et al., 2012). We have shown that the mutation of *Arabidopsis* CHR5 led to a decrease of the transcription level of *LEC1/AFL* genes accompanied with an increase of H3K27me3 on these loci. Furthermore, CHR5 was shown to directly associate with the promoter region of *FUS3* and *ABI3*, and *chr5* mutations resulted in increased nucleosome occupancy near TSS on *FUS3* loci. The data indicated that CHR5 regulates embryo identity gene expression via modulating nucleosome positioning. Only recently was reported the effect of nucleosome positioning on gene transcription

during plant development and responsive to stress. BRM, another SNF2 chromatin remodeling factor, was reported to repress *ABI5* and *ABI3* expression by directing the nucleosome occupancy during postgermination development (Han et al., 2012). H2A.Z-containing nucleosomes mediate the thermosensory responsive in *Arabidopsis*, linking the perception of temperature through DNA-nucleosome fluctuations (Kumar and Wigge, 2010). Furthermore, H2A.Z is enrichment across gene bodies which is negatively correlated with gene transcription levels and positively correlated with gene responsiveness—the degree to which a gene is differentially expressed in responsive to environmental and developmental stimuli (Coleman-Derr and Zilberman, 2012).

Owing to the rapid progress of high-throughput array and sequencing techniques, it is possible to detect the global nucleosome positioning and identify the position of individual nucleosome at a specific time. Given these reasons, it will be very important to investigate the genome-wide target genes of CHR5 and its effect on nucleosome positioning and global gene activities. Comparison of these data will help us to better understanding chromatin remodeling mediated by CHR5 in gene transcription during the plant development. In addition, the function of CHR5 in gene expression related to stress responses could be evaluated to study the role of CHR-dependent nucleosome positioning in inducible gene expression, which would be of significance for plant adaptation to environmental constraints. Finally, it will of great importance to study the interplay between CHR5 and other chromatin modifiers (such as enzymes involved in histone modifications) and remodelers (such as, BRM, other CHD members and proteins involved in H2A.Z deposition) in genome-wide nucleosome dynamics related to plant growth and development.

4.2 The function of JMJ15 in plant development

In plant development, *JMJ15* was particularly expressed in shoot meristem and during lateral root initiation, raising the hypothesis that JMJ15 may regulate meristem function, and lateral root formation. A more detailed examination of these aspects during the plant development will be needed. On the other hand, as the stress-responsive genes account for the largest proportion of regulated genes in *jmj15* overexpression mutants, the function of JMJ15 in response to salt and other stress stimuli would be chosen in a future analysis.

JMJ15 represses the expression of *ARR15*, *ARR5*, *WRKY33*, *CYP707A3* and other genes, which may account for some of the phenotypic changes seen in the *jmj15* overexpression mutants. It would be important to study whether JMJ15 could directly associate with these genes and regulate their expression via demethylating H3K4 on these loci. We produced transgenic overexpression plants of FLAG-HA-tagged JMJ15 by using the 35S promoter. These transgenic lines will be used for ChIP with anti-HA antibody to examine if JMJ15 directly binds to these genes *in vivo*. On the other hand, ChIP experiment will also be carried out to test if there is any change of histone modification on these loci in *jmj15* mutants. In fact, it's also worth identifying the genome-wide targets and histone methylation of JMJ15 by ChIP-seq and ChIP-chip. Then comparison of genomic targets and histone modification in combination with gene expression profiling will unravel the role of JMJ15 in the regulation of gene expression in *Arabidopsis*. Because the expression of *JMJ15* is restricted in a limited numbers of tissues, it will be usefully to perform the above experiments using specific tissues/organs instead of the whole plant.

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APPENDIX

The Role of Histone Methylation and H2A.Z Occupancy during Rapid Activation of Ethylene Responsive Genes

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Abstract

Ethylene signaling pathway leads to rapid gene activation by two hierarchies of transcription factors with EIN3/EIL proteins as primary ones and ERF proteins as secondary ones. The role of chromatin modifications during the rapid gene activation is not known. In this work we studied trimethylated histone H3 lysine 4 (H3K4me3) and lysine 27 (H3K27me3), two opposite histone methylation marks for gene activity, during the induction course of three ethylene-responsive genes (*ERF1*, *AtERF14* and *ChiB*). We found that the three genes displayed different histone modification profiles before induction. After induction, H3K4me3 was increased in the 5' region and the gene body of *ERF1*, while H3K27me3 was decreased in the promoter of *AtERF14*. But the modification changes were later than the gene activation. Analysis of other rapidly inducible *ERF* genes confirmed the observation. In addition, histone H2A.Z occupancy on the three genes and the association of the H3K27me3-binding protein LHP1 with *AtERF14* and *ChiB* were not affected by the inductive signal. However, the mutation of genes encoding H2A.Z and LHP1 attenuated and enhanced respectively the induction of target genes and altered H3K4me3. These results indicate that the induction of ethylene-responsive genes does not require immediate modulation of H3K4me3 and H3K27me3 and dissociation of LHP1 and H2A.Z from the targets, and suggest that the chromatin structure of the genes before induction is committed for transcriptional activation and that H3K4me3 is not required for ethylene-responsive gene activation, but may serve as a mark for gene activity.

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Introduction

In addition to transcription factors chromatin structure plays an important role in the regulation of gene expression. The basic unit of chromatin is nucleosome that is formed by histone octamer containing two copies of H3, H4, H2A and H2B wrapped around by 147 base pairs of DNA. Chromatin structure change includes histone modifications and DNA methylation, histone variant deposition and chromatin remodeling. Histone modifications, especially H3K4 trimethylation and H3K27 trimethylation, have been largely reported to be tightly associated with gene transcription activity [1,2]. H3K4me3 is associated with highly expressed and/or housekeeping genes whereas H3K27me3 marks under-expressed and/or repressed tissue-specific genes [1,2]. Both modification marks could be recognized by different chromatin factors through specific protein domains. For example, the Plant Homeodomain (PHD) of ING2 (Inhibitor of Growth 2) can bind to H3K4me3 and the chromodomain of Polycomb proteins in animal cells and LIKE HETEROCHROMATIN PROTEIN1 (LHP1) in *Arabidopsis* can bind to H3K27me3 [3,4]. The recognitions may serve as a mechanism by which histone modifications regulate gene expression. Histone variant H2A.Z is another important regulator of gene expression which is deposited into nucleosome by SWR complex. Recent analysis in various species has revealed that activation of H2A.Z-regulated genes was accompanied by eviction of H2A.Z or replacement of

H2A.Z with H2A by INO80 complex [5,6,7]. Other studies have suggested that H2A.Z may act as an epigenetic mark to promote gene reactivation [8,9].

In plants, H3K4me3 and the H3K27me3/LHP1 module have been shown to mediate developmental genes expression such as *FLC* (*FLOWERING LOCUS C*), *AG* (*AGAMOUS*), *FUS3* (*FUSCA 3*) and *FT* (*FLOWERING LOCUS T*) [10,11,12,13]. However, how these modifications affect rapidly induced gene activation was not clear. Ethylene is a plant hormone participating in different processes including germination, flower and leaf senescence, fruit ripening, leaf abscission, root nodulation, programmed cell death, and response to stress and pathogen attack. Genetic and molecular analyses have revealed a response pathway from perception to a series of MAP kinase and finally transduced to two hierarchies of transcription regulation [14]. The primary transcription regulation is that transcription factors EIN3 (ETHYLENE-INSENSITIVE3)/EIL1 (ETHYLENE-INSENSITIVE3-LIKE 1) directly bind to EREBP (ethylene-responsive element binding protein) genes such as *ERF1* (*ETHYLENE RESPONSE FACTOR 1*) to activate their expression. Subsequently EREBP proteins activate downstream effector genes (e.g. *ChiB*, *basic chitinase* and *PDF1.2*, *Plant Defensin 1.2*). However, it was not known whether the rapid activation of ethylene-responsive genes involves change of chromatin structure. Here, we chose *ERF1* and *AtERF14* (*Arabidopsis thaliana* Ethylene-responsive element binding factor 14) as well as 5 other *ERF* genes as primary and *ChiB* as secondary regulation

targets to analyze whether chromatin structures of these target genes changed during rapid induction by ethylene. We used 1-aminocyclopropane-1-carboxylic acid (ACC) which is converted to ethylene by 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) in plants to treat 12 day-old seedlings. Increase of H3K4me3 and decrease of H3K27me3 were observed during the treatment, but the changes of both marks were much later than the gene activation. H2A.Z occupancy and LHP1 binding did not respond to the treatment indicating that the gene induction by ethylene signaling did not require immediate change of the cognate chromatin structure. However, mutation of genes encoding H2A.Z and LHP1 affected the induction of ethylene-responsive genes, suggesting that the committed chromatin

structure of these genes before induction is important for the transcriptional activation.

Results

Histone methylation profile and H2A.Z deposition over ethylene-responsive genes before induction

To assess the chromatin structure of ethylene-responsive genes before induction we tested H3K4me3, H3K27me3 and H2A.Z deposition in the promoter, the 5' region and the gene body of *ERF1*, *AtERF14* and *ChiB* (Fig. 1A). *RBCS-1A* (*RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN 1A*), *AG* and *HSP70* (*heat shock protein 70*) were used as positive controls

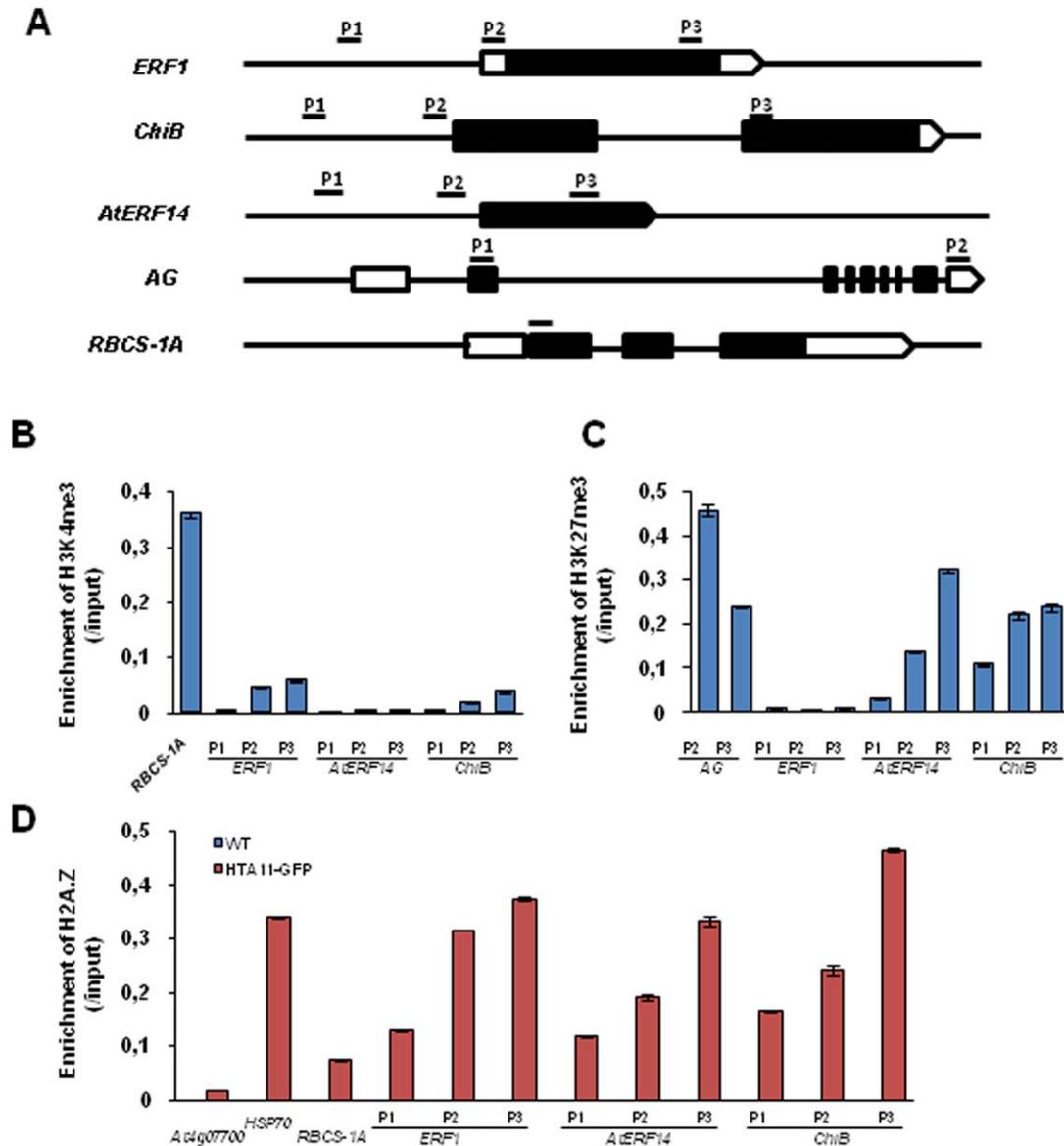


Figure 1. Chromatin status of *ERF1*, *AtERF14* and *ChiB* before ACC induction. (A) Diagrams of the gene structure of *ERF1*, *AtERF14*, *ChiB*, *AG*, and *RBCS1A*. The solid boxes indicate the coding regions, and the open boxes indicate untranslated regions (UTRs). The solid bars indicate the regions in which primers were designed for CHIP tests. (B) H3K4me3 on the three genes before induction. The relative enrichments were calculated by compared to input. *RBCS* was used as a positive control. The regions (P1, P2 and P3) shown in A represent promoter, 5' region and gene body respectively. (C) H3K27me3 on the three genes before induction. *AG* was used as a positive control. (D) H2A.Z abundance on the three genes before induction. *HSP70* was used as a positive control and *At4g07700* was used as a negative control. Bars represent mean values \pm SD from three repeats.

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respectively for H3K4me3, H3K27me3 and H2A.Z deposition [1,5,13]. The *At4g07700* locus was used as negative control for H2A.Z deposition [5]. Moderate levels of H3K4me3 were detected in the 5' region and the gene body, but not the promoter, of *ERF1* and *ChiB* compared to that of *RBCS-1A* (Fig. 1B). In contrast, H3K4me3 was not detected over *AtERF14* (Fig. 1B). H3K27me3 was enriched in the 5' region and the gene body of both *AtERF14* and *ChiB* but not in *ERF1* (Fig. 1C). Analysis of five additional *ERF* genes (*ORA59*, *TDR1*, *AtERF1*, *AtERF2* and *AtERF11*) revealed that *TDR1* displayed a high level of H3K27me3 but a low level of H3K4me3, while the other four genes showed a high level of H3K4me3 but a low level of H3K27me3 (Fig. S2). Similar to what found in *ERF1* and *ChiB*, H3K4me3 levels on the promoter of these genes were relatively low (Fig. S2). This analysis revealed that the ethylene-inducible genes displayed different histone modification profiles before induction.

To test whether H2A.Z was present in the chromatin of these genes, chromatin fragments isolated from H2A.Z-GFP transgenic plants were precipitated with GFP antibody. We found that H2A.Z was incorporated into chromatin over the three genes with highest levels in the gene bodies and lowest levels in the promoters (Fig. 1D). The presence of H2A.Z was detected also over the five additional *ERF* genes (Fig. S2).

Histone methylation dynamics during induction of ethylene responsive genes

In order to study histone modification dynamics during gene activation, we chose five time points to monitor ACC induction time course of ethylene-responsive genes by quantitative RT-PCR. The induction of *ERF1* by ACC was early, which was 4 folds after 1 hour and elevated to 40 folds after 8 hours (Fig. 2A). For *AtERF14*, the expression began to increase after 2 hours and reached to 12 folds after 8 hours (Fig. 2A). The induction of *ChiB* was moderate, only 2 to 3 folds after 8 hours (Fig. 2A). *ORA59*, *AtERF1*, *AtERF2* and *AtERF11* were induced as early as *ERF1*, while the induction of *TDR1* was delayed (Fig. S2).

H3K4me3 and H3K27me3 are two opposite histone modification marks associated with gene transcription activity. However, it is still not clear whether the two modifications are involved in gene activation process. Therefore, we tested the levels of these modifications over ethylene responsive genes during the ACC induction process. For *ERF1*, H3K4me3 in the 5' region and the gene body began to increase only after 4 hours, which was later than the initial increase of gene expression (Fig. 2B). Similarly, the increase of H3K4me3 over *ORA59*, *AtERF1*, *AtERF2* and *AtERF11* was also later than gene activation (Fig. S2). This suggested that the induction of *ERF1* did not require a concurrent increase of H3K4me3. The late increase might indicate that H3K4me3 served as a mark of elevated transcription activity of the genes. H3K4me3 remained undetectable over *AtERF14* and *TDR1* and did not change over *ChiB* during the induction (Fig. 2B) (Fig. S2). These results indicated that H3K4me3 was not necessary for the induction of *AtERF14* and *TDR1*. However, it was not clear whether the basal levels of H3K4me3 over *ERF1* and *ChiB* before induction was required for the induction of the genes.

Due to the low level of H3K27me3 over *ERF1* before induction we did not expect that there would be any change during the induction. So we tested H3K27me3 over *AtERF14* and *ChiB*. H3K27me3 was not much changed in the gene body of both genes during induction, but was decreased in the promoter of the genes, especially *AtERF14* (Fig. 2C). However the decrease of H3K27me3 was delayed compared to the gene induction, suggesting that rapid gene activation did not require or lead to

immediate demethylation of H3K27me3 and that the presence of H3K27me3 did not prevent the induction process. Analysis of H3K27me3 over *ORA59*, *TDR1*, *AtERF1*, *AtERF2* and *AtERF11* confirmed the results (Fig. S2).

Negative function of LHP1 on the induction of *AtERF14* and *ChiB*

H3K27me3 is recognized and bound by LHP1 that is suggested to be an H3K27me3 effector. To explore the role of LHP1 in rapid gene activation, we analyzed the induction of ethylene responsive genes in the *lhp1* mutant. For *AtERF14* and *ChiB* that displayed high levels of H3K27me3 the induction by ACC was clearly enhanced in the mutant (Fig. 3A), indicating that LHP1 had a repressive function on induction of the two genes. However, we also detected an elevated expression of *ERF1* in *lhp1* (Fig. 3A). Considering that there was a low level of H3K27me3 over *ERF1* we speculated that this might be an indirect effect of increased expression of *AtERF14*, as it has been reported that overexpression of *AtERF14* could lead to increased expression of *ERF1* [15]. In addition, we tested H3K4me3 levels over the target genes in *lhp1* in comparison with the wild type. We found that in *lhp1* H3K4me3 was increased in the 5' region and the gene body, but not the promoter, of *ERF1* and *ChiB*. The increased H3K4me3 levels may be also a consequence of increased transcription activity of the genes as mentioned before. However, H3K4me3 remained undetectable over *AtERF14* despite the increased expression of this gene in *lhp1* (Fig. 3B). The early induction of 4 of the 5 additional *ERF* genes was found to be enhanced in the *lhp1* mutant. Except *TDR1* that had no H3K4me3, the other three genes displayed increased H3K4me3 (Fig. S3).

To study whether LHP1 was bound to *ChiB* and *AtERF14*, ChIP analysis of the *lhp1* mutant complemented by *LHP1::LHP1-MYC* was performed by using anti-MYC antibodies [16]. The analysis revealed that LHP1 was associated with *ChiB* and *AtERF14* as well as with *AG*, but not with *ERF1* (Fig. 4). Importantly, ACC treatment did not lead to dissociation of LHP1 from these genes (Fig. 4). Therefore, although the *lhp1* mutation had an effect on the induction of ethylene-induced genes, the presence of the H3K27me3 /LHP1 module on the genes was irresponsive to the inductive signal.

Requirement of H2A.Z for the induction of *ERF1* and *ChiB*

In Arabidopsis, H2A.Z has been shown to be involved in multiple responses such as temperature and phosphate starvation [5,7]. It either activates or represses target genes expression by eviction from nucleosomes occupying around the transcription start site. The presence of H2A.Z was detected over the three ethylene-inducible genes before ACC induction (Fig. 1D). During ACC treatment, no immediate decrease of H2A.Z abundance over ethylene-responsive genes was detected, albeit a slight decrease was observed after induction (Fig. 5; Fig. S2), suggesting that a clear H2A.Z eviction was not required for the initial induction of the genes.

In order to study whether H2A.Z was required for the induction, we tested the expression of these genes in *hta9/hta11* double mutants that have a reduced level of H2A.Z [17]. In the mutant the induction of *ERF1* was reduced, while that of *AtERF14* was not clearly affected (Fig. 6A). The effect of the mutations on the induction of *ChiB* was detected after 8 hours, but was more severe after 24 hours (Fig. 6A). These results indicated that H2A.Z was involved in the induction of *ERF1* and *ChiB*. Then we tested H3K4me3 over *ERF1* and *ChiB* during ACC induction in the *hta9/hta11* mutants. Increase of H3K4me3 over *ERF1* observed in wild type was delayed in the mutants, but the basal levels of H3K4me3 over both *ERF1* and *ChiB* were not affected (Fig. 6B).

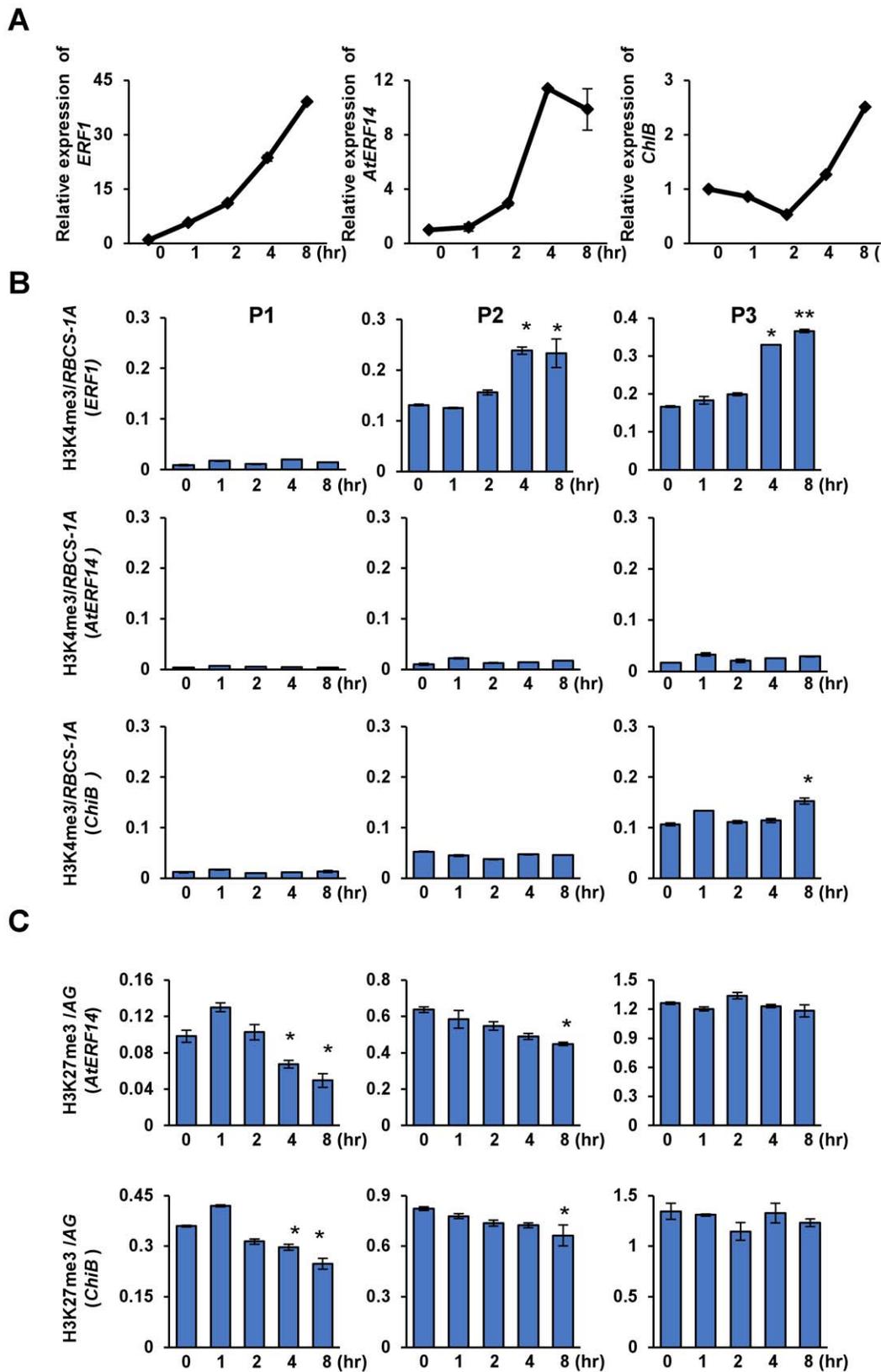


Figure 2. Expression and histone modification changes of three ethylene responsive genes during ACC induction. (A) Induction time course of the three genes by ACC. Twelve day-old seedlings were treated with 50 μ M ACC and harvested at the indicated time points. Relative fold changes were determined by normalization with *ACTIN2* transcript levels. (B) H3K4me3 detected on the three genes during ACC induction. The relative enrichments were calculated by first comparing to input and then to the reference gene *RBCS-1A*. The three regions were analyzed for ChIP. (C) H3K27me3 detected on the three genes during ACC induction. The relative enrichments were calculated by first comparing to input and then to

the reference gene *AGAMOUS* (*AG*). The three regions were analyzed for ChIP. Bars represent mean values \pm SD from three repeats. Significance of H3K4me3 and H3K27me3 differences between after ACC treatment (at different time points) and before treatment (0) was tested by two-tailed Student's t-test, * $p < 0.05$, ** $p < 0.005$.
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We speculated that H2A.Z had no effect on H3K4me3 and the delayed increase of H3K4me3 over *ERF1* might be a result of decreased transcription activity of the gene in the mutants.

Discussion

H3K4me3 serves as a mark of gene transcription activity

Genome-wide analysis in plants indicates that H3K4me3 is associated with actively transcribed genes. But how it affects gene expression remains unclear [1,18]. Some researchers have

proposed that this modification may be recognized and bound by specific proteins which act as effectors to control transcription [3,19,20]. But other studies have suggested that H3K4me3 could serve as a memory or a mark of active genes [21]. In this study we tried to find out the role of H3K4me3 during activation of ethylene-inducible genes. Our results indicated that elevated H3K4me3 was not necessary for the ethylene-induced gene activation but may serve as a mark of transcription activity of the genes. First, H3K4me3 was not detected over *AtERF14* and *TDR1* before induction and was not increased after ACC treatment

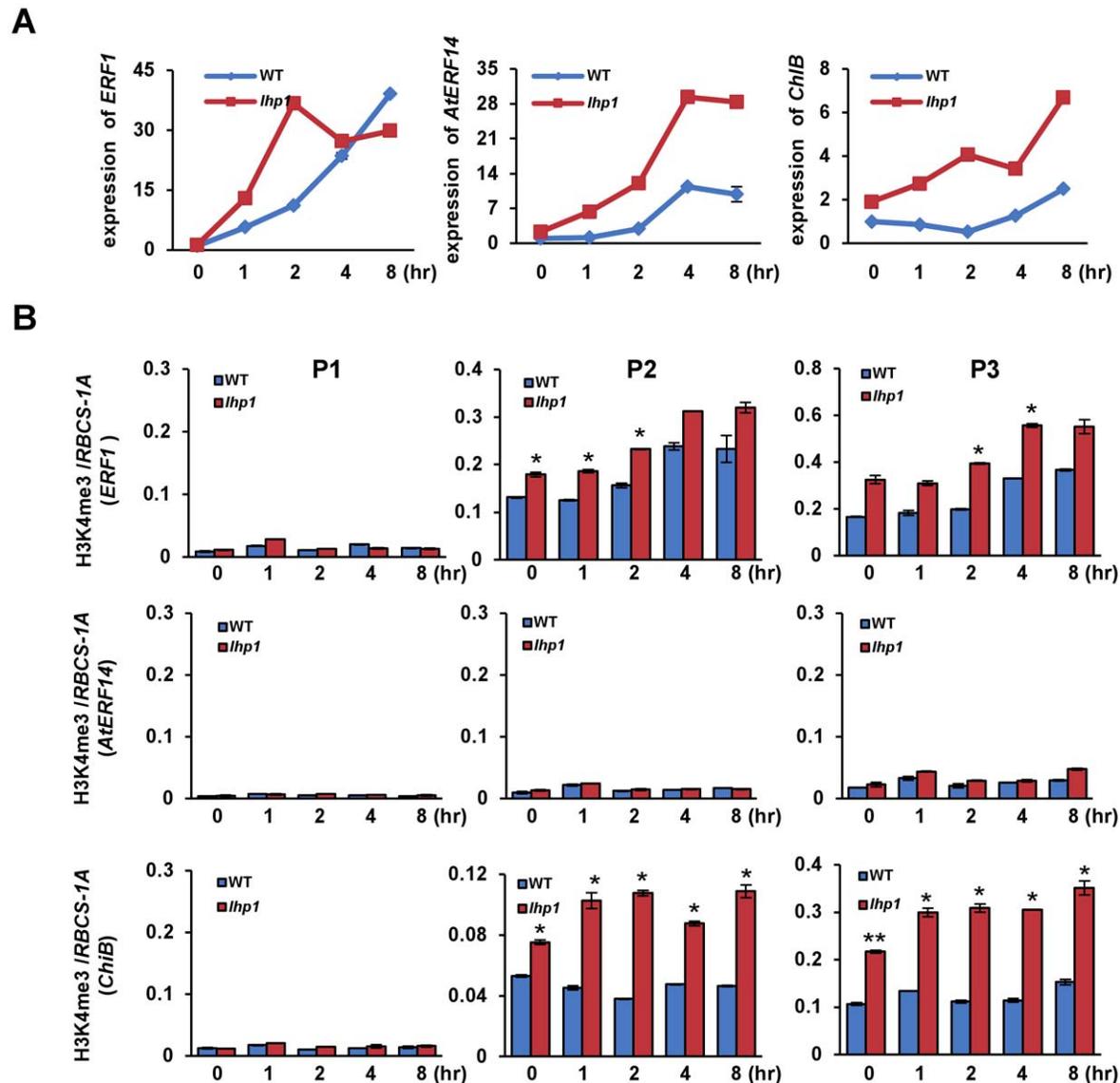


Figure 3. The role of LHP1 on the induction of ethylene responsive genes. (A) Transcript levels of the three genes at different time points during ACC induction in wild type (WT) and *lhp1* plants. Relative fold changes were determined by normalization with *ACTIN2* transcripts. (B) H3K4me3 levels on *ChIB*, *ERF1* and *AtERF14* in wild type (WT) and *lhp1* mutants during treatment. The relative enrichments (to *RBCS-1A*) on three regions of the three genes are presented. Bars represent mean values \pm SD from three repeats. Significance of H3K4me3 differences between WT and *lhp1* at different time points was determined by two-tailed Student's t-test, * $p < 0.05$, ** $p < 0.005$.
doi:10.1371/journal.pone.0028224.g003

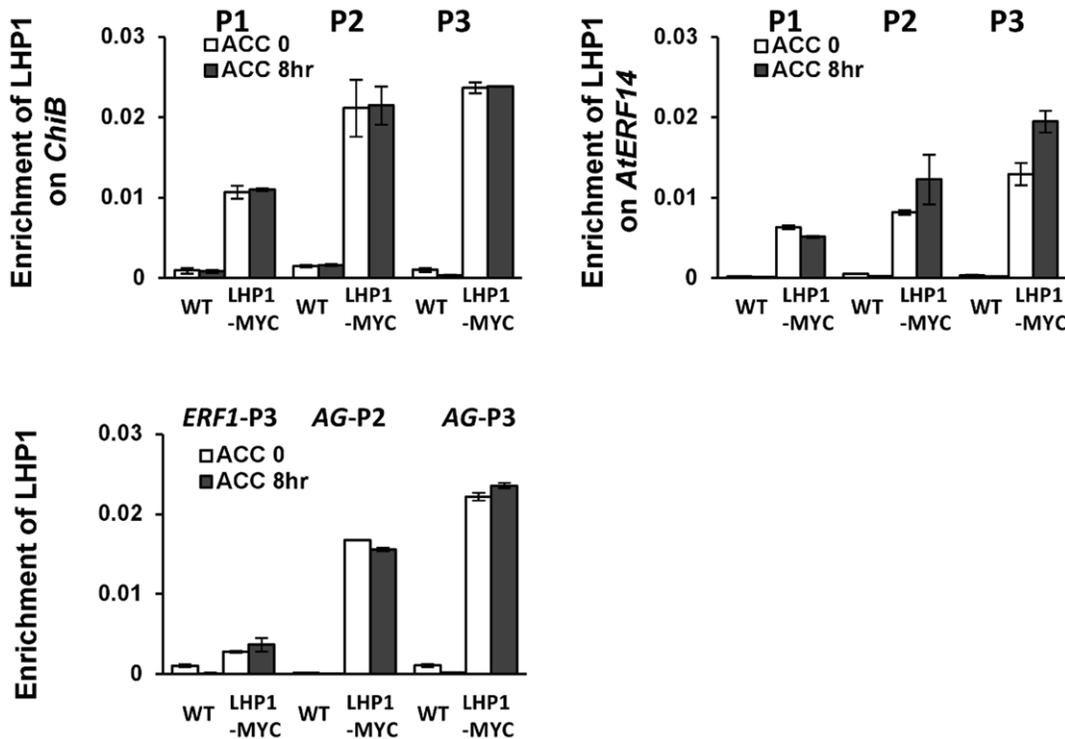


Figure 4. LHP1 binding to different genes before and after ACC treatment. WT and *lhp1* mutants complemented by *LHP1::LHP1-MYC* were used for ChIP analysis with anti-MYC antibodies. The relative enrichments were normalized with input. Bars represent mean values \pm SD from three repeats.

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(Fig. 1B, Fig. 2B; Fig. S2). Second, although some increase of H3K4me3 was detected in the 5' region and/or the gene body of *ERF1*, *ORA59*, *AtERF1*, *AtERF2* and *AtERF11* after induction, the increase of H3K4me3 lagged behind that of gene activation (Fig. 2B; Fig. S2). Finally, the level of H3K4me3 was associated with that of gene expression in *lhp1* and *hta9/hta11* mutant and wild type plants. For instance, H3K4me3 was increased when the induction of *ERF1* and *ChiB* was enhanced in *lhp1* over these genes (Fig. 3). Conversely, the increase of H3K4me3 was delayed when the induction of the genes was attenuated by the mutation of H2A.Z genes (Fig. 6). Although there was no concomitant increase of H3K4me3 with activation of gene expression, it is not excluded the possibility that the basal level of H3K4me3 over *ERF1* and *ChiB* might contribute to the chromatin commitment of these genes for the induction.

Repressive function of the H3K27me3/LHP1 module on inducible genes

The observations that H3K27me3 did not change over *AtERF14*, *ChiB* and *TDR1* during ACC induction are consistent with recent results showing that although H3K27me3 on the floral time repressor *FLC* is inversely correlated with transcriptional activity, the abundance of this mark is not diminished in the first 12h following activation of transcription [22]. Analysis of cold-inducible genes has detected H3K27me3 to decline only one to several days after application of the inductive signals [23]. These observations suggest that transcription activation may not involve immediate demethylation of H3K27me3, or the presence of H3K27me3 is not sufficient to impair transcriptional activation during induction. This is supported by a recent result showing that

vernalization-mediated induction of *VERNALISATION INSENSITIVE 3 (VIN3)* does not lead to any decrease of H3K27me3 on the locus 40 days after exposure to cold temperature [24,25].

The observations that the binding of LHP1 to *AtERF14* and *ChiB* was not affected by ACC induction and that the mutation of *LHP1* led to increased induction of the genes suggest that the H3K27me3/LHP1 module is required for the repression of the genes and the repressive effect could be lifted by additional events during the induction. The constitutive association of LHP1 with these targets is reminiscent of the data showing that LHP1 remains to be associated with *VIN3* chromatin many days after induction by vernalization [25], indicating that the LHP1 binding in that conditions does not lead to gene silencing. These observations collectively suggest that additional elements associated with the H3K27me3/LHP1 module, which can be inactivated by inductive signals, might be involved in H3K27me3/LHP1-mediated gene silencing.

Involvement of H2A.Z in the induction of gene expression

Our data showing that deposition of histone variant H2A.Z over the eight ethylene-responsive genes was not evicted after ACC induction are in agreement with the findings that H2A.Z is present in both silent and active *FLC* chromatin (Fig. 5; Fig. S2) [8]. Probably, the presence of H2A.Z may mark these genes for induction, supporting the notion that H2A.Z serves to mark active gene and poise silent genes for reactivation [8,9]. Recent results have shown that H2A.Z is required for both gene activation and repression in responding to warmer temperature [5]. In contrast to the observations on the ethylene-responsive genes, H2A.Z-containing nucleosomes are found to be lost from both up-

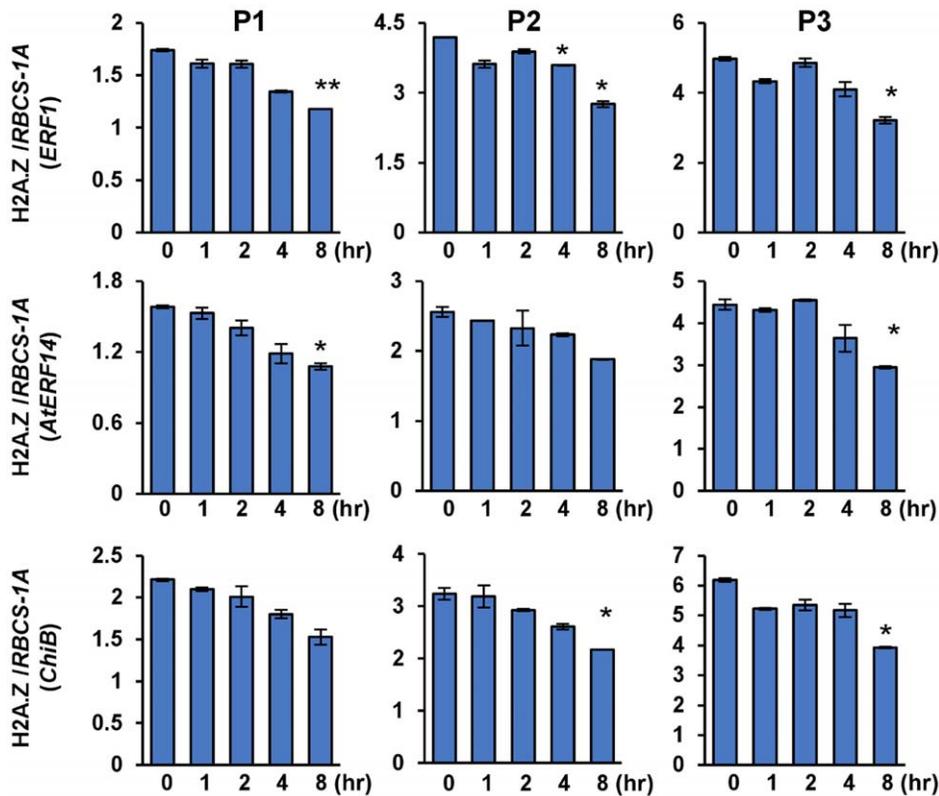


Figure 5. H2A.Z deposition over ethylene responsive genes during ACC induction. H2A.Z-GFP transgenic plants were used for ChIP analysis with GFP antibody. The enrichments on three regions are shown. Bars represent mean values \pm SD from three repeats. Significance of H2A.Z level difference between induced (at different time points) and non induced (0) was determined by two-tailed Student's t-test, * $p < 0.05$, ** $p < 0.005$.

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regulated and down-regulated genes after an increase of temperature. Therefore, the role of H2A.Z in chromatin structure and in gene activity is complex, which may be dependent on the chromatin context of the gene. In addition, our results showed that mutation of H2A.Z genes had an obvious negative effect on the induction of *ERF1* and *ChiB* but not *AtERF14*. Considering the undetectable level of H3K4me3 over *AtERF14* and moderate levels over *ERF1* and *ChiB* we speculate that H2A.Z may have a coordinated effect with H3K4me3 on the activation of these genes during ACC induction.

Chromatin structure is considered as an important regulator of transcription in addition to transcription factors especially for the developmental genes. In this work we tried to figure out whether chromatin modifications take place during activation of rapidly inducible genes. Our work revealed that histone modifications including H3K4me3 and H3K27me3 and presence of chromatin proteins such as LHP1 and H2A.Z did not display any immediate change upon ACC treatment. However, mutation of LHP1 and H2A.Z genes had an effect on the induction suggesting that basal chromatin structure before induction is important for the induction.

Materials and Methods

Plant material and Exogenous ACC treatment

The mutants used in this study are *lhp1* [26], *hta9/hta11* [17], *lhp1* complemented by LHP1::MYC [16] and H2A.Z-GFP transgenic plants [5]. Arabidopsis seeds were surface-sterilized and growth at 22°C with a 16 h light/8 h dark (long day) cycle.

Twelve days after germination 50 μ M ACC solution was added. The samples were harvested at indicated time points.

RNA extraction and reverse transcription

Total RNA was extracted from twelve day-old seedling using Trizol (Invitrogen). Four μ g total RNA were treated first with 1 unit of DNase I (Promega) and then reverse transcribed in a total volume of 20 μ L with 0.5 μ g oligo(dT)₁₅, 0.75 mM dNTPs, 2.5 mM MgCl₂, 1 μ L ImProm-II reverse transcriptase (Promega). The resulting products were tested by Real-Time PCR with gene specific primers (Table S1).

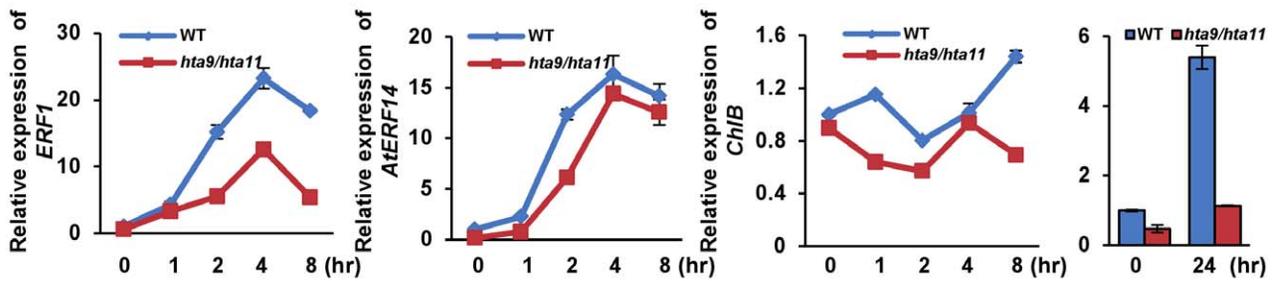
Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) experiment was performed most as described in [27]. One gram of 12 day-old seedlings before and after ACC treatment were harvested and crosslinked in 1% formaldehyde under vacuum. Nuclei were then extracted with extraction buffers. Chromatin was fragmented to 200–2000 bp by sonication and ChIP was performed using antibodies: c-Myc (Sigma, M4439), H3K4me3 (Cell Signaling, 9751S) H3K27me3 (Millipore, 07-449) and GFP antibody (Abcam, ab290). The precipitated and input DNAs were then analyzed by real-time PCR with gene specific primer sets (Fig. S1, Table S1). At least three biological repeats were performed for the ChIP experiments.

Real-Time PCR

Real-time PCR was performed in a total volume of 20 μ L with 1.0 μ L of the reverse transcription or ChIP products, 0.25 μ M

A



B

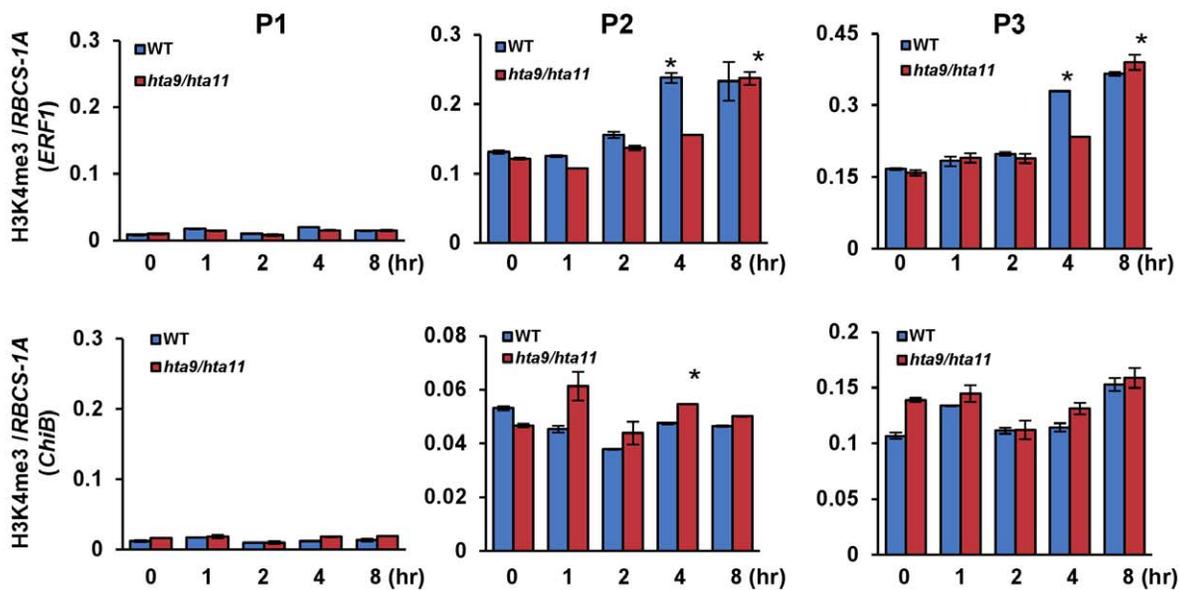


Figure 6. Function of H2A.Z in the ACC induction of *ERF1*, *AtERF14* and *ChiB*. (A) Transcript levels of the three genes at different time points during ACC induction in wild type (WT) and *hta9/hta11* mutants. Relative fold changes were determined by normalizing with *ACTIN2* transcripts. (B) H3K4me3 levels on *ERF1* and *ChiB* during ACC induction in WT and *hta9/hta11*. The relative enrichments (to *RBCS-1A*) on three regions are shown. Bars represent mean values \pm SD from three repeats. Significance of H3K4me3 difference between WT and *hta9/hta11* at different time points was determined by two-tailed Student's t-test, * $p < 0.05$, ** $p < 0.005$. doi:10.1371/journal.pone.0028224.g006

primers, and 10 μ l SYBR Green Master mix (Roche) on a LightCycler 480 real-time PCR machine (Roche) according to the manufacturer's instructions. All primers were annealed at 60°C and run 45 cycles. The ChIP enrichment for GFP, H3K27me3 and H3K4me3 was quantified by comparing the thresholdcycle (C_t) of the ChIP samples with that of the input and then normalized with the levels of control genes: $2^{(C_t \text{ of input} - C_t \text{ of sample ChIP})} / 2^{(C_t \text{ of input} - C_t \text{ of control ChIP})}$. The expression level of target genes was normalized with that of *ACTIN*: $2^{(C_t \text{ of actin} - C_t \text{ of target})}$.

Supporting Information

Figure S1 Genes used as controls in this study were not affected by ACC treatment. The expression of *ACTIN2*, *AGAMOUS* (*AG*) and *RBCS1A* was not affected by ACC treatment. For *ACTIN2*, three biological replication of ACC induction were performed. Data represent average means and the expression before ACC induction was set as 1. The expressions of *AG* and *RBCS-1A* were normalized with that of *ACTIN2*. (TIF)

Figure S2 Expression, histone methylation and H2A.Z deposition of five additional ethylene responsive factor (ERF) genes during ACC induction. RNA levels (A), H3K4me3 (B, C), H3K27me3 (D) and H2A.Z (E) of *ORA59* (At1g066160), *TDR1* (At3g23230), *AtERF1* (At4g17500), *ATERF2* (At4g47220) and *ATERF11* (At1g28370) were measured at the different time points during ACC treatment as indicated. Bars represent mean values \pm SD from three repeats. For ChIP experiments, primers corresponding to the promoter (B) and gene bodies (C-E) were used. Significance of H3K4me3, H3K27me3 and H2A.Z induction compared to that before treatment (0) was determined by two-tailed Student's t-test, * $p < 0.05$, ** $p < 0.005$. (TIF)

Figure S3 Expression and H3K4me3 of additional ethylene responsive factor (ERF) genes between WT and *lhp1* during ACC induction. RNA levels (upper) and H3K4me3 (lower) were measured during ACC treatment. Bars represent mean values \pm SD from three repeats. For ChIP experiments, primers corresponding to the gene bodies were used.

Significance of H3K4me3 levels between WT and *lhp1* before and after ACC treatment was determined by two-tailed Student's *t*-test, **p*<0.05, ***p*<0.005.

(TIF)

Table S1 Sequences of primers used in this study.

(DOC)

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Author Contributions

Conceived and designed the experiments: YH DXZ. Performed the experiments: YH YS NCES. Analyzed the data: YH DXZ. Wrote the paper: YH DXZ.

RESUME

Le remodelage de la chromatine et la modification des histones jouent des rôles très importants dans l'établissement et la reprogrammation de l'état de l'expression génique. Il reste largement inconnu concernant les mécanismes de la régulation de ces processus chromatiniens dans le contrôle de l'expression génique impliquée dans le développement de la plante et son adaptation à l'environnement. Mon sujet de thèse se focalise sur l'analyse fonctionnelle d'un facteur de remodelage de la chromatine de type Chromodomain/Hélicase/DNA-binding 1 (CHD1) d'*Arabidopsis*, appelé CHR5 et une histone déméthylase qui est spécifiquement impliquée dans la déméthylation de l'histone H3 lysine 4 (H3K4), appelée JMJ15. Dans la première partie de cette étude, nous avons montré que le gène CHR5 est activé au cours de l'embryogénèse et que son expression se maintient élevé dans les tissus/organes en développement. L'analyse de mutants révèle que la perte de fonction de ce gène fait réprimer l'expression de gènes régulateurs de la maturation de l'embryon tels que LEC1, ABI3 et FUS3 pendant le développement des graines, et fait baisser l'accumulation des protéines de réserve. L'analyse de double mutants a permis de démontrer une fonction antagoniste entre CHR5 et PKL, une protéine du groupe « CHD3 », dans l'activité du promoteur de gènes régulateurs du développement de l'embryon et l'accumulation de réserve de graine. Nous avons montré que la protéine CHR5 s'associe directement avec les promoteurs d'ABI3 et FUS3 et que la mutation du gène CHR5 conduit à l'augmentation de présence de nucléosome dans la région du départ de transcription. Ces résultats suggèrent que CHR5 est impliquée dans le positionnement de nucléosome pour stimuler l'expression de gènes de la maturation de l'embryon, ce qui est contrebalancé par l'action de PKL au cours du développement de l'embryon. La deuxième partie de cette étude a permis de montrer que l'expression du gène de l'histone déméthylase JMJ15 manifeste une forte spécificité tissulaire. L'analyse de mutants du gène a permis de l'identification de 2 allèles de gain de fonction (avec surexpression du gène), et un allèle de perte de fonction. La surexpression du gène réduit la croissance d'hypocotyle et de tige de la plante avec accumulation de lignine dans la tige, mais la perte de fonction du gène ne produit pas de phénotype apparent. Par ailleurs, la surexpression du gène renforce la tolérance de la plante au stress salin, alors la perte de fonction du gène rend la plante plus sensible. L'analyse du transcriptome a révélé beaucoup plus de gènes réprimés qu'activés par la surexpression du gène JMJ15. Ces gènes réprimés sont préférentiellement marqués par la H3K4me2 ou H3K4me3, parmi lesquels beaucoup codent de facteurs de transcription. Ces données suggèrent que l'induction de JMJ15 pourrait réguler le programme de l'expression génique qui coordonne la restriction de la croissance de la plante et la tolérance au stress. Ces travaux de thèse ont permis d'identifier quelques nouveaux éléments dans la compréhension de la fonction de régulateurs chromatiniens dans l'expression génique de la plante.

SUMMARY

Chromatin remodeling and histone modification play important roles in the establishment and dynamic regulation of gene expression states. However, little is known regarding the regulatory mechanism of chromatin modification and remodeling that control gene expression involved in plant development and responses to environmental cues. My thesis work concerns functional analysis of an *Arabidopsis* Chromodomain/Helicase/DNA-binding 1 (CHD1) type chromatin remodeling gene known as CHR5 and a histone demethylase gene that specifically removes methyl groups from methylated histone H3 lysine 4 (H3K4me), called JMJ15 in regulating chromatin structure or in resetting chromatin modifications that control the expression of plant developmental and stress responsive genes. In the first part of the study we found that *CHR5* expression is activated during embryogenesis and remained to be expressed in developing organs/tissues. Analysis of mutants revealed that loss-of-function of the genes led to decreased expression of key embryo maturation genes *LEC1*, *ABI3* and *FUS3* in developing seeds and reduced seed storage protein accumulation. Analysis of double mutants revealed an antagonistic function between *CHR5* and *PKL*, a CHD3 gene, in embryo gene promoter activity and seed storage protein accumulation. *CHR5* was directly associated with the promoters of *ABI3* and *FUS3* and *chr5* mutations led to increased nucleosome occupancy near the transcriptional start site. The results suggest that *CHR5* is involved in nucleosome occupancy to regulate embryo identity genes expression, which is counterbalanced by *PKL* during embryo development. The second part of this study showed that expression of *JMJ15* was restricted to a few tissues during vegetative growth. The *jmj15* gain-of-function mutations reduced the length of seedling hypocotyls and inflorescence stems with higher accumulation of lignin in the stem, while the loss-of-function mutants did not show any visible phenotype. The gain-of-function mutants enhanced salt tolerance, whereas the loss-of-function mutants were more sensitive to salt. Transcriptomic analysis revealed a much higher number of genes down-regulated in *JMJ15* over-expression plants, which are highly enriched for H3K4me3 and H3K4me2. Among the down-regulated genes, many encode transcription regulators of stress responsive genes. The data suggest that increased *JMJ15* levels may regulate the gene expression program that may coordinate plant growth restrains and enhances stress tolerance. Taken together, my thesis work brought a few new elements to the current understanding of chromatin regulators function in plant gene expression.